ADVANCED BIOREMEDIATION TECHNOLOGIES AND PROCESSES FOR THE TREATMENT OF SYNTHETIC ORGANIC COMPOUNDS (SOCs)

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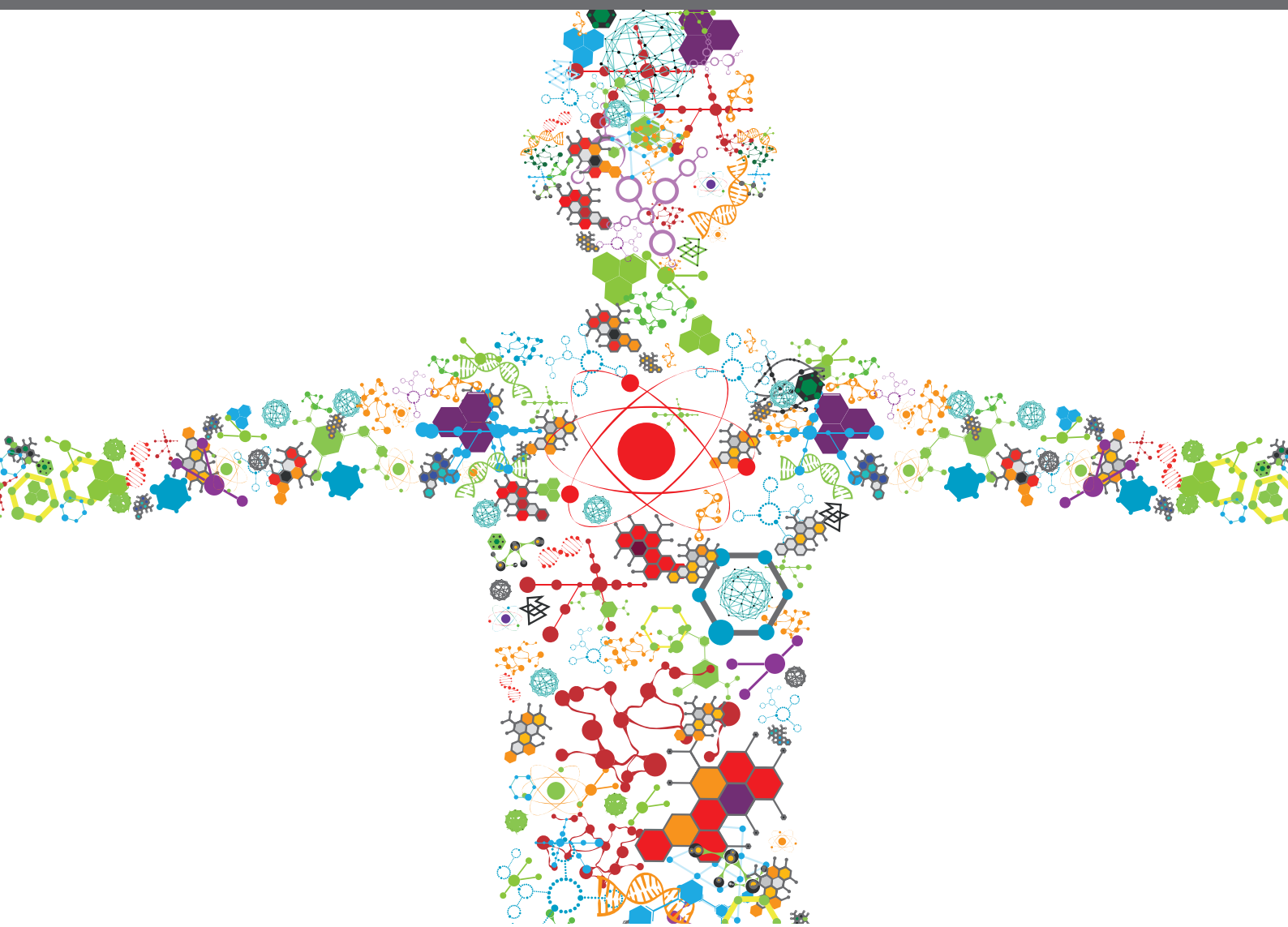

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ADVANCED BIOREMEDIATION TECHNOLOGIES AND PROCESSES FOR THE TREATMENT OF SYNTHETIC ORGANIC COMPOUNDS (SOCs)

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Table of Contents

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EDITORIAL
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Editorial: Advanced Bioremediation Technologies and Processes for the Treatment of Synthetic Organic Compounds

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Editorial on the Research Topic

Advanced Bioremediation Technologies and Processes for the Treatment of Synthetic Organic Compounds

Synthetic organic compounds (SOCs) are the primary pollutants of aquatic and terrestrial ecosystems. This group of chemicals includes pesticides, herbicides, and pharmaceutical products and their transformed products; industrial chemicals (such as plasticizers, dyes, and dye intermediates), organophosphate and brominated flame retardants, volatile organic compounds (VOCs) (i.e., organic solvents), polycyclic aromatic hydrocarbons, per- and polyfluoroalkyl substances (PFAS), plastic polymers, and many more (Tijani et al., 2016; Ahmed et al., 2018; Ilyas and van Hullebusch, 2020). The SOCs are commonly found in urban wastewater, agricultural runoffs, and industrial waste streams (Postigo and Barceló, 2015). SOCs pollute various habitats through diffusion or as point source pollutants. SOCs exert multi-dimensional effects in the polluted environment by altering the physicochemical properties of the habitats where they are released, representing a threat to humanity (Naidu et al., 2021). The environmental distribution and persistence of many SOCs were previously unknown, but with the development of newer detection methods, SOCs have been found in the environment at much higher concentrations. Due to their bio-toxic properties, many SOCs are now recognized as priority pollutants, requiring immediate solutions for their removal from polluted ecosystems (Lohmann et al., 2007; Walker, 2019).

Soon after realizing the adverse effects of SOCs on the biosphere, different treatment technologies and processes, including hybrid integrated systems, have been developed. Due to the exponential rise in the generation of SOCs, and concomitant heterogeneous compositions of these xenobiotics in the polluted environment, traditional treatment technologies were found to have varying degrees of efficacy. The accumulating volume and heterogeneity of the xenobiotic wastes that are generated are the driving force for the emergence of next-generation technologies in waste treatment. In recent years, promising multi-dimensional treatment technologies have been developed such as hybrid integrated treatment systems coupling biological and physicochemical processes, membrane bioreactors, microbial electrochemical technologies, phyto-reactors, *in-situ* treatment methods (e.g., bioventing, biostimulation, and bioaugmentation), novel pre-treatment approaches, and innovative bioreactor/bioprocess designs for treatment of SOCs (Jain et al., 2020; Trellu et al., 2020).

Therefore the aim of this Research Topic is to collate the recent advances in the development of bioremediation processes and technologies for the treatment of synthetic organic compounds

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(SOCs). This research topic has published thirteen articles including six original studies, one brief research report, and six review articles (including one systematic review paper).

In their review, Mishra et al. explain the remarkable role of microorganisms and their catabolic potential, with genes, enzymes, and degradation pathways, in the biodegradation process of xenobiotic compounds. The successful execution of omics technologies with bioinformatics tools shows the way to next-level research in the bioremediation potential of microorganisms and exploits their capability to remove xenobiotic contamination.

Another review article (by Arora) described the competence of the genus *Bacillus* for the degradation of xenobiotic compounds along with the detoxification of potentially toxic metals. The author has listed 154 species of *Bacillus* and reported their use in the biodegradation and biotransformation of various pesticides, herbicides, and insecticides along with chlorophenol, nitrophenol, and chloronitrophenol, the decolorization and biotransformation of dye compounds, the biodegradation of different polycyclic and heterocyclic aromatic hydrocarbons, explosives, drugs, natural aromatic compounds, and other xenobiotic compounds like crude oils, plastic bags, etc., and the detoxification of potentially toxic metals. Several advanced technologies, like genome-editing tools used in bioremediation, have been also discussed. The author concludes that the construction of a genetically engineered Bacilli strain or genomic editing tool will help in developing efficient *Bacillus* strains for bioremediation.

Surkatti et al. studied the organic degrading bacteria from gas-to-liquid process water (GTL). They have isolated and identified the native bacteria from GTL process water, which were further used for biodegradation of organic contaminants from local GTL process water. The authors have used techniques from three areas of environmental microbiology and biochemistry along with bioremediation. Three distinct bacterial species namely, *Alcaligenes faecalis*, *Stenotrophomonas* sp. and *Ochrobactrum* sp. were identified. They observed that the bioremediation potential of mixed bacterial strains was much better than the three individual strains, though individually the treatment efficiency reached up to 60% reduction in chemical oxygen demand (COD).

Mahapatra and Phale provide insights into the ecological aspects of field application and microbial strain optimization for efficient bioremediation of polycyclic aromatic hydrocarbons (PAHs) following a system biology approach. In another review by Shahsavari et al. the chemical properties, sources, and fate of per- and polyfluoroalkyl substances (PFAS) contamination in the environment are discussed. This review systematically addresses the current status of PFAS biodegradation as well as challenges faced in their remediation.

Plastics or Low-Density Polyethylene (LDPE) have been one of the major global pollutants for last the several years. Due to its highly recalcitrant properties, its natural turnover rate in the environment is negligible. In an experimental study, Dey et al. demonstrated the degradation of LDPE using an enriched microbial community. The culturable portion of the community consists of *Stenotrophomonas* sp. and *Achromobacter* sp. The microbial growth in presence of LDPEs, high cell surface hydrophobicity of the microbial community, and substantial weight reduction of the treated LDPEs were the primary indicators that signified plastic degradation.

Maurya et al. reviewed the current research on enzymatic remediation of Polyethylene Terephthalate (PET) based polymers. The review provides a brief overview of the various approaches of biocatalysis used for effective recycling of PET, along with the factors affecting the hydrolysis rate and the challenges faced during this step. The authors suggest that further research should be focused on identifying thermostable PET-hydrolyzable enzymes capable of hydrolyzing hcPET with broad substrate specificity.

Dhakar et al. have demonstrated the construction of a genome-scale metabolic model following automatic and manual protocols and its application for improving metabolic potential through interactive simulations. In an experimental study, Ferreira et al. studied the phytoremediation efficiency of four plant species for soil samples contaminated with Tebuthiuron (herbicides) and the effect of vinasse during the treatment.

In a review by Jaiswal et al. SOC management from paper mill/pulp industrial effluents is discussed in detail. Authors have described various SOCs present in pulping and paper processing effluents. The review describes various methods to reduce SOC production from pulp and paper industries, and their treatment strategies were also discussed in brief along with their challenges and limitations. It further describes the biotechnological applications such as genetically modified biological agents for sustainable remediation of SOCs from paper industries.

In a brief research report by Mazioti et al. treatment of real bilge wastewater with zero valent iron (ZVI) and activated charcoal using three different biological methods: (i) anaerobic digestion with granular sludge and ZVI addition for enhancement of methane production, (ii) activated charcoal addition to biological treatment (aerobic and anaerobic) for significant reduction of COD, and (iii) combination of ZVI and anaerobic charcoal addition for high-performance treatment. The study found that a combination of ZVI and activated charcoal could have greater bioremediation performance amongst the three approaches used for the treatment of real bilge wastewater.

In a research article on total petroleum hydrocarbon (TPH) degradation, Garousin et al. have accessed various methods of bioremediation (i.e., bio-stimulated microcosm, bacterialized microcosm, combinatorial approach of bio-stimulated microcosm and bacterialized microcosm, and natural attenuation) using *Bacillus altitudinis* HRG-1 in age-old petroleum contaminated soils. The study reports that a combination of bio-stimulated microcosm and bacterialized microcosm was the most effective method, where 38.2% TPH was reduced under experimental conditions over a 60-day period. The author concludes that biostimulation alone was insufficient, and that proper application of suitable microbes is essential to reduce the contaminant load from polluted soils.

Askri et al. through their experimental study, demonstrated that bioelectricity can be generated during the treatment of real textile effluents using microbial halothermotolerant bioanodes from hypersaline sediment and textile dyeing wastewater. The authors claimed to produce a reproducible bioelectric current of about 12.5 ± 0. 2 A/m^2 with a simultaneous reduction in COD with 91 ± 3% efficiency.

This research topic highlights the microbial degradation of various SOCs by different approaches, including integrated methods. The technologies reported include traditional bioremediation approaches using pure microbial cultures as well as mixed cultures, integrated biological treatment, and advanced phytoremediation approaches, as well as genomics and metabolic studies to understand the biodegradation of SOCs. This research topic also discusses the current status of the research advances in the treatment of SOCs, the limitations and challenges for effective removal of SOCs, and offers a direction for future research in achieving optimal biotechnological solutions in the treatment of SOCs.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Bacilli-Mediated Degradation of Xenobiotic Compounds and Heavy Metals

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Xenobiotic compounds are man-made compounds and widely used in dyes, drugs, pesticides, herbicides, insecticides, explosives, and other industrial chemicals. These compounds have been released into our soil and water due to anthropogenic activities and improper waste disposal practices and cause serious damage to aquatic and terrestrial ecosystems due to their toxic nature. The United States Environmental Protection Agency (USEPA) has listed several toxic substances as priority pollutants. Bacterial remediation is identified as an emerging technique to remove these substances from the environment. Many bacterial genera are actively involved in the degradation of toxic substances. Among the bacterial genera, the members of the genus *Bacillus* have a great potential to degrade or transform various toxic substances. Many *Bacilli* have been isolated and characterized by their ability to degrade or transform a wide range of compounds including both naturally occurring substances and xenobiotic compounds. This review describes the biodegradation potentials of *Bacilli* toward various toxic substances, including 4-chloro-2-nitrophenol, insecticides, pesticides, herbicides, explosives, drugs, polycyclic aromatic compounds, heavy metals, azo dyes, and aromatic acids. Besides, the advanced technologies used for bioremediation of environmental pollutants using *Bacilli* are also briefly described. This review will increase our understanding of *Bacilli*-mediated degradation of xenobiotic compounds and heavy metals.

Keywords: 4-Chloro-2-nitrophenol, naproxen, polycyclic aromatic hydrocarbons, cypermethrin, ibuprofen, *Bacillus*

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INTRODUCTION

The genus *Bacillus* belongs to the family *Bacillaceae* that comprises of 293 species/subspecies (Patel and Gupta, 2020). This genus is characterized by a group of rod-shaped, Gram-positive, aerobic, or facultatively anaerobic, endospore-forming bacteria (Patel and Gupta, 2020). Members of the genus *Bacillus* are ubiquitous; they have been isolated from a variety of sources including soil, sewage sludge (Demharter and Hensel, 1989), ocean sediments (Ruger et al., 2000), saline water (Smibert and Krieg, 1994). They have the exceptional ability to grow very rapidly in high densities as well as to tolerate adverse environmental conditions. The genus *Bacillus* includes both non-pathogenic (free-living) and pathogenic (parasitic) species. Few examples of non-pathogenic species are *Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, and *B. pumilus*, which are closely related to each other. The pathogenic strains include *B. anthracis*, which causes anthracis in human beings and *B. cereus* that causes food poisoning (Claus and Berkeley, 1986).

Bacilli constitute a versatile group of bacteria, which have many applications in the field of health, environment, and agriculture. They produce several secondary metabolites including antibiotics and biosurfactants (Caulier et al., 2019). Furthermore, they are potential sources of industrial enzymes including lipases, proteases, alpha-amylase, and the BamH1 restriction enzyme (Latorre et al., 2016). Few species of *Bacillus* including *B. thuringiensis*, and some strains of *B. sphaericus* have insecticidal properties (Palma et al., 2014). Using the genetic engineering approach, the genes encoding insecticidal proteins in *B. thuringiensis* have been incorporated into corn and cotton plants to generate insect-resistance genetically modified crops (Jouzani et al., 2017). Some *Bacillus* species are ideal candidates for biological control due to their antagonistic activities against fungal and some bacterial pathogens (Wulff et al., 2002).

Bacilli are considered as potential bioremediator agents, which are capable of degrading several toxic substances (Arora et al., 2016; Singh and Singh, 2016; Xiao et al., 2017). Earlier studies have also been reported degradation of various xenobiotic compounds and heavy metals by the members of genus *Bacillus* (Birolli et al., 2016; Upadhyay et al., 2017; Arora et al., 2018; Díez-Méndez et al., 2019). Wang et al. (2019) reported the efficient biodegradation of petroleum hydrocarbons by *B. subtilis* BL-27. Viesser et al. (2020) isolated new petroleum-degrading strains of *B. thuringiensis* and *B. subtilis* from the rhizosphere of *Panicum aquaticum*. Both of these strains were able to utilize petroleum hydrocarbons as their sole source of carbon and energy. Bonifer et al. (2019) reported that *B. pumilus* B12 degrades poly-lactic acid that is the second most common biodegradable polymer found in commercial plastics. The transformation of 4-chloro-2-nitrophenol was extensively studied in many *Bacilli* (Arora et al., 2018). The ability of *Bacilli* to degrade polycyclic aromatic compounds, drugs, dyes, explosives have also been reported in the literature (Singh and Singh, 2016; Górny et al., 2019). These data indicated that *Bacilli* play a significant role in the biodegradation of toxic substances.

So far, several reviews have been published dealing with the biotechnological application of *Bacilli* (Bunk et al., 2010; Kumar et al., 2013; Jouzani et al., 2017; Sansinenea, 2019). Kumar et al. (2013) reviewed the significance of *Bacilli* for the production of biofuels, polyhydroxyalkanoates, and bioactive molecules. Sansinenea and Ortiz (2011) described the importance of secondary metabolites produced by *Bacilli*. Bunk et al. (2010) summarized the industrial applications of *Bacillus megateriunm* and other *Bacilli*. Sansinenea (2019) discussed the plant growth-promoting activities of *Bacilli*. Even though *Bacill i*are highly involved in biodegradation of various natural and xenobiotic compounds, a review on the biodegradation potential of *Bacilli* is rare. In the last decade, several researchers have been investigated the degradation abilities of *Bacilli* toward many toxic compounds. This review aims to summarize the role of *Bacilli* in the biodegradation process of various xenobiotic compounds and heavy metals.

ROLE OF *BACILLUS* SPECIES IN BIODEGRADATION

Table 1 summarizes the role of various *Bacilli* in biodegradation of dyes, pesticides, herbicides, chlorophenols, nitrophenols, chloronitrophenols, heavy metals, drugs, explosives, crude oil waste, plastics, alkaline lignin, and other natural compounds. One of the following processes may involve in the degradation of toxic compounds by *Bacilli*: (i) Complete mineralization of toxic compounds, (ii) Co-metabolism of xenobiotics compounds. The mineralization involves complete utilization of toxic compounds by a *Bacillus* strain which utilized them as its sole source of carbon energy and converts them into CO_2 and water (Arora et al., 2018). In the co-metabolism, *Bacilli* transform chemical compounds into other compounds which generally less toxic than parent compounds. Co-metabolism-based bioremediation is a non-growth linked biological process in which bacteria convert environmental pollutants to other substances in the presence of carbon source or growth substrate (Hazen, 2010). In this process, bacteria do not depend on the pollutants for growth and use non-specific enzymes to degrade environmental pollutants that do not support their growth (Hazen, 2010). In this section, the biodegradation potential of *Bacilli* toward a variety of xenobiotic compounds and heavy metals is discussed.

Bacilli-Mediated Degradation of 4-Chloro-2-Nitrophenol

4-Chloro-2-nitrophenol is a chloro derivative of nitrophenol that is widely used for the synthesis of dyes, pesticides, drugs, and chemicals (Arora et al., 2018). Due to its wide range of applications, this compound has been detected in a variety of sources including industrial effluents. It is highly toxic to living beings and may cause methemoglobinemia in human beings. So far, several physicochemical and biological methods have been used for the 4-chloro-2-nitrophenol degradation (Bruhn et al., 1988; Beunink and Rehm, 1990; Saritha et al., 2007; Gharbani et al., 2010; Hashemi et al., 2017; Arora et al., 2018). In this sub-section, the role of *Bacillus* species in the 4-chloro-2-nitrophenol degradation is summarized.

Many *Bacilli* have been characterized for their ability to decolorize the yellow color of 4-chloro-2-nitrophenol in the presence of additional carbon source (Arora et al., 2018). A marine bacterium, *Bacillus* sp. MW-1 (Arora and Jain, 2012), and a soil bacterium, *Bacillus subtilis* RKJ 700 (Arora, 2012) decolorized and transformed 4-chloro-4-nitrophenol into 5-chloro-2-methylbenzoxazole via detoxification mechanism. In this mechanism, 4-chloro-2-nitrophenol initially reduced to 4-chloro-2-aminophenol, which is further acetylated to 4-chloro-2-acetaminophenol. The next step involves the conversion of 4-chloro-2-acetaminophenol to 5-chloro-2-methylbenzoxazole (**Figure 1**). Recently, ten bacterial strains belonging to *Bacillus* isolated from a wastewater sample showed decolorization of 4-chloro-2-nitrophenol in the presence of glucose. One of a bacterium, identified as *Bacillus aryabhattai* strain PC-7

TABLE 1 | A list of members of the genus *Bacillus*, which have biodegradation potential.

S. No.	Bacteria strain	Compounds	Remarks	References
A. Decolorization and transformation of Dyes				
1.	*Bacillus aryabhattai* LN01	Toluidine Blue	Decolorized Toluidine Blue in Lacaase-like and azoreductase-like reactions	Díez-Méndez et al., 2019
2	*Bacillus aryabhattai* LN08	Toluidine Blue, Remazol, and Brilliant Blue	Decolorized Toluidine Blue in Lacaase-like reaction and Remazol Brilliant Blue in Peroxidase-like reaction	Díez-Méndez et al., 2019
3.	*Bacillus aryabhattai* LN09	Congo Red, Toluidine Blue, and Remazol Brilliant Blue	Decolorized Congo Red in Laccase-like reaction; Toluidine Blue in Azoreductase-like reaction and Remazol Brilliant Blue in Peroxidase like reaction	Díez-Méndez et al., 2019
4.	*Bacillus aryabhattai* LN10	Toluidine Blue	Decolorized Toluidine Blue in Laccase-like and Azoreductase-like reactions	Díez-Méndez et al., 2019
5.	*Bacillus aryabhattai* LN15	Congo Red, and Toluidine Blue	Decolorized Congo Red and Toluidine Blue in Laccase-like and Azoreductase-like reactions	Díez-Méndez et al., 2019
6.	*Bacillus aryabhattai* LN16	Congo Red	Decolorized Congo Red by Peroxidase-like reaction	Díez-Méndez et al., 2019
7.	*Bacillus megaterium* LN30	Congo Red	Decolorized Congo red by Azoreductase-like reaction	Díez-Méndez et al., 2019
8.	*Bacillus aryabhattai* LN37	Congo Red, Toluidine Blue, and Remazol Brilliant Blue	Decolorized Congo red in Laccase like reaction; Remazol Brilliant Blue in Azoreductase-like reaction and Toluidine Blue in Laccase-like and Azoreducatase like reactions	Díez-Méndez et al., 2019
9.	*Bacillus aryabhattai* LN39	Remazol Brilliant Blue, and Toluidine Blue	Decolorized Remazol Brilliant Blue and Toluidine Blue in Azoreductase-like reaction.	Díez-Méndez et al., 2019
10.	*Bacillus aryabhattai* LN41	Remazol Brilliant Blue and Toluidine Blue	Decolorized Remazol Brilliant Blue and Toluidine Blue in Azoreductase like reduction	Díez-Méndez et al., 2019
11.	*Bacillus aryabhattai* LN49	Remazol Brilliant	Decolorized Remazol Brilliant Blue in Laccase-like reaction	Díez-Méndez et al., 2019
12.	*Bacillus aryabhattai* LN61	Toluidine Blue	Decolorized Toluidine Blue in Azoreductase-like reaction	Díez-Méndez et al., 2019
13.	*Bacillus aryabhattai* LN84	Congo Red, and Remazol Brilliant Blue	Decolorized Congo Red in Laccase-like reaction and Remazol Brilliant Blue in Azoreductase and Laccase-like reactions	Díez-Méndez et al., 2019
14.	*Bacillus aryabhattai* LN87	Toluidine Blue	Decolorized Toluidine Blue in Azoreductase and Laccase-like reactions	Díez-Méndez et al., 2019
15.	*Bacillus aryabhattai* LN88	Toluidine Blue and Remazol Brilliant Blue	Decolorized Toluidine Blue in Peroxidase-like and Azoreducase-like reactions and Remazol Brilliant Blue in Azoreucatase-like reactions	Díez-Méndez et al., 2019
16.	*Bacillus aryabhattai* LN90	Congo Red, Toluidine Blue, and Remazol Brilliant Blue	Decolorized Congo red in Laccase-like reaction; Toluidine Blue in Laccase-like and Azoredutase-like reactions and Remazol Brilliant Blue in Azoreductase-like reaction.	Díez-Méndez et al., 2019
17.	*Bacillus* sp. VUS	Brown 3REL	Transformed into 6,8-dichloro-quinazoline-4-ol and cyclopentanone.	Dawkar et al., 2008
18.	*Bacillus* sp. OY1-2	Red B dye	Rapid biodegradation of Red B dye was observed in anoxic conditions as compared to aerobic conditions.	Li et al., 2004
19.	*Bacillus firmus*	Reactive Blue 160	Decolorized dye (500 mg/l) and detoxify it.	Barathi et al., 2020
20.	*Bacillus megaterium* KY848339.1	Acid red 337 dye	Degraded it via small aliphatic compounds and CO2	Ewida et al., 2019
21.	*Bacillus* sp. BDN2	Reactive Blue 160	Degraded 65% dye	Balapurea et al., 2014
22.	*Bacillus* sp. BDN7	Reactive Blue 160	Degraded 80% within 12 h	Balapurea et al., 2014
23.	*Bacillus* sp. BDN8	Reactive Blue 160	Degraded 75% within 18 h	Balapurea et al., 2014
24.	*Bacillus* megaterium NCIM 2054	Disperse Red 73 dye	61% dye decolorization within 48 h	Kadam et al., 2014
25.	*Bacillus cereus* HJ-1	Reactive Black B	Decolorized dye and detoxify it.	Liao et al., 2013
26.	*Bacillus* sp. YZU1	Reactive Black 5	95% dye decolorization was observed in 120 h	Wang et al., 2013
27.	*Bacillus* sp. AK1	Metanil Yellow	Degraded 200 mg/l dye within 27 h	Anjaneya et al., 2011
28.	*Bacillus* odyssey SUK3	Reactive blue 59	Decolorized dye (50 mg/l) completely within 60 h	Patil et al., 2008

(Continued)

TABLE 1 | Continued

S. No.	Bacteria strain	Compounds	Remarks	References
29.	*Bacillus* cereus DC11q	Malachite green	Degraded to 4,4′-bis(dimethylamino)benzophenone and benzophenone	Deng et al., 2008
30.	*Bacillus cereus*DC11q	Acid Blue 25	95–98% dye (100 μM) decolorization within 6 h under anaerobic conditions	Deng et al., 2008
31.	*Bacillus cereus* DC11q	Basic Blue X-GRRL	Degraded via reduction of azo bonds.	Deng et al., 2008
32.	*Bacillus fusiformis* KMK5	Disperse Blue 79, and Acid Orange 10	Complete mineralization of dyes at the concentration of 1.5 g/L was observed within 48 h	Kolekar et al., 2008
33.	*Bacillus subtilis* IFO 13719	Crystal Violet	Decolorized via 4,4'-bis(dimethylamino)benzophenone	Yatome et al., 1991
34.	*Bacillus megaterium*	Azo dye	Decolorized 98% dye	Shah et al., 2013
35.	*Bacillus cereus*	Azo dye	Decolorized 95% dye	Shah et al., 2013
36.	*Bacillus pseudomycoides*	Acid Black 24	96% of dye decolorization was achieved within 24 h.	Kumar et al., 2019
37.	*Bacillus subtilis*	Disperse yellow 211	80% dye (100 mg/) decolorization observed under optimum conditions.	Sharma et al., 2009
38.	*Bacillus subtilis*	Crystal violet	Decolorized dye (100 mg/l) effectively at pH 8 and temperature 35° C	Kochher and Kumar, 2011
39.	*Bacillus cohnii* MTCC 3616	Direct Red-22	95% dye decolorization (5,000 mg l^{-1}) was observed at 37° C and pH 9 in 4 h	Prasad and Rao, 2013
40.	*Bacillus firmus*	CI Direct Red 80	Decolorized 50 mg/L of dye under anoxic conditions within 12 h	Ogugbue et al., 2012
41.	*Bacillus licheniformis*	Reactive Red 2	Transformed it into 2, 4-dichloro-6-[(1H-indazol-5-ylimino)-methyl]-phenol, benzene sulfonamide, 1H indole and urea as final metabolites	Sudha and Balagurunathan, 2013
42.	*Bacillus megaterium*	Remazol Blue	Decolorized up to 5 mg/ml	Joshi et al., 2013
43.	*Bacillus subtilis*	RED M5B	Decolorization by the activity of peroxidase	Gunasekar et al., 2013
44.	*Bacillus* sp. VUS	Orange T4LL	transforms it into 4-methyl-2-o-tolylazo-benzene-1,3-diamine and [3-(phenyl-hydrazono)-cyclohexa-1,4-dienyl]-methanol	Dawkar et al., 2010
45.	*Bacillus flexus*	Remazol Black	Decolorized 100% of dye within 24 h	Saini et al., 2018
46.	*Bacillus flexus*	Direct Blue	Decolorized 100% of dye within 24 h	Saini et al., 2018
47.	*Bacillus flexus*	Acid Orange	Decolorized 100% of dye within 24 h	Saini et al., 2018
B. Biodegradation of Pesticides, Herbicides, and Insecticides				
48.	*Bacillus subtilis* strain 1D	Cypermethrin	Completely metabolized via cyclododecylamine, phenol, 3-(2,2-dichloroethenyl 2,2-dimethyl cyclopropane carboxylate,1-decanol, chloroacetic acid, acetic acid, cyclopentan palmitoleic acid, and decanoic acid	Gangola et al., 2018
49.	*Bacillus* sp. strain SG2	Cypermethrin	Metabolized via Phenoxybenzaldehyde,2,2,3,3 tetramethylcyclopropanecarboxylic acid 4-propylbenzoate, 4-propylbenzaldehyde, phenol M-tert-butyl, and 1-dodecanol,	Pankaj et al., 2016
50.	*Bacillus subtilis* BSF01	Cypermethrin	Metabolized via Phenoxybenzaldehyde, 2,2,3,3 tetramethylcyclopropanecarboxylic acid	Xiao et al., 2015
51.	*Bacillus* sp. AKD1	Cypermethrin	Transformed Cypermethrin in presence of heavy metals	Tiwary and Dubey, 2016
52.	*Bacillus* sp. ISTDS2	Cypermethrin	Metabolized Cypermethrin in soil microcosm via formation of cyclopropane, carboxylic acid, hydroxyacetonitrile, and benzene ethanamine	Sundaram et al., 2013
53.	*Bacillus licheniformis* B-1	Cypermethrin	Degraded via 3-phenoxybenzoic acid	Lai et al., 2012
54.	*Bacillus thuringiensis* ZS-19	Cyhalothrin	Degraded via α-hydroxy-3-phenoxy-benzeneacetonitrile, 3-phenoxyphenyl acetonitrile, N-(2-isoproxy-phenyl)-4-phenoxy-benzamide, 3-phenoxybenzaldehyde, 3-phenoxybenzoate, and phenol	Chen et al., 2015
55.	*Bacillus cereus* PU	Malathion	Degraded via malathion monocarboxylic and dicarboxylic acid	Singh et al., 2012

(Continued)

TABLE 1 | Continued

S. No.	Bacteria strain	Compounds	Remarks	References
56.	*Bacillus thuringiensis* MOS-5	Malathion	Degraded via malathion monocarboxylic and dicarboxylic acid	Zeinat et al., 2008
57.	*Bacillus megaterium* MCM B-423	Monocrotophos	Degraded into carbon dioxide, ammonia, and phosphates	Bhadbhade et al., 2002
58.	*Bacillus* sp. N1	Metribuzin	Used as a nitrogen source.	Zhang et al., 2014
59.	*Bacillus alkalinitrilicus*	Imidacloprid	Degraded via 6-chloronictinic acid nitrosamine	Sharma et al., 2014
60.	*Bacillus subtilis* Y242	Chlorpyrifos	96% degraded within 48 h	El-Helow et al., 2013
61.	*Bacillus pumilus* NY97-I	Carbendazim	87.76% degradation	Zhang et al., 2009
62.	*Bacillus cereus* WD-2	Prochloraz-manganese	90.7% degradation at pH 8.	Jiang et al., 2019
63.	*Bacillus* sp. DG-02	Fenpropathrin	Transformed into 3,4-dihydroxybenzoic acid, 3,4-dimethoxyphenol, and phenol	Chen et al., 2014
64.	*Bacillus aryabhattai* strain VITNNDJ5	Monocrotophos	Degraded via three routes.	Dash and Osborne, 2020
65.	*Bacillus firmus*	Fipronil	Degraded via fipronil sulfide, fipronil sulfone and fipronil amide.	Mandal et al., 2013
66.	*Bacillus* sp. TAP-1	Triazophos	Co-metabolized via hydrolyzing insecticide triazophos	Tang and You, 2012
67.	*Bacillus pumilus* W1	Organophosphates	Hydrolysis of organophosphates by enzyme encoding by *opdA*	Ali et al., 2012
68.	*Bacillus subtilis* DR-39	Profenofos	4-Bromo-2-chlorophenol was identified as a metabolite	Salunkhe et al., 2013
69.	*Bacillus subtilis* CS-126,	Profenofos	4-Bromo-2-chlorophenol was identified as a metabolite	Salunkhe et al., 2013
70.	*Bacillus subtilis* TL-171	Profenofos	4-Bromo-2-chlorophenol was identified as a metabolite	Salunkhe et al., 2013
71.	*Bacillus subtilis* TS-204	Profenofos	4-Bromo-2-chlorophenol was identified as a metabolite	Salunkhe et al., 2013
72.	*Bacillus* sp. strain C5	Methyl Parathion	Hydrolyzedmethyl parathion to 4-nitrophenol and other metabolites	Hao et al., 2014
73.	*Bacillus pumilus* C2A1	Chlorpyrifos	Degraded via 3,5,6-trichloro-2-pyridinol	Anwar et al., 2009
74.	*Bacillus subtilis* MTCC 8561	Endosulfan and Endosulfan sulfate	Used as sulfur source and transformed to endosulfan diol and endosulfan lactone	Kumar et al., 2014
75.	*Bacillus subtilis* HB-6	Atrazine	Mineralized via hydroxyatrazine, cyanuric acid, and urea	Wang et al., 2014
76.	*Bacillus badius* ABP6	Atrazine	Optimum conditions of the atrazine degradation were determined	Khatoon and Rai, 2020
77.	*Bacillus megaterium* strain Q3	Quinclorac	Transformed to 3, 7-dichloro-8-methyl-quinoline, 3-chlorin-8-quinoline-carboxylic and 8-quinoline-carboxylic	Liu et al., 2014
78.	*Bacillus licheniformis* CY-012	Fenvalerate	Co-metabolized via α-isopropyl-4-chlorobenzene acetic acid, 4-chlorobenzene acetic acid, 3-phenoxybenzyl alcohol, phenol, and benzoic acid.	Tang et al., 2018
79.	*Bacillus* sp. 4T	Esfenvalerate	Transformed to (i) 3-2-(4-chlorophenyl)-3-methylbutyric acid), (ii) phenoxybenzoic acid, (iii) 2-(3-hydroxyphenyl)acetic acid	Birolli et al., 2016
80.	*Bacillus* sp. 2B	Esfenvalerate	Transformed to (i) 3-2-(4-chlorophenyl)-3-methylbutyric acid), (ii) phenoxybenzoic acid, hydroxy phenoxybenzoic acid and 3-hydroxybenzoic acid(iii) 2-(3-hydroxyphenyl)acetic acid	Birolli et al., 2016
81.	*Bacillus* sp. P5CBNB	Esfenvalerate	Transformed to (i) 3-2-(4-chlorophenyl)-3-methylbutyric acid), (ii) phenoxybenzoic acid, hydroxy phenoxybenzoic acid	Birolli et al., 2016
82.	*Bacillus* sp. CBMAI 1833	Esfenvalerate	Transformed to (i) 3-2-(4-chlorophenyl)-3-methylbutyric acid), (ii) phenoxybenzoic acid, hydroxy phenoxybenzoic acid (iii) 2-(3-hydroxyphenyl)acetic acid	Birolli et al., 2016

(Continued)

TABLE 1 | Continued

S. No.	Bacteria strain	Compounds	Remarks	References
83.	*Bacillus* sp. DG-2	3-phenoxybenzoic acid	Degraded via 3-(2-methoxyphenoxy) benzoic acid, protocatechuate, phenol, and 3,4-dihydroxy phenol.	Chen et al., 2012a
C. Biodegradation and Biotransformation of Chlorophenol, Nitrophenol, and Chloronitrophenol				
84.	*Bacillus licheniformis* strain SL10	2,4-Dichlorophenol	Degradation occurred via meta cleavage pathway of catechol or chlorocatechol	Chris Felshia et al., 2020
85.	*Bacillus* sp. MW-1	4-chloro-2-nitrophenol	Transformed to 5-chloro-2-methyl benzoxazole	Arora and Jain, 2012
86.	*Bacillus* subtilis RKJ 700	4-chloro-2-nitrophenol	Transformed to 5-chloro-2-methyl benzoxazole	Arora, 2012
87.	*Bacillus* cereus PC-1	4-chloro-2-nitrophenol	Decolorized up to concentration of 1.0 mM	Arora et al., 2016
88.	*Bacillus toyonensis* PC-2	4-chloro-2-nitrophenol	Decolorized up to a concentration of 0.9 mM	Arora et al., 2016
89.	*Bacillus thuringiensis* PC-3	4-chloro-2-nitrophenol	Decolorized up to a concentration of 1.0 mM	Arora et al., 2016
90.	*Bacillus firmus* PC-4	4-chloro-2-nitrophenol	Decolorized up to a concentration of 0.8 mM	Arora et al., 2016
91.	*Bacillus koreensis* PC-5	4-chloro-2-nitrophenol	Decolorized up to concentration of 0.6 mM	Arora et al., 2016
92.	*Bacillus megaterium* PC-6	4-chloro-2-nitrophenol	Decolorized up to a concentration of 1.5 mM	Arora et al., 2016
93.	*Bacillus aryabhattai* PC-7	4-chloro-2-nitrophenol	Decolorized up to concentration of 2.0 mM	Arora et al., 2016
94.	*Bacillus aerophilus* PC-8	4-chloro-2-nitrophenol	Decolorized up to concentration of 0.6 mM	Arora et al., 2016
95.	*Bacillus siamensis* PC-9	4-chloro-2-nitrophenol	Decolorized up to concentration of 0.8 mM	Arora et al., 2016
96.	*Bacillus amyloliquefaciens* PC-10	4-chloro-2-nitrophenol	Decolorized up to concentration of 0.9 mM	Arora et al., 2016
97.	*Bacillus subtilis* MF447840	4-chlorophenol	Degraded 4-chlorophenol up to of 1,000 mg/L	Sandhibigraha et al., 2020
98.	*Bacillus cereus* PU	Trinitrophenol	Used trinitrophenol as nitrogen source and degraded via Hydride-Meisenheimer complex.	Singh et al., 2011
D. Biodegradation of Polyaromatic hydrocarbons				
99.	*Bacillus subtilis* 3KP	Naphthalene and Phenanthrene	Metabolized via hydroxy-2-naphthoic acid, salicylic acid, and pyrocatechol	Ni'matuzahroh et al., 2017
100.	*Bacillus fusiformis*	Naphthalene	Degraded via o-phthalic acid and benzoic acid,	Lin et al., 2010
101.	*Bacillus cereus* RKS4	Naphthalene	Catechol and 2-naphthol were identified as major metabolites of naphthalene degradation.	Sonwani et al., 2019
102.	*Bacillus* sp. SBER3	Anthracene and Naphthalene	Degraded 83.4% of anthracene and 75.1% of and naphthalene in 6 days.	Bisht et al., 2014
103.	*Bacillus subtilis* DM-04	Pyrene	Used it as its carbon source and energy	Das and Mukherjee, 2007
104.	*Bacillus subtilis* BM-1	Fluorene	Degrade 86% of 50 mg/L fluorine with 21 days	Salam and Obayori, 2014
105.	*Bacillus amyloliquefaciens* BR1	Fluorene	Degrade 82% of 50 mg/L fluorine with 21 days	Salam and Obayori, 2014
106.	*Bacillus subtilis* BTM4i	Benza-pyrene	Utilized as a sole source of carbon and energy and degradation ability was chromosomally coded.	Lily et al., 2010
107.	*Bacillus pumilus* (MTCC 1002)	Pyrene	Co-metabolize 64% of 50 μg/ml pyrene via 9-methoxyphenanthrene and phthalate	Khanna et al., 2011
E. Biotransformation and detoxification of heavy metals				
108.	*Bacillus* sp. strain FM1	Chromium	Completely reduced 100 mg/L Cr(VI) within 48 h	Masood and Malik, 2011

(Continued)

TABLE 1 | Continued

S. No.	Bacteria strain	Compounds	Remarks	References
109.	*Bacillus* sp. strain KSUCr9a	Chromium	rapidly reduce up to 100 μM of Chromium within 24 h	Ibrahim et al., 2012
110.	*Bacillus sphaericus* AND 303	Chromium	300 μM Cr(VI) reduction by cell extracts (4.56 mg protein/mL) of strain AND303	Pal et al., 2005
111.	*Bacillus* sp. FY1	Chromium	Reduced 78–85% of Cr(VI) (100–200 mg/l) within 24 h	Xiao et al., 2017
112.	*Bacillus* sp. MNU16	Chromium	Reduced 75% of Cr(VI) of 50 mg/L within 72 h.	Upadhyay et al., 2017
113.	*Bacillus amyloliquefaciens* CSB 9	Chromium	Reduced Cr(VI) to Cr (III) that was immobilized to the bacterial cell surface and subsequent intercellular accumulation of Cr (III) along with the formation of coagulated cell precipitate	Das et al., 2014
114.	*Bacillus cereus* S612	Chromium	Reduced chromate under aerobic conditions	Wang et al., 2015
115.	*Bacillus cereus*	Chromium	Reduced Cr(VI) to Cr (III). Cr(III) precipitates were accumulated on bacterial surfaces	Chen et al., 2012c
116.	*Bacillus* sp. ES 29	Chromium	A copper (Cu2+) stimulated soluble Cr(VI)-reducing enzyme reduced Cr(VI) to Cr (III)	Camargo et al., 2003
117.	*Bacillus* sp. MH778713	Chromium	Accumulated up to 100 mg Cr(VI)/g of cells and tolerate up to 15,000 mg/L Cr (VI)	Ramírez et al., 2019
118.	*Bacillus cereus* TN10	Chromium	Detected chromate transporters in the genome	Hossain et al., 2020
119.	*Bacillus cereus* 12-2	Lead	Transformed Pb(II) into nanosized rod-shaped Ca2.5Pb7.5(OH)2(PO4)6 crystal	Chen et al., 2016
120.	*Bacillus* sp. KK-1	Lead	Converted Pb(NO3)2 into lead sulfide (PbS) and lead silicon oxide (PbSiO3)	Govarthanan et al., 2013
121.	*Bacillus* cereus BPS-9	Lead	Bioaccumulation of lead by biosorption	Sharma and Shukla, 2021
122.	*Bacillus megaterium*	Selenium	Reduced Se(IV) to red element Se (III)	Mishra et al., 2011
123.	*Bacillus subtilis*	Selenium	Proposed physiological mechanisms regulating the selenite reduction	Garbisu et al., 1995
124.	*Bacillus selenatarsenatis* SF-1	Selenium	Reduced selenate to selenite through anaerobic respiration, and subsequently into elemental selenium	Kuroda et al., 2015
125.	*Bacillus selenitireducens* MLS10	Selenium	Enzyme respiratory selenite [Se(IV)] reductase (Srr) was characterized.	Wells et al., 2019
126.	*Bacillus cereus* CM100B	Selenium	Produced selenium nanoparticles by transformation of toxic selenite (SeO32-) anions into red elemental selenium (Se0) under aerobic conditions	Dhanjal and Cameotra, 2010
127.	*Bacillus mycoides* strain SelTE01	Selenium	Reduced selenite (SeO32-) anions into red elemental selenium (Se0) with the formation of selenium nanoparticles.	Lampis et al., 2014
128.	*Bacillus thuringiensis*	Uranium	Transformation from U(VI) into nano-uramphite	Pan et al., 2015
129.	*Bacillus licheniformis* SPB-2	Copper	Reduced [Co(III)–EDTA]– to [Co(II)–EDTA]2– which was further absorbed by strain SPG-2	Paraneeiswaran et al., 2015
130.	*Bacillus firmus* strain TE7	Chromium and Arsenic	Reduced Cr(VI) to Cr (III) and oxidized As(III) to As(V)	Bachate et al., 2013
131.	*Bacillus* sp. strain Arzi	Mb	Reduced molybdate to molybdenum blue	Othman et al., 2013
132.	*Bacillus thuringiensis* OSM29	Ni and Cu	Biosorption capacity of the strain OSM29 for the metallic ions was highest for Ni (94%) which was followed by Cu (91.8%).	Oves et al., 2013
F. Degradation of Natural Compounds				
133.	*Bacillus macerans* JJ-lb	Protocatechuate	Completely mineralized	Crawford et al., 1979
134.	*Bacillus* sp.	3-Hydroxybenzoate	Completely mineralized via protocatechuate	Mashetty et al., 1996

(Continued)

TABLE 1 | Continued

S. No.	Bacteria strain	Compounds	Remarks	References
135.	*Bacillus brevis* PHB-2	4-Hydroxybenzoate	Completely mineralized via protocatechuate	Crawford, 1976
136.	*Bacillus circulans* strain 3	4-Hydroxybenzoate	Completely mineralized via protocatechuate	Crawford, 1976
137.	*Bacillus laterosporus*PHB-7a	4-Hydroxybenzoate	Completely mineralized via gentisate	Crawford, 1976
138.	*Bacillus* sp. B-1	Cinnamic acid	Degraded via benzoic acid	Peng et al., 2003
139.	*Bacillus* sp. B-1	4-Coumaric acid	Degraded via 4-hydroxybenzoic acid	Peng et al., 2003
140	*Bacillus* sp. B-1	Ferculic acid	Metabolized via 4-hydroxy-3-methoxyphenyl-beta-hydroxypropionic acid, vanillin, and vanillic	Peng et al., 2003
141.	*Bacillus pumilus* W1	Cholesterol degradation	Cholesterol as only carbon and energy	Wali et al., 2019
142.	*Bacillus ligniniphilus* L1	Alkaline lignin	Degraded via three different pathway including gentisate pathway, benzoic acid pathway, and the β-ketoadipate pathway	Zhu et al., 2017
G. Degradation of Explosives				
143.	*Bacillus* sp. J8A2	Pentaerythritol tetranitrate	Utilized it as a nitrogen source	Yerson and Christian, 2013
144.	*Bacillus* sp. SF	Trinitrotoluene	Transformed to hydroxylaminodinitrotoluene, 4-amino-2,6-dinitrotoluene; 2-amino-4,6-dinitrotoluene, different azoxy compounds, 2,6-diaminonitrotoluene and 2,4-diaminonitrotoluene.	Nyanhongo et al., 2008
145.	*Bacillus cereus*	Trinitrotoluene	Transformed into 2,4-dinitrotoluene and 4-aminodinitrotoluene derivates,	Mercimek et al., 2013
146.	*Bacillus* sp. ATCC51912	Propylene glycol dinitrate	Sequentially denitrated to propylene glycol mononitrate and propylene glycol	Sun et al., 1996
147.	*Bacillus* sp. ATCC51912	Glycerol trinitrates	Sequential denitration of glycerol trinitrates to glycerol via glycerol dinitrate isomers and glycerol mononitrate isomers	Meng et al., 1995
H. Degradation of Drugs				
148.	*Bacillus thuringiensis* B1	Naproxen	Degraded via salicylic acid and catechol	Górny et al., 2019
149.	*Bacillus thuringiensis* B1	Ibuprofen	Degraded it via hydroxyibuprofen	Marchlewicz et al., 2017
150.	*Bacillus drentensis* S1	Acetaminophen	Degraded via 2-isopropyl-5-methylcyclohexanone and phenothiazine	Chopra and Kumar, 2020
I. Degradation of other Xenobiotic compounds				
151.	*Bacillus salamalaya* 139SI	Crude oil waste	Degraded 88% of the total petroleum hydrocarbons within 42 days in mineral media containing 1% of crude oil waste.	Ismail and Dadrasnia, 2015
152.	*Bacillus* sp. BCBT21	Plastic bags	Produced extracellular hydrolase enzymes including lipase, carboxymethyl cellulase, xylanase, chitinase, and protease	Dang et al., 2018
153.	*Bacillus pumilus*B12	Poly-lactic acid	Degraded plylacitc acid film within 48-h by the release of L-lactate monomers	Bonifer et al., 2019
154.	*Bacillus* sp. strain 4	Pyridine	Used it sole C, N and energy source	Watson and Cain, 1975

decolorized 4-chloro-2-nitrophenol up to a concentration of 2.0 mM and transformed it into 5-chloro-2-methylbenzoxazole (Arora et al., 2016).

Besides *Bacillus* spp., several other bacteria are also capable of transforming 4-chloro-2-nitrophenol to 5-chloro-2-methylbenzoxazole. These bacteria belong to the genera *Pseudomonas, Leuconostoc*, and *Paenibacillus* (Arora et al., 2016). The memberes of genus *Bacillus* were unable to completely mineralize 4-chloro-2-nitrophenol, but they transformed 4-chloro-2-nitrophenol via a detoxification mechanism. The complete degradation of 4-chloro-2-nitrophenol was studied using an *Exiguobacterium* sp. PMA (Arora et al., 2012), a co-culture of *Enterobacter* cloacae and an *Alcaligenes* sp. TK-2 (Beunink and Rehm, 1990), and the genetically engineered bacterium, *Pseudomonas* sp. N31 (Bruhn et al., 1988).

Bacilli-Mediated Degradation of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are those aromatic compounds which contain two or more fused aromatic rings in linear, angular, or cluster arrangements (Masih and

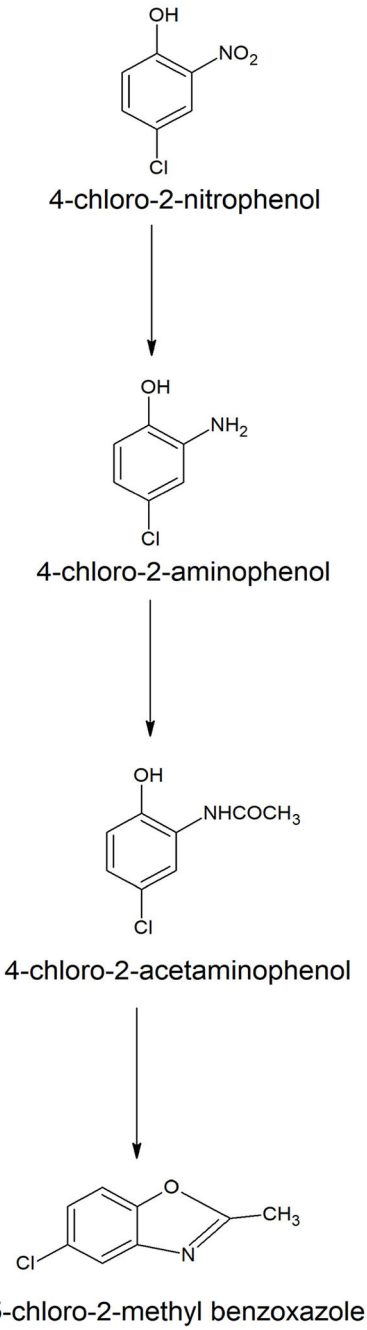

FIGURE 1 | Biotransformation pathway of 4-chloro-2-nitrophenol in *Bacillus* spp. (Arora, 2012).

Taneja, 2006). Examples are naphthalene, anthracene, fluorene, phenanthrene, fluoranthene, pyrene, and benzo[a]pyrene (Abdel-Shafy and Mansour, 2016). PAHs are toxic to the living world and some of them are considered as possible carcinogens. Therefore, the USEPA has listed 16 PAHS in its priority list of pollutants (Zelinkova and Wenzl, 2015). Major sources of PAHs pollution include fuel combustion, automobiles, spillage of petroleum products, waste incinerators, and industrial effluents (Abdel-Shafy and Mansour, 2016). In this section, *the Bacilli*-mediated degradation of a few PAHs is summarized.

Naphthalene is the simplest example of polycyclic aromatic compounds. An early study on naphthalene degradation by *B. cereus* ATCC14579 showed the complete transformation of naphthalene to 1-naphthol (Cerniglia et al., 1984). A possible degradation pathway of naphthalene was studied in *B. fusiformis* BFN that was isolated from oil refining wastewater sludge (Lin et al., 2010). The naphthalene degradation was initiated with 1, 2-dioxygenation, resulting in the formation of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene that dehydrogenated to 1,2-dihydroxynaphthalene. The *ortho*-ring cleavage of 1,2-dihydroxynaphthalene produced *o*-phthalic acid via the formation of trans-2-carboxybenzalpyruvic acid and 2-formyl benzoic acid (**Figure 2**). The phthalic acid decarboxylated to benzoic acid that further metabolized carbon dioxide and water. Ni'matuzahroh et al. (2017) studied the degradation of naphthalene and phenanthrene by *B. subtilis* 3KP that degraded them via the formation of 1-hydroxy-2-naphthoic acid, salicylic acid, and pyrocatechol. Sonwani et al. (2019) reported that *B. cereus* RKS4 degraded naphthalene via the formation of 2-naphthol and catechol. Annweiler et al. (2000) studied the degradation of naphthalene in *B. thermoleovorans* Hamburg 2 under thermophilic conditions (60° C). *B. thermoleovorans* Hamburg 2 utilized naphthalene as the sole source of carbon and energy and degraded it via formation of 1-naphthol, 2-naphthol, 2,3-dihydroxynaphthalene, 2-carboxycinnamic acid, phthalic acid, and benzoic acid, coumarin, 3-(2-Hydroxyphenyl)-propanoic acid, 2,3-dihydrocoumarin, 2-hydroxybenzoic acid (salicylic acid) and 2-carboxycinnamic acid.

Anthracene is an integral part of many carcinogenic PAHs; therefore it has been detected easily in several contaminated sites of PAHs. Many *Bacilli* have been identified and characterized for degradation of anthracene. Examples are *Bacillus* sp. SBER3 (Bisht et al., 2014), *B. cereus* JMG-01 (Das et al., 2017), *B. licheniformis* MTCC 5514 (Swaathy et al., 2014), *B. cereus* S13 (Bibi et al., 2018), and *B. badius* D1 (Sarwade and Gawai, 2014). Das et al. (2017) studied the degradation pathway of anthracene for *B. cereus* JMG-01 that degraded 98% of 500 ppm anthracene. The anthracene degradation was initiated with the formation of naphthalene and naphthalene-2-methyl. In the next step, a dioxygenase enzyme catalyzed oxidation of naphthalene-2-methyl to benzene acetic acid. Further, benzene acetic acid underwent ring cleavage to produce phthalic acid and benzaldehyde. Benzaldehyde converted to catechol that degraded via either *ortho* or *meta* ring cleavage. Swaathy et al. (2014) reported the existence of two degradation pathways in biosurfactant mediated biodegradation of anthracene by *B. licheniformis* (MTCC 5514). One pathway proceeded with the formation of naphthalene, naphthalene 2-methyl, phthalic acid, and benzene acetic acid. Another pathway was initiated with dioxygenation of anthracene to produce di-hydroxy anthracene, which, further transformed to anthraquinone by a dioxygenase enzyme (**Figure 3**). Anthraquinone was further degraded with the formation of phthalic acid, benzaldehyde or benzoic acid,

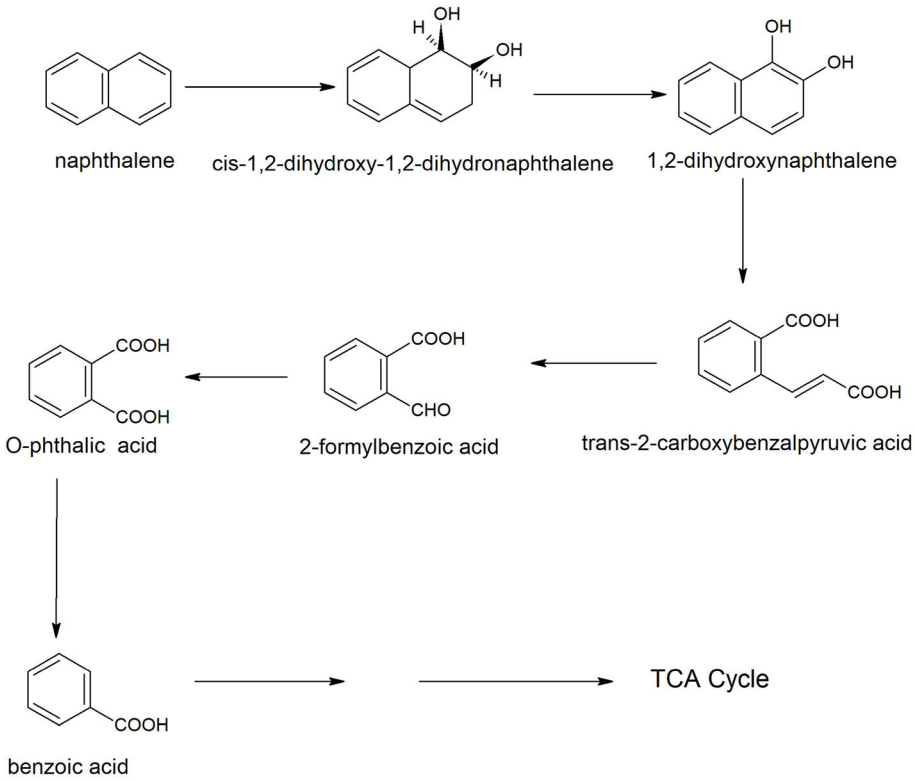

FIGURE 2 | A degradation pathway of naphthalene in *Bacillus fusiformis* strain BFN (Lin et al., 2010).

and catechol. Metabolites of both of the pathways (9, 10-dihydroxyanthracene, anthraquinone, benzene acetic acid, and catechol) were also reported in the anthracene degradation pathway of *B. cereus* S13 that utilized it as the sole source of carbon and energy (Bibi et al., 2018). Another pathway of anthracene was studied in an alkaliphilic bacterium *B. badius* D1 that was able to degrade anthracene at a concentration of 50 mg/100 ml at pH 9.0 (Sarwade and Gawai, 2014). In this pathway, anthracene was initially oxidized to 1, 2-dihydoxyanthracene that further oxidized (3Z)-4-[3-hydroxy (2-naphthyil)-2-oxobut-3-enoic acid with subsequent conversion to 2-hydroxynaphthoic acid, Further oxidation resulted in the formation of phthalic acid that was degraded via formation of simple aliphatic compounds.

Bacilli-Mediated Degradation of Pyrethroid Insecticides

Pyrethroid insecticides are synthetic pyrethroids which are analogs to natural pyrethrins extracted from Chrysanthemum cinerariaefolium (Cycoń and Piotrowska-Seget, 2016). Representative compounds of these pesticides are cyhalothrin, fenpropathrin, deltamethrin, cypermethrin, cyfluthrin, and bifenthrin (Zhan et al., 2020). They are used to control a broad spectrum of pests in households and agriculture fields. Due to their wide range of applications in agriculture fields, they have been spread into soil and water and create environmental problems because of their toxic nature (Zhan et al., 2020). Many *Bacilli* have been isolated and characterized for the degradation of several pyrethroids (Chen et al., 2012b; Cycoń and Piotrowska-Seget, 2016; Bhatt et al., 2020). In this section, *Bacilli*-mediated degradation of various pyrethroids is discussed.

The degradation of cypermethrin is well-studied in some *Bacilli* including *Bacillus* sp. SG2 (Pankaj et al., 2016), *B. subtilis* BSF01 (Xiao et al., 2015), *B. subtilis* strain 1D (Gangola et al., 2018), *Bacillus* sp. AKD1 (Tiwary and Dubey, 2016), *Bacillus* sp. ISTDS2 (Sundaram et al., 2013) and *B. licheniformis* B-1 (Lai et al., 2012). The initial steps of degradation pathways of cypermethrin are common in *Bacillus* sp. SG2 and *B. subtilis* BSF01 (Xiao et al., 2015; Pankaj et al., 2016). Cypermethrin was initially transformed into two metabolites: α-hydroxy-3-phenoxy-benzene acetonitrile and 3-(2,2-dichloroethenyl)-2,2-dimethyl cyclopropanecarboxylate). The unstable compound, α-hydroxy-3-phenoxy-benzene acetonitrile was spontaneously transformed into 3-phenoxybenzaldehyde (**Figure 4**). Further degradation of 3-phenoxybenzaldehyde proceeded via a different route in *Bacillus* sp. SG2 and *B. subtilis* BSF01. In *B. subtilis* BSF01, the degradation of 3-phenoxybenzaldehyde proceeded via the formation of 3-phenoxybenzoic acid and 3, 5-dimethoxyphenol (Xiao et al., 2015). However, in *Bacillus* sp. SG2, 3-phenoxybenzaldehyde was further converted to 4-propylbenzaldehyde and then to 4-hydroxybenzoate that was transformed to phenyl ester of *o*-phenoxy benzoic acid (Pankaj et al., 2016). The phenyl ester of *o*-phenoxy benzoic

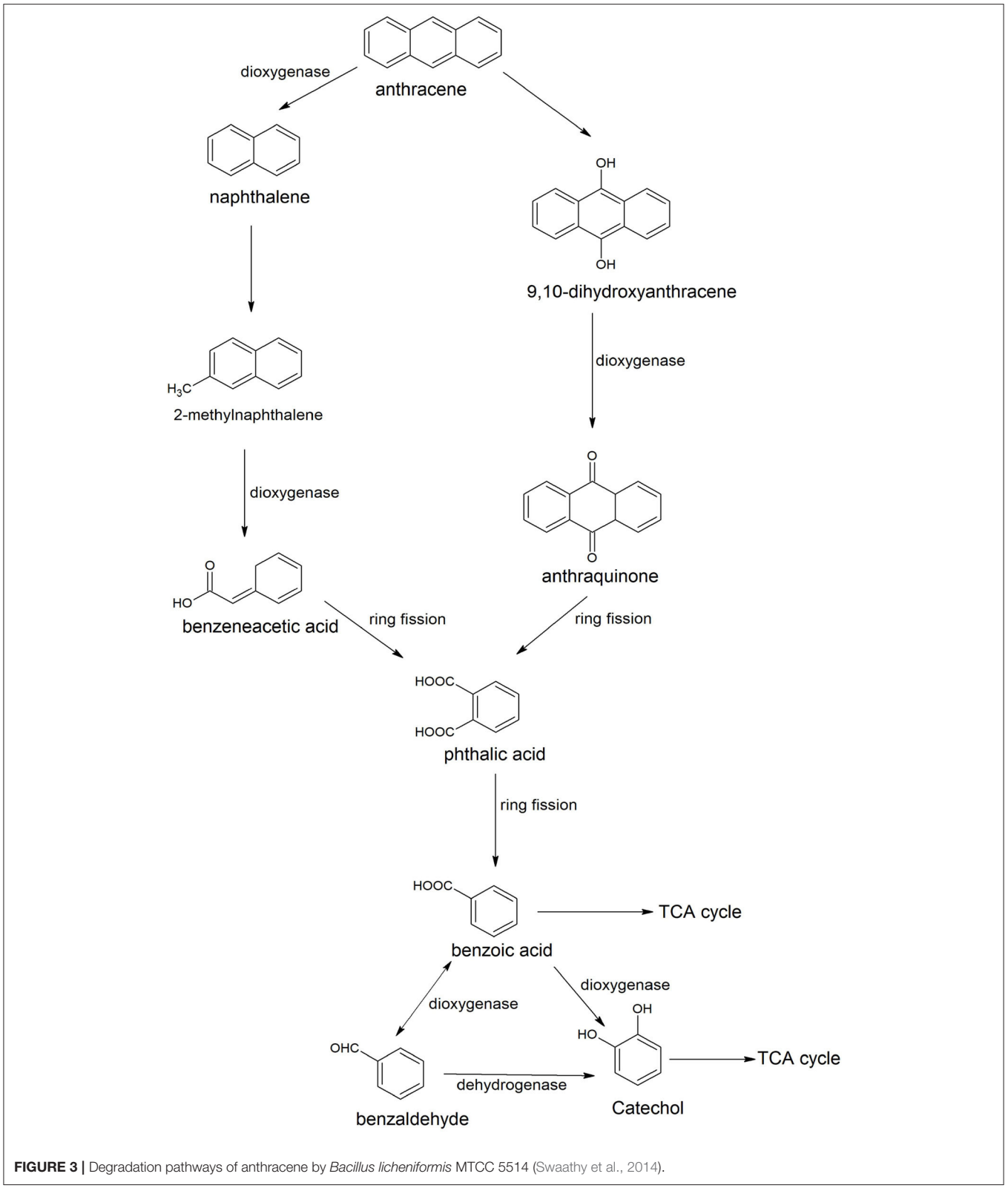

FIGURE 3 | Degradation pathways of anthracene by *Bacillus licheniformis* MTCC 5514 (Swaathy et al., 2014).

acid was degraded via the formation of phenol-M-tert-butyl, phenol, and aliphatic hydrocarbons or short-chain compounds. Another pathway of degradation of cypermethrin was studied in *B. subtilis* strain 1D (Gangola et al., 2018). In this pathway, cypermethrin was initially transformed into 3-(2, 2-dichloro ethenyl)-2,2-dimethyl-cyclopropanecarboxylate and

cyclododecylamine due to hydrolysis of the ester linkage (**Figure 5**). The unstable compound, cyclododecylamine oxidized to phenol which reacted with water to form cyclopentane that transformed into aliphatic compounds like acetic acid and decanoic acid. Another metabolite, 3-(2, 2-dichloro ethenyl)-2,2-dimethyl-cyclopropanecarboxylate was hydrolyzed to form chloroacetic acid (Gangola et al., 2018).

The degradation pathway of cyhalothrin [(RS)-α-Cyano-3-phenoxybenzyl-(Z)-(1RS,3RS)-(2-chloro-3,3, 3-trifluoro propenyl)-2,2-dimethylcyclopropanecarboxylate)] was studied in *B. thuringiensis* ZS-19 that initiated degradation of cyhalothrin by cleavage of the carboxyl ester linkage through hydrolysis to form α-hydroxy-3-phenoxy-benzeneacetonitrile and (1RS,3RS)-*trans*-2,2-dimethyl-(2-methyl-1-propenyl)cyclopropane-1-carboxylic acid (Chen et al., 2015). The α-hydroxy-3-phenoxy-benzeneacetonitrile was converted to 3-phenoxybenzote acid via 3-phenoxyphenyl acetonitrile, N-(2-isoproxy-phenyl)-4-phenoxy-benzamide, and 3-phenoxybenzaldehyde (**Figure 6**). Further degradation of 3-phenoxybenzoate was proceeded through cleavage of diaryl bond to produce and phenol that was degraded via aromatic ring cleavage (Chen et al., 2015).

The degradation pathway of fenpropathrin(α-cyano-3-phenoxybenzyl 2,2,3,3-tetramethylcyclopropanecarboxylate) was studied in *Bacillus* sp. DG-02, isolated from a soil sample collected from the aerobic pyrethroid-manufacturing wastewater treatment system of China (Chen et al., 2014). Initially, fenpropathrin was converted to α-hydroxy-3-phenoxybenzeneacetonitrile and 2, 2, 3, 3-tetramethylcyclopropanecarboxylic acid phenyl ester due to cleavage of the carboxyl ester linkage (**Figure 7**). In the next step, unstable compound α-hydroxy-3-phenoxybenzeneacetonitrile was spontaneously transformed into 3-phenoxybenzaldehyde, which oxidized to 3-phenoxybenzoate. Subsequent degradation of 3-phenoxybenzoate produced 3, 4-dihydroxybenzoic acid, 3, 4-dimethoxyphenol, and phenol (Chen et al., 2014).

Bacilli-Mediated Degradation of Organophosphorus Pesticides

Organophosphorus pesticides are a large group of chemicals that widely used for protecting crops, livestock from various pests (Sidhu G. K. et al., 2019). Commonly used organophosphates are malathion, parathion, methyl parathion, chlorpyrifos, diazinon, fenitrothion, dichlorvos, ethion, and monocrotophos (Sidhu G. K. et al., 2019). These compounds act as an inhibitor of an acetylcholinesterase enzyme that hydrolyzes the neurotransmitter acetylcholine found in both the peripheral and central nervous systems (Robb and Baker, 2020). This inhibition mechanism involves the phosphorylation of the serine hydroxyl group present on the active site of acetylcholinesterase (Robb and Baker, 2020). In this section, the role of *Bacilli* for the degradation of organophosphorus pesticides is discussed.

Many reports have been published dealing with the potential applications of *Bacilli* to degrade organophosphorus pesticides. Bhadbhade et al. (2002) reported mineralization of monocrotophos to carbon dioxide, ammonia, and phosphates by *B. megaterium* MCM B-423, isolated from soil exposed to monocrotophos. The enzymes, phosphatase, and esterase were involved in the monocrotophos degradation pathway, which proceeds via acetic acid, methylamine, and one unidentified metabolite. Dash and Osborne (2020) studied degradation pathways of monocrotophos by *B. aryabhattai* strain VITNNDJ5 in artificially contaminated soil and reported that *B. aryabhattai* may be degraded monocrotophos via three routes; one route proceeds with the hydrolysis of monocrotophos into dimethyl phosphate that was degraded further into phosphoric acid and acetic acid esters by hydrolase and monooxygenase enzymes. The second degradation pathway was initiated with the demethylation of monocrotophos to N-(hydroxymethyl) acetamide that was further degraded into acetamide. Acetamide converted into acetic that entered the TCA cycle. In the third route, monocrotophos, monocrotophos converted into orthophosphoric acid and acetic acid via formation of phosphonoacetate intermediate.

Another *Bacillus* sp. TAP-1 that was isolated from sewage sludge of a wastewater treating system of organophosphorus pesticide was capable of hydrolyzing high concentrations of triazophos (50–400 mg/l) (Tang and You, 2012). Salunkhe et al. (2013) reported the biodegradation of an organophosphorus insecticide, profenofos by four *B. subtilis* strains, namely, DR-39, CS-126, TL-171, and TS-204, isolated from grapevines or grape rhizosphere and 4-bromo-2-chlorophenol was identified as a metabolite. A marine *Bacillus* sp. strain C5 isolated from the China Bohai Sea produced an extracellular esterase that hydrolyzed methyl parathion to 4-nitrophenol and other metabolites (Hao et al., 2014). Anwar et al. (2009) reported that *B. pumilus* C2A1 isolated from a soil sample collected from the cotton field, degraded chlorpyrifos, and its first hydrolysis metabolite 3,5,6-trichloro-2-pyridinol. Strain C2A1 degraded maximum amounts of chlorpyrifos at alkaline pH (8.5) and high inoculums bacterial density. Pailan et al. (2015) isolated organophosphates-degrading bacterium, *B.* aryabhattai strain SanPS1 from a soil sample of an agricultural field located at Narigram in Burdwan district of West Bengal, India. Strain SanPS1 degraded parathion via the formation of 4-nitrophenol and 4-nitrocatechol.

Bacilli-Mediated Degradation of Organochlorine Pesticides

Organochlorine pesticides are a group of chlorinated compounds, which include DDT, methoxychlor, endosulfan, dieldrin, chlordane, toxaphene, mirex, kepone, lindane, and benzene hexachloride (Jayaraj et al., 2016). These compounds are widely distributed to the environment due to applications. In this section, *Bacilli*-mediated degradation of organochlorine pesticides is discussed.

B. subtilis MTCC 8561 utilized endosulfan and endosulfan sulfate as its sulfur sources and degraded both of them via the formation of endosulfan diol and endosulfan lactone (Kumar et al., 2014). Awasthi et al. (2003) also reported the degradation of alpha and beta isomers of endosulfan via the formation of

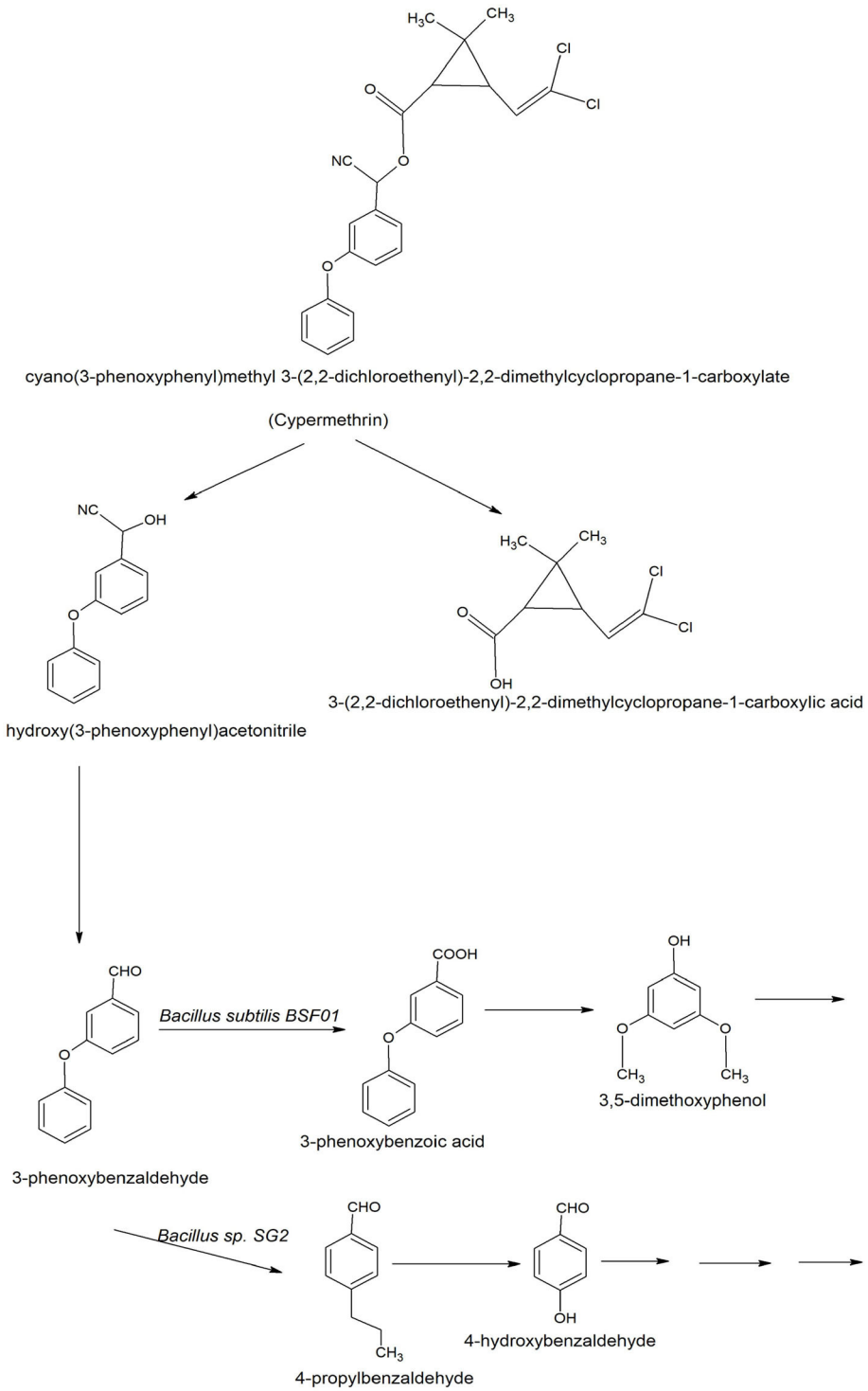

FIGURE 4 | Degradation pathways of cypermethrin in *Bacillus* sp. SG2 and *Bacillus subtilis* BSF01 (Xiao et al., 2015; Pankaj et al., 2016).

endosulfan diol and endosulfan lactone using the co-culture of *Bacillus* sp. MTCC 4444 and *Bacillus* sp. MTCC 4445. Seralathan et al. (2014) postulated the role of cytochrome P450 BM3 of *B. megaterium* in biotransformation of endosulfan through *in silico* prediction approach. Kumar and Philip (2006) reported that the anaerobic degradation of endosulfan, endosulfan ether, and endosulfan lactone using mixed bacterial culture containing two strains of *B. circulans* and one strain of *Staphylococcus* sp. All

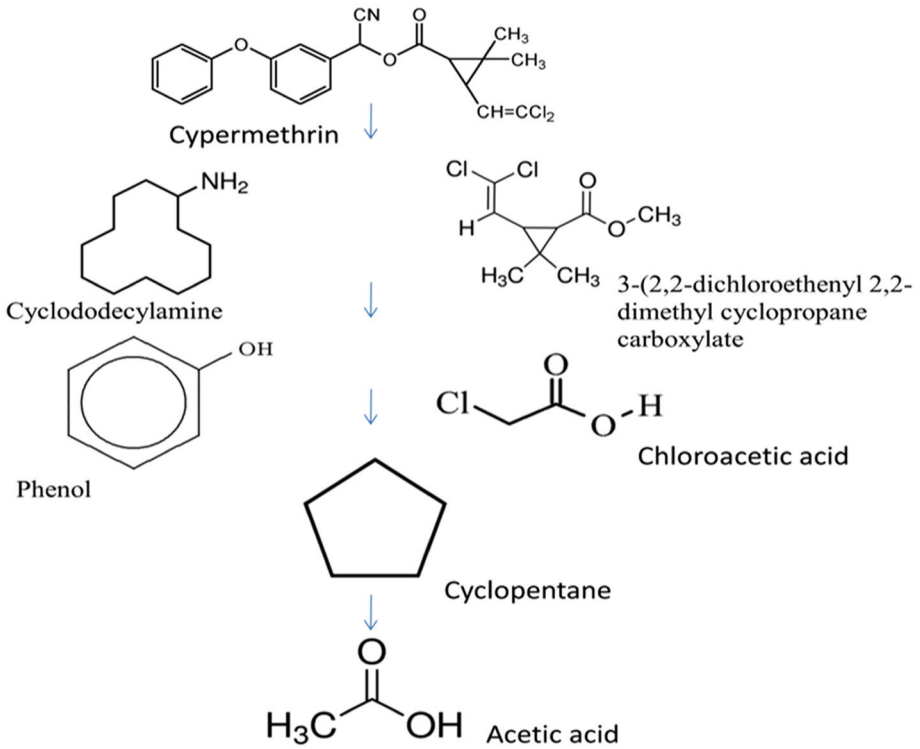

FIGURE 5 | Degradation pathways of cypermethrin in *Bacillus subtilis* strain 1D (adapted from Gangola et al., 2018).

three strains metabolized endosulfan via hydrolysis pathway with the formation of carbenium ions and/or ethylcarboxylates, which further converted into simple hydrocarbons (Kumar and Philip, 2006).

Bacilli-Mediated Degradation of Herbicides

Herbicides are chemical substances that are generally used to control the growth of unwanted plants (Herrera-Herrera et al., 2016). These are known as weed killers and divided into two categories: contact herbicides and systematic herbicides. Contact herbicides are localized in action and affect only the part of the plant that they touch (Herrera-Herrera et al., 2016). Examples are diclofop, dinoseb, diquat, and paraquat (Herrera-Herrera et al., 2016). Systemic herbicides may be translocated to other parts of the plants. Examples are atrazine, quinclorac, glyphosate 2,4-dichlorophenoxyacetic acid (2,4-D), and simazine (Herrera-Herrera et al., 2016). In this section, the role of *Bacilli* for the degradation of herbicides is discussed.

Bacillus subtilis HB-6 isolated from industrial wastewater utilized atrazine as its sole nitrogen source for growth and mineralized it via formation of hydroxyatrazine, cyanuric acid, and urea (Wang et al., 2014). The atrazine-degrading genes, *trzN, atzB*, and *atzC* which encode the enzymes to converting atrazine to cyanuric acid were detected in strain HB-6 (Wang et al., 2014). Liu et al. (2014) studied the degradation of a highly selective auxin herbicide, quinclorac (3,7-dichloro-8-quinoline-carboxylic) by *B. megaterium* Q3 isolated from the root of tobacco grown in quinclorac contaminated soil. Strain Q3 transformed quinclorac to 3, 7-dichloro-8-methyl-quinoline, 3-chlorin-8-quinoline-carboxylic and 8-quinoline-carboxylic (Liu et al., 2014).

Bacilli-Mediated Degradation of Drugs

Ibuprofen and naproxen are known as non-steroidal anti-inflammatory drugs and widely used to control mild to moderate pain, fever, inflammation, menstrual cramps, and types of arthritis (Marchlewicz et al., 2017). Due to the high consumption of these drugs, they have been detected in the effluents of several biological wastewater treatment systems as environmental pollutants (Marchlewicz et al., 2017). In this section, the *Bacillus*-medited degradation of ibuprofen and naproxen is discussed.

To date, only one species of *Bacillus*, i.e., *B. thuringiensis* B1 was able to degrade both Ibuprofen and naproxen (Marchlewicz et al., 2017; Górny et al., 2019). The effective degradation of both of these drugs occurred in the presence of glucose. *B. thuringiensis* B1 was able to degrade ibuprofen and naproxen up to concentrations of 25 mg/Land 12 mg/L, respectively.

The degradation pathways of ibuprofen and naproxen were studied in *B. thuringiensis* B1. The first step of the ibuprofen degradation is hydroxylation of ibuprofen into 2-hydroxyibuprofen by aliphatic monooxygenase (Marchlewicz et al., 2017). The second step was the conversion of 2-hydroxyibuprofen to 2-(4-hydroxyphenyl-) propionic acid that was further transformed into 1,4-hydroquinone by acyl-CoA synthase/thiolase activity (**Figure 8**). In the next step,

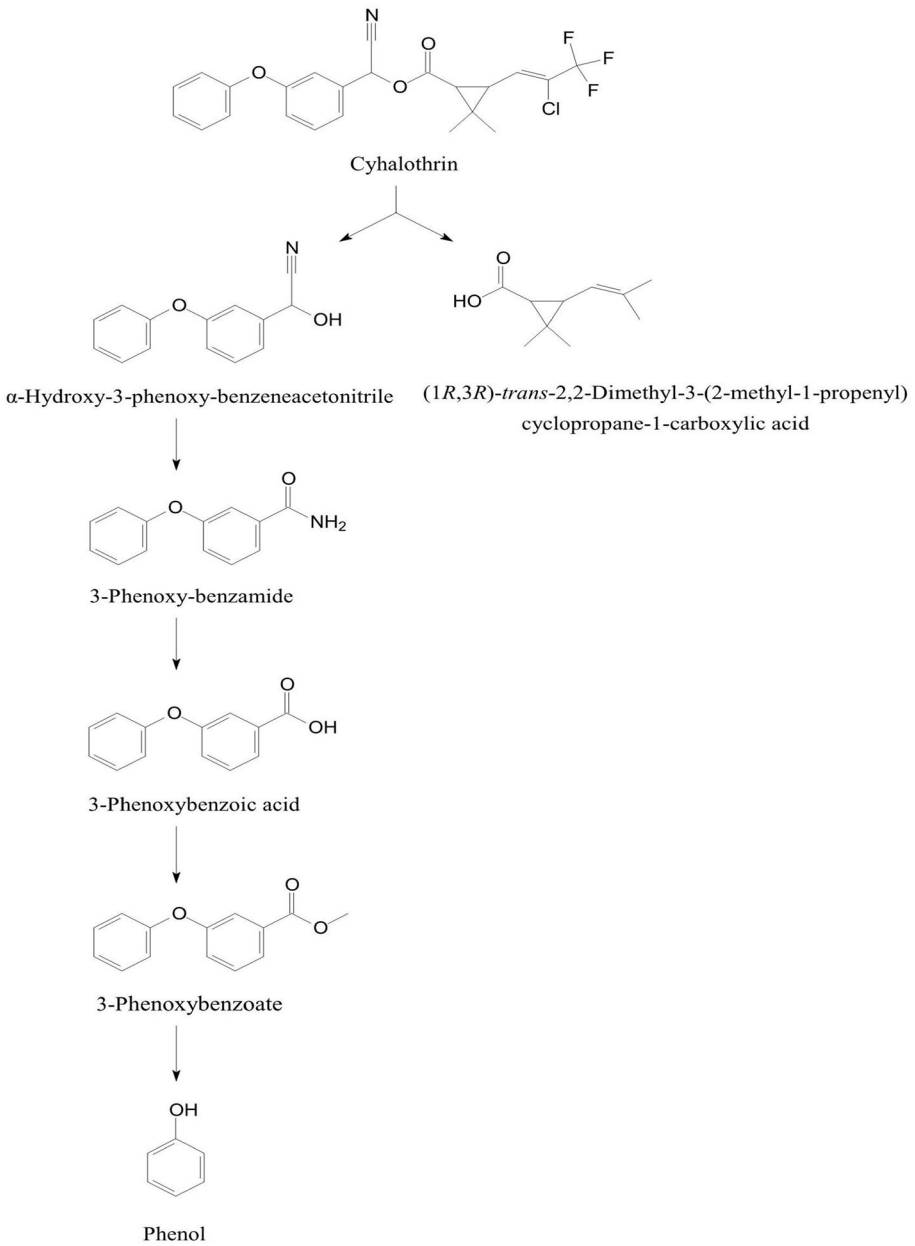

FIGURE 6 | Degradation pathway of cyhalothrin in *Bacillus thuringiensis* ZS-19 (adapted from Chen et al., 2015).

a hydroquinone monooxygenase catalyzed conversion of 1,4-hydroquinone to 2-hydroxy-1,4-quinol which cleaved to 3-hydroxy-*cis*, *cis*-muconic acid by hydroxyquinol 1,2-dioxygenase (Marchlewicz et al., 2017).

The degradation of naproxen was initiated with the transformation of naproxen into o-desmethylnaproxen by the action of tetrahydrofolate dependent *O*-demethylase (Górny et al., 2019). The next step involved the formation of 2-formyl-5-hydroxyphenylacetic that was converted to salicylic acid (**Figure 9**). Salicyclic acid hydroxylated to catechol or gentisic acid or can be cleaved to 2-oxo-3, 5-heptadienedioic acid (Górny et al., 2019).

Bacilli-Mediated Transformation of Heavy Metals

The bacterial remediation of heavy metals involves removals of heavy metals from aqueous solution and soil through biosorption, bioaccumulation, or biotransformation (Dixit et al., 2015). Biosorption is one of the important mechanisms for the removal of heavy metals, which involves the interaction of

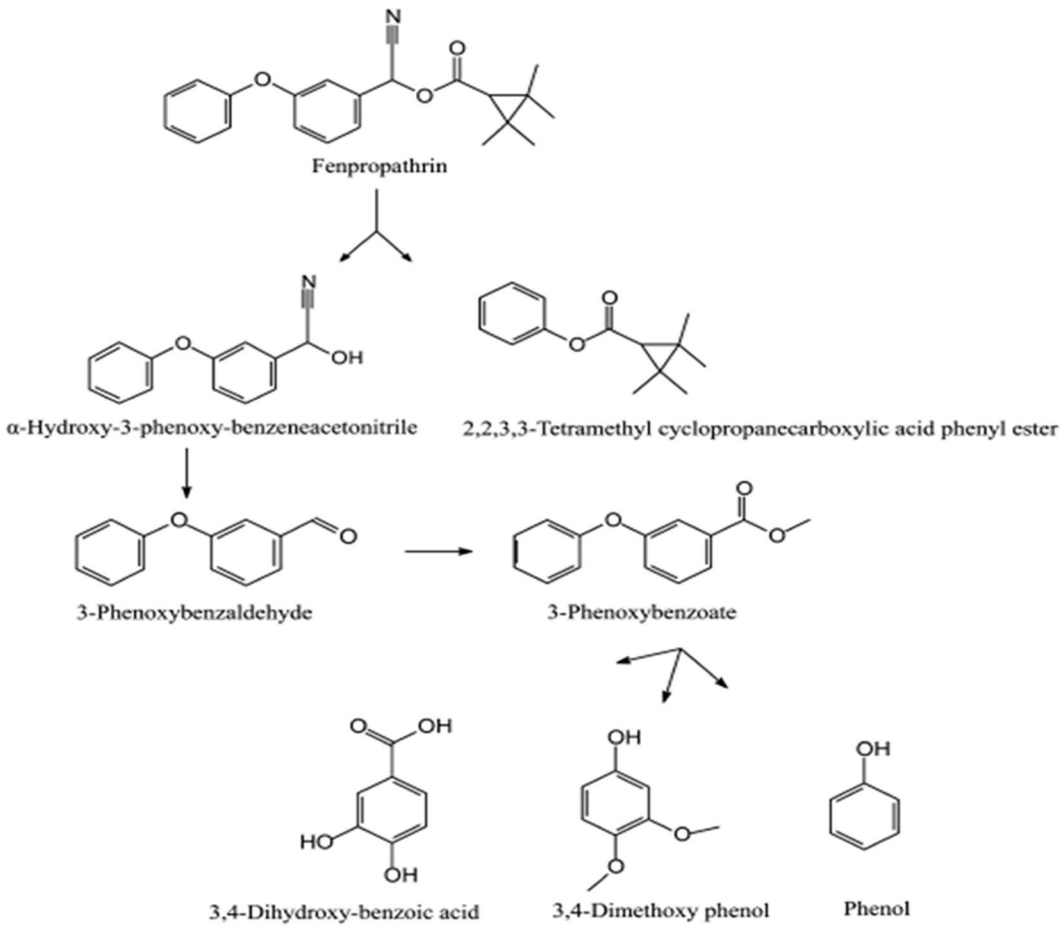

FIGURE 7 | Degradation pathway of fenpropathrin in *Bacillus* sp. DG-02 (Reprinted (adapted) from Chen et al., 2014). Copyright (2014) American Chemical Society.

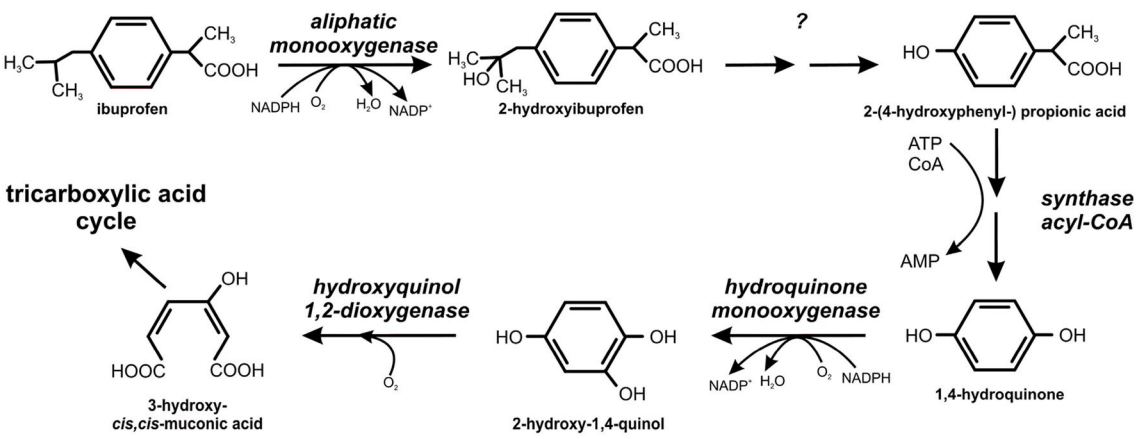

FIGURE 8 | Degradation pathway of ibuprofen in *Bacillus thuringiensis* B1 (adapted from Marchlewicz et al., 2017).

heavy metals with the functional groups present on bacterial surfaces (Igiri et al., 2018). Bioaccumulation is a metabolism-driven process in which the heavy metal ions pass across the cell membrane into the cytoplasm, accumulating inside the cells (Diep et al., 2018). Biotransformation involves conversation of one form of heavy metal to another form (Juwarkar and Yadav,

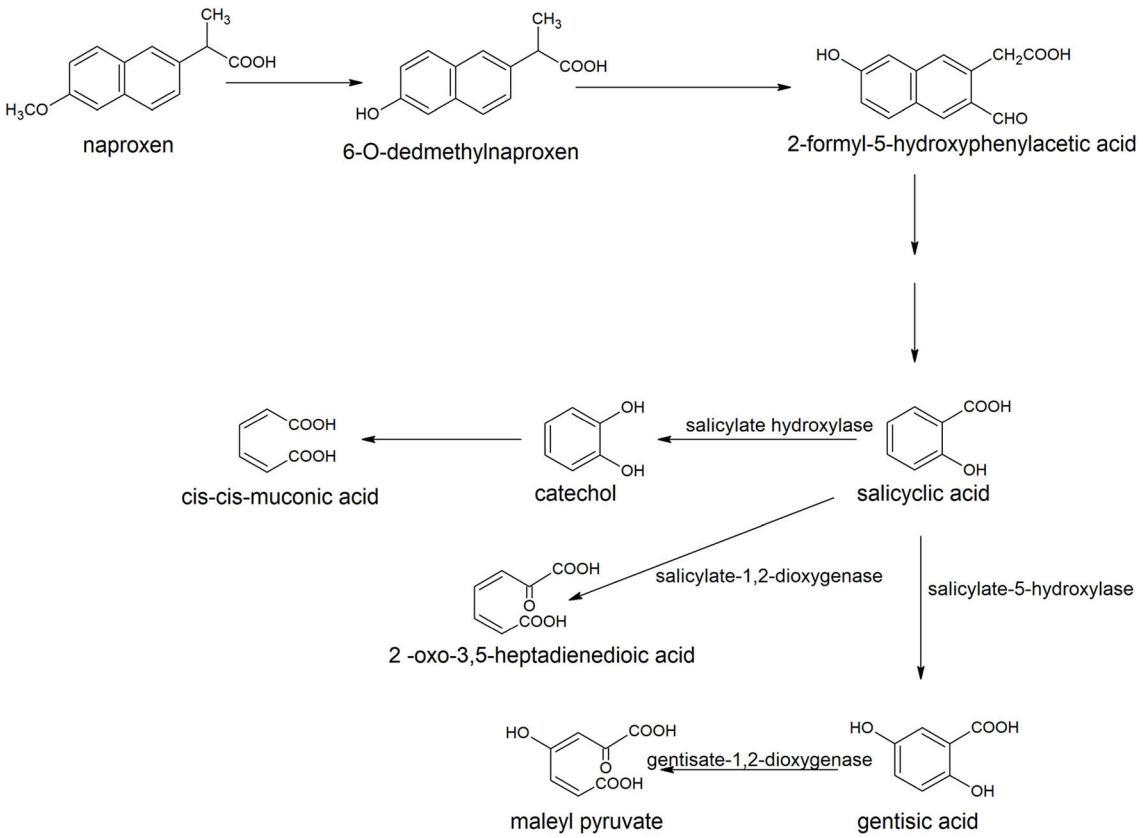

FIGURE 9 | Degradation pathway of naproxen in *Bacillus thuringiensis* B1 (Górny et al., 2019).

2010). In this subsection, the role of *Bacilli* in the bioremediation of various heavy metals is summarized.

Many *Bacilli* have been characterized for the bioreduction of chromium from Cr(VI) to Cr(III). Examples are *Bacillus* sp. strain FM1 (Masood and Malik, 2011), *Bacillus* sp. strain KSUCr9a (Ibrahim et al., 2012), *B. sphaericus* AND 303 (Pal et al., 2005), *Bacillus* sp. FY1 (Xiao et al., 2017), *Bacillus* sp. MNU16 (Upadhyay et al., 2017), *B. amyloliquefaciens* (Das et al., 2014), and *B. cereus* S612 (Wang et al., 2015). Several mechanisms have been proposed for chromium reduction and removal. Chen et al. (2012c) investigated the Cr(VI) uptake mechanism in *B. cereus* that reduced Cr(VI) into Cr(III). The reduced Cr(III) was coordinated with carboxyl and amido functional groups of the bacterial cell and the Cr(III) precipitates were accumulated on bacterial surfaces. Das et al. (2014) studied the mechanism of Cr(VI) reduction in *B. amyloliquefaciens* strain CSB 9 isolated from chromite mine soil of Sukinda, India. The reduced product Cr (III) was removed via surface immobilization and accumulated inside the bacterial cells. *Bacillus* sp. ES 29 produced copper (Cu^{2+}) stimulated soluble Cr(VI)-reducing enzyme that reduced Cr(VI) to Cr(III)(Camargo et al., 2003).

The lead transformation from toxic Pb(II) to non-toxic lead compounds has been investigated in a few *Bacillus* strains. Chen et al. (2016) studied the transformation of Pb(II) into nanosized rod-shaped $Ca_{2.5}Pb_{7.5}(OH)_2(PO_4)_6$ crystal in B. cereus 12-2, isolated from lead-zinc mine tailings. Initially, bacterial cells rapidly absorbed Pb(II) through the synergy of electrostatic attraction, ionic exchange, and chelating activity of functional groups present in bacterial cells. In the next step, enzyme-mediated Pb(II) transformation to rod-shaped crystalline minerals occurred inside the bacteria. Govarthanan et al. (2013) isolated and characterized an autochthonous bacterium, *Bacillus* sp. KK-1 for biomineralization of Pb in mine tailings. Strain KK-1 can convert $Pb(NO_3)_2$ into lead sulfide (PbS) and lead silicon oxide ($PbSiO_3$). The ability of strain KK-1 to remove Pb was investigated in mine tailings. Strain KK-1 significantly reduced the exchangeable fraction of Pb and induced calcite in the precipitation of Pb ions.

The selenium reduction from Se(IV) to Se (III) is well-studied in *Bacillus* strains. Mishra et al. (2011) reported the reduction of Se(IV) to red-element Se (III) by two strains of B. megaterium. Garbisu et al. (1995) studied the physiological mechanisms regulating the selenite reduction in *B. subtilis*. They concluded that the reduction mechanism involves an inducible detoxification system, which deposited elemental selenium between the cell wall and the plasma membrane. Another mechanism was observed in a selenate reducing bacterium, *B. selenatarsenatis* SF-1, isolated from selenium-contaminated

sediment (Kashiwa et al., 2001). Strain SF-1 reduced selenate to selenite and subsequently to non-toxic insoluble elemental selenium using lactate as an electron donor and selenate as an electron acceptor in an anaerobic condition. Elemental selenium was deposited both inside and outside of the cells. *B. selenitireducens* produced enzymes to reduce the oxidized forms of arsenic and selenium to their less toxic reduced forms (Wells et al., 2019). *B. cereus* CM100B and *B. mycoidesstrain* SeITE01 produced selenium nanoparticles (SNs) by transformation of toxic selenite (SeO_3^{2-}) anions into red elemental selenium (Se^0) under aerobic conditions. In this mechanism, initially, SeO_3^{2-} enzymatically reduced to selenium through redox reactions by the bacterial enzymes (membrane reductase) and later, selenium nanoparticles were generated due to the result of an Ostwald ripening mechanism (Dhanjal and Cameotra, 2010; Lampis et al., 2014).

The uranium transformation from U(VI) into nano-uramphite was studied in two *B. thuringiensis* strains isolated from uranium mine (Pan et al., 2015). The initial step involves the adsorption of U(VI) on the bacterial surface through coordinating with phosphate, -CH_2, and amide groups. The next step involves the formation and accumulation of needle-like amorphous uranium compounds.

Paraneeiswaran et al. (2015) reported that *B. licheniformis* SPB-2 reduced $[Co(III)–EDTA]^-$ to $[Co(II)–EDTA]^{2-}$ which was further absorbed by strain SPG-2. B. firmus strain TE7, isolated from tannery effluent reduced Cr(VI) to Cr (III) and oxidized As(III) to As(V) (Bachate et al., 2013). *Bacillus* sp. strain A.rzi isolated from a metal-contaminated soil reduced molybdate to molybdenum blue (Othman et al., 2013). *B. thuringiensis* OSM29 isolated from the rhizosphere of cauliflower grown in soil irrigated consistently with industrial effluents was capable of removing several heavy metals including cadmium, chromium, copper, lead and nickel via biosorption (Oves et al., 2013). The biosorption capacity of the strain OSM29 for the metallic ions was highest for Ni (94%) which was followed by Cu (91.8%).

Bacilli-Mediated Transformation of Azo Dyes

Azo dyes are a large group of synthetic aromatic compounds which contain one or more azo groups (-N=N-) between organic residues. Based on the number of azo linkages, azo dyes are classified as monoazo, disazo, trisazo, and polyazo (Benkhaya et al., 2020). Few examples of azo dyes are Metanil Yellow, Navy Blue 2GL, Dye Orange T4LL, Reactive Red 2, Direct Red-22, Turquoise Blue dye, and Acid Black 24. These are widely used in the textile industry that is a major source of dye contamination. During the dyeing process, the textile industry discharged ∼10% of the dyes into the wastewater (Easton, 1995). Apart from the textile industry, azo dyes are also used in food, paper printing, color photography, leather, and cosmetic industries (Chang and Lin, 2001). They are widely distributed in the environment due to improper discharge of dye into wastewater. These dyes are highly toxic to plants by inhibiting their photosynthesis. In the environment, they may generate mutagenic and carcinogenic amines due to microbial transformation (Chung and Cerniglia, 1992; Weisburger, 2002; Asad et al., 2007). Dye removal is an essential step for the treatment of dye-containing wastewater (Banat et al., 1996). Microbial dye degradation process has two steps; First is dye decolorization in which azoreductase-mediated cleavage of the azo bond (—N=N—) to give aromatic amines. The second step involves the degradation of aromatic amines into non-toxic compounds. In this sub-section, the role of *Bacilli* in dye decolorization is summarized.

Many *Bacillus* strains have been characterized for decolorization of wastewater containing various azo dyes. Anjaneya et al. (2011) studied the decolorization of metanil yellow using a sulfonated azo dye decolourizing bacterium, *Bacillus* sp. AK1 that was isolated from dye contaminated soil sample collected from Atul Dyeing Industry, Bellary, India. *Bacillus* sp. AK1 decolorized metanil yellow (200 mg L^{-1}) completely within 27h and transformed it into metanillic acid and *p*-aminodiphenylamine by the action of the azoreductase enzyme. Dawkar et al. (2009) studied the effects of inducers on the decolorization of a textile azo dye, navy blue 2GL by a *Bacillus* sp. VUS isolated from textile effluent contaminated soil. Strain VUS decolorized azo dye navy blue 2GL within 48 h under the static anoxic condition in yeast extract medium, whereas in the presence of $CaCl_2$ it decolorized it only within 18 h. They reported that $CaCl_2$ induced the activities of the enzymes involved in the decolorization of navy blue 2GL. 4-Amino-3-(2-bromo-4, 6-dinitro-phenylazo)-phenol and acetic acid 2-(-acetoxy-ethylamino)-ethyl ester were detected as the transformation products of dye decolorization. *Bacillus* sp. VUS also decolorized dye orange T4LL in static anoxic condition within 24 h and transformed it into 4-methyl-2-o-tolylazo-benzene-1,3-diamine and [3-(phenyl-hydrazono)-cyclohexa-1,4-dienyl]-methanol. Another bacterium, *B. licheniformis* decoulorized Reactive Red 2 and transformed it into 2, 4-dichloro-6-[(1H-indazol-5-ylimino)-methyl]-phenol, benzene sulfonamide, 1H indole and urea as final metabolites (Sudha and Balagurunathan, 2013). *B. firmus* immobilized within tubular polymeric gel completely decolorized 50 mg/L of CI Direct Red 80 under anoxic conditions within 12 h by transforming it into aromatic amine (Ogugbue et al., 2012). These aromatic amines were further degraded aerobically by the same strain within the subsequent 12 h.

Saleem et al. (2014) studied the effects of the various carbon sources, pH, temperature, and nitrogen sources on decolorization of pulp and paper industrial effluents by *B. cereus*. They observed that the optimum temperature and pH for decolorization were 45° C and 6.5, respectively. Maximum decolorization was observed when carbon and nitrogen sources were sucrose (0.5%) and ammonium sulfate (1%), respectively. Sharma et al. (2009) optimized process variables for decolorization of disperse yellow 211 by *B. subtilis* using Box–Behnken design and observed that the optimum conditions for maximum decolorization were 100 mg l^{-1} initial dye concentration, 7.0 pH and 32.5° C temperature. A crystal violet decolourizing bacterium, *B. subtilis* decolorized crystal violet (100 mg/L) effectively at pH 8 and temperature 35° C when starch and peptone were used as carbon and nitrogen sources, respectively (Kochher and Kumar, 2011). Gunasekar et al. (2013) reported the decolorization of reactive

dye RED M5B by *B. subtilis* and observed that decolorization was due to the action of enzyme peroxidase produced by the organisms during its growth. Joshi et al. (2013) reported the decolorization of turquoise blue dye (Remazol Blue BB) by *B. megaterium* isolated from a sample collected from dye industries. This organism can decolorize turquoise blue dye up to a concentration of 5 mg/ml. Prasad and Rao (2014) reported decolorization of Acid Black 24 by *B. halodurans* MTCC 865 which was able to decolorize Acid Black within 6 hat pH 9 and 37° C with 5% NaCl under static conditions. Prasad and Rao (2013) reported aerobic decolorization of the textile azo dye Direct Red-22 by an obligate alkaliphilic bacterium *B. cohnii* MTCC 3616. This strain was able to decolorize Direct Red-22 (5,000 mg l^1) with 95% efficiency at 37° C and pH 9 in 4 h under static conditions.

Bacilli-Mediated Degradation of Natural Aromatic Acids

Aromatic acids are a class of chemical compounds in which an organic acid attached to the aromatic ring. Examples are phenolic acids (3-Hydroxybenzoic acid. 4-Hydroxybenzoic acid and Salicylic acid) and Hydroxycinnamic acids (cinnamic, 4-coumaric, and ferulic acids). In this subsection, the role of *Bacilli* in biodegradation of various aromatic acids is summarized. *B. macerans* JJ-lb degraded protocatechuate via ring cleavage and subsequent enzymatic decarboxylation of the ring fission product (Crawford et al., 1979). Initially, protocatechuate-2,3-dioxygenase catalyzes the ring cleavage of protocatechuate to 5-carboxy-2-hydroxymuconic semialdehyde that is further decarboxylated to 2-hydroxymuconic semialdehyde. Mashetty et al. (1996) reported the degradation of 3-hydroxybenzoate by a *Bacillus* sp. that utilized it as the sole source of carbon and energy. This strain metabolized 3-hydroxybenzoic acid via protocatechuic acid that was further degraded via both the *ortho*- and *meta*-cleavage pathway. The enzyme activities for 3-hydroxybenzoate 4-hydroxylase, protocatechuate 3,4-dioxygenase, and protocatechuate 4,5-dioxygenase were detected in cell-free extracts. Crawford (1976) reported degradation pathways of 4-hydroxybenzoate in *B. brevis* PHB-2, *B. circulans* strain 3, and *B. laterosporus* PHB-7a. *B. brevis* PHB-2 and *B. circulans* strain 3 degraded 4-hydroxybenzoate via protocatechuate that was further degraded through *ortho* cleavage pathway or *meta* cleavage pathway. *B. laterosporus* PHB-7a converts 4-hydroxybenzoate to gentisate, which is further degraded by the glutathione-independent gentisic acid pathway. Peng et al. (2003) reported the degradation of cinnamic, 4-coumaric, and ferulic acids by thermophilic *Bacillus* sp. B-1. Strain B-1 degraded cinnamic acid via benzoic acid that was further degraded via catechol and its ring cleavage. The 4-coumaric acid degradation proceeded via 4-hydroxybenzoic acid that was further degraded via gentisic acid and its ring cleavage. The ferulic acid metabolized via 4-hydroxy-3-methoxyphenyl-beta-hydroxypropionic acid, vanillin, and vanillic acid as the intermediates. *Bacillus* sp. DG-2 degraded 3-phenoxybenzoic acid via 3-(2-methoxyphenoxy) benzoic acid, protocatechuate, phenol, and 3,4-dihydroxy phenol.

Bacilli-Mediated Degradation of Explosives

Bacilli play a critical role in the degradation of explosives such as nitrate esters, 2,4,6-Trinitrotoluene (TNT), Trinitrophenol (TNP). Denitration is the main step for the biodegradation of nitrate esters. Meng et al. (1995) studied the biotransformation of glycerol trinitrate by *Bacillus* sp. ATCC51912 that sequentially denitrated glycerol trinitrate to glycerol via the formation of glycerol dinitrate and glycerol mononitrate isomers. Similarly, *Bacillus* sp. ATCC51912 denitrated propylene glycol dinitrate to propylene glycol via propylene glycol mononitrate (Sun et al., 1996). Yerson and Christian (2013) isolated pentaerythritol tetranitrate (PETN)-degrading bacterium, *Bacillus* sp. J8A2 from mining environment. Strain J8A2 utilized PETN as its nitrogen source. Bacterial degradation of PENT generally initiated with sequential denitration of PENT to pentaerythritol via the intermediary formation of tri-, di-, and mononitrate pentaerythritol. An NADPH-dependent PETN reductase enzyme isolated from *Bacillus* sp. was capable of liberating nitrite from nitrate esters with the oxidation of NADPH.

Bacillus sp. can use TNP as a sole nitrogen source under aerobic conditions (Singh et al., 2011).TNPs has three electron-withdrawing nitro groups that prevent an initial oxidative attack on the aromatic ring. Therefore, the initial steps of TNP degradation are reductive. *Bacilli* degraded TNP by via hydrogenation to form a Meisenheimercomplex, hydride σ-complex (Singh et al., 2011).

Degradation of 2,4,6-Trinitrotoluene (TNT) by *Bacillus* sp. occurs also via the reductive route. *B. cereus* transformed TNT to 2,4-dinitrotoluene and 4-aminodinitrotoluene derivates and degraded 77% of 75 mg L^{-1}, TNT within 96 h (Mercimek et al., 2013). Nyanhongo et al. (2008) reported that *Bacillus* sp. SF transformed TNT via an initial reduction mechanism to produce hydroxylaminodinitrotoluenes, 4-amino-2,6-dinitrotoluenes, 2-amino-4,6-dinitrotoluenes, different azoxy compounds, 2,6-diaminonitrotoluenes, and 2,4-diaminonitrotoluenes.

PILOT SCALE STUDIES USING *BACILLI*

For biodegradation purposes, a pilot study plays a vital role before conducting the big scale degradation studies in fields. Chopra and Kumar (2020) examined the degradation of acetaminophen (N-acetyl-para-aminophenol) by *B. drentensis* strain S1 within the the pilot-scale anaerobic batch reactor. The ideal conditions include temperature 40° C, pH 7, 300 mg/L acetaminophen, and agitation speed 165 rpm (Chopra and Kumar, 2020). 2-Isopropyl-5-methylcyclohexanone and phenothiazine were identified metabolites of the acetaminophen degradation. Sonwani et al. (2019) studied the degradation of naphthalene in a pilot-scale integrated aerobic treatment plant and catechol and 2-naphthol were detected as the major intermediate metabolites. Fujita et al. (2002) studied the removal of toxic soluble selenium (selenite/selenate) using *Bacillus* sp. SF-1 in a continuous flow bioreactor under an anoxic condition. The outcomes indicated that both selenite and selenate were reduced to elemental selenium at long cell retention times. Sundar et al. (2011) successfully demonstrated the removal of

trivalent chromium using *Bacillus* biofilms through a continuous flow reactor. Pan et al. (2014) used a mixture of planktonic cells and biofilms of *B. subtilis* for successful removal of Cr(IV) from Cr(IV)-containing wastewater in 10-L pilot-scale experiment. Kim et al. (2014) treated 80 tons of groundwater containing heavy metals using immobilized dead cells of *B. drentensis* in pilot-scale study and results demonstrated over 93% removal of Cu, Cd, Zn, and Fe. Narayanan et al. (2015) reported the production of laccase from *B. subtilis* MTCC 2414 for the study of decolorization of Yellow GR, Orange 3R, and T-Blue. They used guaiacol as a substrate under Submerged Fermentation Conditions for the production of laccase, which was immobilized with sodium alginate. The immobilized laccase exhibited optimum activity at pH 7 and temperature 35° C. Results of their studies showed that immobilized laccases degraded Yellow GR (81.72%), Orange 3R (77.2%), and T-Blue (78.55%) at higher efficiency as compared to free laccase. Several researchers investigated the pilot scale-production of commercial compounds using various wastes as substrates (Mohapatra et al., 2017). Yezza et al. (2004) studied the production of *Bacillus thuringiensis*-based biopesticides in fermenters using wastewater sludge as raw materials and results demonstrated high production of pesticides. Mohapatra et al. (2017) studied bioconversion of fish solid waste into polyhydroxybutyrate using the *Bacillus subtilis*-based submerged fermentation process. Barros et al. (2008) reported the production of biosurfactant by *Bacillus subtilis* on a pilot scale using cassava wastewater as substrate.

ADVANCED TECHNOLOGIES FOR BIOREMEDIATION OF XENOBIOTIC COMPOUNDS AND HEAVY METALS USING *BACILLI*

This section briefly describes various current technologies used to enhance the bioremediation of xenobiotic compounds and heavy metals.

Metagenomics

Several xenobiotic-degrading enzymes stay undiscovered in light of the fact that a greater part of bacteria (99%) remain uncluturable in laboratory (Arora et al., 2010). In such a case, metagenomics plays a vital role to investigate novel microbial enzymes from whole network of microbial community. The metagenomic approach includes (i) the isolation and purification of DNA from a sample, (ii) cloning of DNA into appropriate vectors, (iii) the transformation of host cells with construct and (iv) functional and sequence based screening of constructed clones (Arora et al., 2010). The sequence-based approaches depend on already known sequences of the target gene and utilize bioinformatics tools. However, the function-based approaches do not include the involvement of metagenomic derived sequences and, in this way, may prompt to the invention of novel genes with desired functions. Several enzymes involved in biodegradation of various xenobiotic compounds have been identified by metagenomic studies of several environmental samples. Sidhu C. et al. (2019) identified novel 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC-SD3) and catechol 2,3-dioxygenase (C23O-RW1) from the metagenomic DNA isolated from sludge and river water samples. These enzymes were clones, expressed and purified to monitor their abilities to degrade various aromatic compounds. BphC-SD3 specifically oxidized 2,3-dihydroxybiphenyl, catechol, and 3-methylcatechol, whereas C23O-RW1 oxidized catechol, 4-chlorocatechol, 2,3-dihydroxybiphenyl and 3-methylcatechol. Suenaga et al. (2007) studied extradiol dioxygenases diversity in activated sludge used to treat coke plant wastewater by a metagenomic approach and identified 38 new extradiol dioxygenases that formed a new subfamily of extradiol dioxygenases. Singh et al. (2010) identified two flavin monooxygenases from an effluent treatment plant sludge metagenomic library which were involved in the oxidation of indole to a mixture of indigo and indirubin pigments. Nagayama et al. (2015) identified a multicomponent hydroxylase involved in the phenol degradation from a metagenomic library derived from soil sample artificially contaminated with aromatic compounds. Choi et al. (2018) identified and characterized the first metagenome-derived toxoflavin-degrading enzyme that was involved in biodegradation of toxoflavin and its derivatives including methyltoxoflavin, fervenulin, and reumycin. Ye et al. (2010) identified a muti-copper oxidase with laccase activity from activity-based functional screening of a metagenomic library from mangrove soil. The characteristic feature of this laccase was its strong alkaline activity and its high solubility.

Rational Designing

This protein engineering approach requires the knowledge of protein structure, function and mechanism to improve enzyme properties. Several xenobiotic-degrading enzymes of *Bacilli* have been improved using rational designing approach. Best studied example is laccase enzyme that catalyzes the oxidation of a variety of xenobiotic compounds, including diphenols, polyphenols, diamines, aromatic amines, and synthetic dyes. Mollania et al. (2011) used rational design approach to increase the thermal stability of laccase enzyme of *Bacillus* sp. HR03. They substituted Glu188 residue with 2 positive (Lys and Arg) and one hydrophobic (Ala) residues to obtain mutants. All variants exhibited strong thermal stability and thermal activation as compared to the wild-type. The 3-fold higher thermal activation and higher T_{50} (5° C) as compared to native enzyme was observed in the case of the Glu188Lys variant (Mollania et al., 2011). Rasekh et al. (2014) increased the tolerance of this laccase toward organic solvents by substitution of the Glu188 residue with non-polar (Ala, Ile, Leu, and Val) and positively charged (Lys and Arg) residues. All variants showed higher C_{50} values (organic solvent concentration at which 50% of enzyme activity remains) as compared to the wild type. Non-polar amino acid substitutions created more efficient mutants as they exhibited significantly increased C_{50} value and decreased thermo inactivation rate in the presence of organic solvents (Rasekh et al., 2014).

Another example of rational design to improve the enzyme activity is cytochrome P450 monooxygenase from *Bacillus*

megaterium 3 (P450 BM3). Carmichael and Wong (2001) reported double mutation in P450 BM3 at R47L and Y51F to enhance its oxidation activity toward phenanthrene and fluoranthene. The mutants showed 40-folds and 10-folds oxidation activity toward phenanthrene and fluoranthene. Li et al. (2001) reported oxidation of polycyclic hydrocarbons such as naphthalene, fluorene, acenaphthene, acenaphthylene, and 9-methylanthracene by triple mutant of P450 BM3 at A74G/F87V/L188Q sites.

Directed Evolution

Directed evolution is an approach of protein engineering to improve the efficiency of proteins without a prior knowledge of amino acid sequences. It is based on the Darwinian principle of evolution and involves (i) the use of rapid molecular manipulations to mutate the target gene and (ii) the subsequent selection of the improved variants by screening (Arora et al., 2010). Using directed evolution, many xenobiotic-degrading genes have been improved for their properties. Best studied example is cytochrome P450 monooxygenase from *Bacillus megaterium* 3 (P450 BM3) that involves in oxidation of various aromatic compounds. Sideri et al. (2013) used directed evolution to generate mutants of P450 BM3 to hydoxylate chrysene and pyrene. Two rounds of random mutagenesis by error–prone PCR were used to generate mutants. Three mutants exhibited hydroxylation of chrysene and pyrene. These mutants hydroxylated chrysene in different positions and hydroxylate pyrene to 1-hydroxypyrene. Santos et al. (2019) reported that directed evolution of P450 BM3 to improve the hydroxylation activity toward six o-heterocycles; benzo-1,4-dioxane, phthalan, isochroman, 2,3-dihydrobenzofuran, benzofuran, and dibenzofuran. They screened in-house libraries of P450 BM3 to generate P450 BM3 CM1 (R255P/P329H) that was further underwent error–prone PCR, generating P450 BM3 GS2 (R255S/P329H/F331L). Another error-prone PCR of P450 BM3 GS-2 generated P450 BM3 GS3 (I122V/R255S/P329H/F331L). In next step, P450 BM3 WT was subjected to single site saturation mutagenesis (SSM) in the four identified positions and double SSM at positions I122 and R255, which provided the most active variants, P450 BM3 R255G and R255L.

Recombinant DNA Technology or Genetic Engineering

Genetic engineering or recombinant DNA technology includes multiple techniques used to cut up and join together DNA from various biological sources, and to introduce the resulting hybrid DNA into an organism so as to create new combinations of heritable genetic material (Rosenberg, 2017). Genetic engineering is a promising technique to enhance the potentials of microorganisms for the bioremediation of environmental pollutants (Ezezika and Singer, 2010). Genetically engineered bacteria are considered as potential candidates for bioremediation applications in soil, groundwater, and activated sludge (Sayler and Ripp, 2000). A list of few genetically engineered bacteria with their bioremediation applications is presented in **Table 2**.

Even though several genetically engineered *Bacilli* have been constructed for various industrial applications (Wang et al., 2006; Drejer et al., 2020), the bioremediation applications of genetically engineered *Bacilli* is very limited. Huang et al. (2015) constructed a genetically engineered *B. subtilis* 168 expressing the arsenite S-adenosylmethionine methyltransferase gene of thermophilic algae for bioremediation of arsenic. This genetically engineered bacterium was able to convert the inorganic As into dimethylarsenate and trimethylarsine oxide via methylation, and also able to volatilize substantial amounts of dimethylarsine and trimethylarsine (Huang et al., 2015). The rate of As methylation and volatilization increased with temperature from 37 to 50° C. However, wild type *B. subtilis* 168 lacks the properties of methylation and volatilization.

Genome-Editing Technologies

Genome-editing technologies are currently using to manipulate DNA by the engineered nucleases or molecular scissors, which have a wide range of applications in research fields of plants, animals, and microorganisms (Jaiswal et al., 2019). The process of genome editing is generally performed by genome editing tools and involves following steps (i) double standard break in targeted gene sequence (ii) repaired by homologous recombination using self-designed guide sequence complementary to targeted gene sequence (iii) error-prone non-homologous end joining (Jaiswal et al., 2019). The aim of using gene-editing tools is to develop a microbe with great potentials. Jaiswal et al. (2019) describe the role of the gene-editing tools such as Transcription-activators like effector nucleases (TALEN), clustered regularly interspaced short palindromic repeats (CRISPR-Cas), and zinc finger nucleases (ZFNs) to design bacteria with improved metabolic capabilities for enhancing the bioremediation of environmental pollutants.

Genomics

Genomic studies are a powerful tool for the study of microorganisms capable of degrading environmental pollutants (Rodríguez et al., 2020). Next-Generation sequencing technology has been widely used for the whole-genome sequences of various organisms. The whole genomes of several xenobiotic-degrading *Bacilli* have been sequenced using Next-Generation sequencing technology, and several genes and proteins involved in biodegradation have been identified through gene predictions and annotation of the *Bacilli* genomes. Hossain et al. (2020) identified chromate transporters in the genome of a chromium-reducing bacterium, *B. cereus* TN10 isolated from tannery effluent. Chromate transporters are involved in chromium resistance and play a role in the efflux of cytoplasmic chromate. He et al. (2010) identified a putative chromate transport operon, two chromate transporters, azoreductase gene, and four nitroreductase genes in *Bacillus cereus* SJ1 which may be involved chromate resistance and chromate reduction. The genome of *B. cereus* S612 contains genes encoding multidrug efflux pumps and reductases that are potentially related to chromium resistance and reduction (Wang et al., 2015). Genome analysis of zearalenone-degrading *Bacillus velezensis* ANSB01E revealed

TABLE 2 | A list of few genetically engineered bacteria invloved in bioremediation.

Genetically engineered bacteria	Compound/heavy metal	Properties/application	References
Bacillus subtilis 168	Arsenic	Expressed the arsenite S-adenosylmethionine methyltransferase gene from thermophilic algae, *Cyanidioschyzon merolae*. This bacterium involved in arsenic methylation and volatilization	Huang et al., 2015
Rhodopseudomonas palustris	Mercury	Expressed mercury transport system and metallothionein for Hg2+ uptake	Deng and Jia, 2011
Escherichia coli	Nickel	Expressed nickel-affinity transmembrane protiens and metallothionein for Ni2+ bioaccumulation	Deng et al., 2013
Pseudomonas putida MC4-5222	1,2,3-Trichloropropane (TCP)	Expressed the haloalkane dehalogenase (DhaA31). More than 95% degradation of TCP was observed	Samin et al., 2014
Pseudomonas fluorescens	Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	Expressed the RDX-metabolizing enzyme XplA to degrade RDX in the rhizosphere	Lorenz et al., 2013
Pseudomonas putida KTUe	Organophosphates, pyrethroids, and carbamates	A scarless genome editing strategy was used to insert four pesticide degrading genes, *vgb*, and *gfp*. This bacterium completely degraded methyl parathion, chlorpyrifos, fenpropathrin, cypermethrin, carbofuran and carbaryl when concentration was 50 mg/L	Gong et al., 2018
Cupriavidus necator JMP134-ONP	Nitrophenols	Inserted ortho-nitrophenol degradation operon (*onpABC* gene cluster). This bacterium was able to degrade two isomers of nitrophenols	Hu et al., 2014

the presence of genes coding peroxiredoxin and alpha/beta hydrolase, which may be involved in zearalenone degradation (Guo et al., 2020).

Bioinformatics Tools

Bioinformatics approaches including biodegradative databases, pathway prediction systems, and protein-structure predicting tools may be used for biodegradation studies (Arora and Bae, 2014). Biodegradative databases provide information about pollutants, their degradation pathways, bacteria, genes, and enzymes in their degradation (Arora and Bae, 2014). Examples of these databases are the EAWAG Biocatalysis/Biodegradation Database (EAWAG-BBD), a database of biodegradative oxygenases (OxDBase), Biodegradation Network-Molecular Biology database (Bionemo), MetaCyc, and BioCyc (Arora and Bae, 2014). The structure of enzymes involved in biodegradation of environmental pollutants in *Bacilli* can be predicted by online structure prediction tools such as Iterative Threading Assembly Refinement server (I-TASSER) (Yang and Zhang, 2015), SWISS-MODEL (Waterhouse et al., 2018), and optimized protein fold RecognitION (ORION) (Ghouzam et al., 2015).

CONCLUSION

Many *Bacilli* have been isolated and characterized for degradation of various environmental pollutants including chloronitrophenols, dyes, drugs, pesticide, explosives, polycyclic aromatic compounds, heterocyclic aromatic compounds, and heavy metals. The biochemical characterization of degradation pathways of various environmental pollutants was extensively studied in *Bacilli.* The genes involved in the degradation of various xenobiotic compounds have been identified from the genome sequences of various xenobiotic degrading *Bacilli*. Further studies on cloning and expression of these genes would be useful to understand the mechanism of biodegradation. The construction of genetically engineered *Bacilli* with improved degradation efficiency will be useful for biodegradation applications. Furthermore, genome editing tools may be used to develop more efficient *Bacilli* for the bioremediation of pollutants. Bioinformatics tools such as databases, pathway prediction systems, and protein structure predicting tools are useful to determine the fate of environmental pollutants in the fields.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Enzymatic Remediation of Polyethylene Terephthalate (PET)–Based Polymers for Effective Management of Plastic Wastes: An Overview

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Globally, plastic-based pollution is now recognized as one of the serious threats to the environment. Among different plastics, polyethylene terephthalate (PET) occupies a pivotal place, its excess presence as a waste is a major environmental concern. Mechanical, thermal, and chemical-based treatments are generally used to manage PET pollution. However, these methods are usually expensive or generate secondary pollutants. Hence, there is a need for a cost-effective and environment-friendly method for efficient management of PET-based plastic wastes. Considering this, enzymatic treatment or recycling is one of the important methods to curb PET pollution. In this regard, PET hydrolases have been explored for the treatment of PET wastes. These enzymes act on PET and end its breakdown into monomeric units and subsequently results in loss of weight. However, various factors, specifically PET crystallinity, temperature, and pH, are known to affect this enzymatic process. For effective hydrolysis of PET, high temperature is required, which facilitates easy accessibility of substrate (PET) to enzymes. However, to function at this high temperature, there is a requirement of thermostable enzymes. The thermostability could be enhanced using glycosylation, immobilization, and enzyme engineering. Furthermore, the use of surfactants, additives such as Ca^{2+}, Mg^{2+}, and hydrophobins (cysteine-rich proteins), has also been reported to enhance the enzymatic PET hydrolysis through facilitating easy accessibility of PET polymers. The present review encompasses a brief overview of the use of enzymes toward the management of PET wastes. Various methods affecting the treatment process and different constraints arising thereof are also systematically highlighted in the review.

Keywords: plastic pollution, polyethylene terephthalate, remediation, PET hydrolases, recycling

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INTRODUCTION

Indiscriminate usage of plastics and related products along with its poor disposal management leads to the widespread presence of plastic waste in the environment. The need for plastic becomes so much prevalent that it is now described as one of the inseparable commodities (Koshti et al., 2018). Various properties such as light weight, heat resistance, high malleability, transparency, hardness,

and tensile strength make plastics as one of the desirable polymers for a variety of applications. This extensive application of plastic resulted in the steady rise of plastic waste in different ecosystems. Plastic is highly recalcitrant and takes about 1,000 years to decompose in nature, and thus, it keeps on accumulating in nature (Webb et al., 2013). This excess accumulation of plastic and associated wastes in the environment possesses various risks to living beings (Ogunola et al., 2018; Saleem et al., 2018).

From the start of the 21st century, the production of plastic has increased tremendously because of high demand, and as a consequence of it, plastic waste generation also tripled in these two decades (Beat Plastic Pollution, 2020). At present, around 0.3 billion of plastic wastes are produced, and 90% of it lands up in the ocean (Schmidt et al., 2017). Since the 1950s, around 8,300 million plastic wastes has been generated, and by 2050, it is expected to reach double-digit billions, if plastic waste is generated at the same pace (Geyer et al., 2017).

Plastics have resistance toward organic solvents, oxidation, and ionizing radiation, making it a priority choice for many industrial applications. From the total plastic production, 33% are used in packaging (Rhodes, 2018). Among various forms of plastics, polyethylene terephthalate (PET)–based plastics are noteworthy as they are widely used in packaging industries because of their durability and thermostability. PET is a semicrystalline, colorless, hygroscopic resin with excellent properties of high wear and tear resistance, high tensile strength, and transparency (Koshti et al., 2018). Owing to these properties, PET is extensively used in packaging industries. Prominently, it is used in plastic bottles of soft drinks, food jars, and plastics films.

Polyethylene terephthalate is synthesized by polycondensation of terephthalic acid (TPA) and ethylene glycol (EG) or transesterification of dimethyl terephthalate and EG forming a polymer of semiaromatic polyesters (Hiraga et al., 2019). TPA and EG monomeric units of PET are linked by ester linkages. It is chemically inert and hydrophobic in character, which creates an almost non-soakable surface (de Castro et al., 2017). Melting temperature (T_m) of PET is noted to be 240–250°C with good hydrolytic stability (Mohsin et al., 2017). PET varies in crystallinity (CrI); for instance, those possessing up to 7% CrI are called low crystalline PET (lcPET) and with 30–35% CrI is high-crystalline PET (hcPET) (Furukawa et al., 2019). The extent of CrI depicts the mobility of ester linkages in PET (Zekriardehani et al., 2017). High CrI indicates more rigidity in the linkages. The glass transition temperature (T_g) of PET is around 70–80°C (Ronkvist et al., 2009). T_g is the temperature at which mobility of the polymer is increased, allowing more accessibility to ester links between monomeric units.

The high demand for PET-based plastics, especially in packaging industries, leads to the total production of 18.8 million tons in 2015 out of total of 269 million tons of total plastic production (Taniguchi et al., 2019). Out of total PET production, only 28.4% is recycled to fiber, sheets, films, and bottles, and the rest is discarded in the environment (Taniguchi et al., 2019). This discarded PET then goes into the open environment and forms a threat to the various life forms.

Like other plastics, PET is usually non-biodegradable and difficult to decompose, especially those with high crystallinity. Hence, most of the PET-based plastic wastes are either incinerated or dumped in landfill sites (Geyer et al., 2017). A very low portion of it is recycled. As mentioned above, accumulation of plastic wastes affects the normal functioning of an ecosystem through various detrimental effects on living forms. Consumption of plastic materials by stray animals and tiny plastic materials floating on water bodies by aquatic animals lead to various alterations in their physiological activities (Bhattacharya and Khare, 2020). Sometimes, this consumption also leads to blockage of the digestive system and clogging of respiratory passage and ultimately results in the mortality of particular animal species (Koshti et al., 2018). In addition to that, the toxic constituents released during the partial decomposition of plastic wastes also add on to soil pollution and affect various life forms. As PET-based plastics are hydrophobic in nature and thus act as adsorption sites for various pollutants such as persistent organic pollutants and heavy metals found in aquatic and terrestrial systems (Bhattacharya and Khare, 2020). These adhered toxins are also transferred through the food chain and possess a risk for high-trophic-level consumers, as these may get biomagnified upon transfer through food chains (Koshti et al., 2018).

At present, majorly employed plastic/PET disposal methods in developing countries are landfilling and incineration. Landfilling cannot be carried out for long because of scarcity of space and increasing cost; similarly, incineration results in an emission of toxic fumes containing various toxicants and fly ash, which requires further disposal (Saleem et al., 2018). However, recycling is considered one of the best ways to manage plastic/PET wastes. Recycling uses less energy and fewer resources and also leads to the lowering of carbon footprint compared to the production of petrochemical-based virgin PET products (Quartinello et al., 2017). Post consumption, PET wastes in many countries (most of the European countries and Japan) are recycled to form new products through the recovery of PET monomers (TPA and EG). Various recycling options *viz.* thermal (used as fuels), material/mechanical (melted and reused once), and chemical/catalytic (degraded to monomers and used for re-synthesis) are usually practiced for management of PET wastes (Kawai et al., 2019).

Material or mechanical recycling is one of the widely used methods for recycling of PET wastes. This process involves sorting and separation of wastes followed by washing for the removal of dirt and contaminants from wastes (Park and Kim, 2014). Thereafter grinding and crushing are accomplished to reduce the particle size; finally, reextrusion and reprocessing are done for the production of new products (Park and Kim, 2014). However, heterogeneity of waste along with the presence of contaminants mainly hinders mechanical processing. Additionally, products formed from mechanically recycled PET wastes are of poor quality because of mechanical stress and photo-oxidation caused by the heat of fusion (Park and Kim, 2014; Kawai et al., 2019). Hence, this method of recycling is generally practiced for the production of low-grade plastics. Similarly, thermal processing/incineration of PET waste is considered to be undesirable as it results in air pollution through the generation of toxic fumes.

Chemical-based recycling involves the degradation of PET into its monomeric, oligomeric, and other chemical forms using various processes involving different harsh chemicals (Park and Kim, 2014). Glycol-based glycolysis, hydrolysis using strong acids and alkali, and aminolysis using primary amines are some of the chemical-based recycling methods (Joo et al., 2018). These methods are not environment-friendly and cost-effective and thus are normally not suggested for the recycling of PET wastes.

In the midst of the aforementioned recycling methods, biocatalytic-based recycling of PET is identified as one of the efficient and eco-friendly strategies for the management of PET wastes. Biocatalytic recycling not only sustainably manages the PET wastes, but also the products formed through this process possess the same properties as the virgin PET. PET hydrolases are identified to play a pivotal role in catalytic-based recycling of PET wastes. However, considering the total PET production, still the total recycling or recovery rate is very low, and most of these wastes enter into landfills and open aquatic environment. Catalytic-based recycling of PET waste is successfully implemented in many countries (France, Japan) and identified as one of the contributing factors in the concept of a circular PET economy (Tournier et al., 2020). Nevertheless, efficient PET hydrolases are very limited in number, and still, only four PET hydrolases (cutinases) have been identified that can significantly degrade PET to its monomers (Kawai et al., 2020). However, other specialized enzymes, *viz*. lipase, esterase, and PETase, have been used by several researchers for the hydrolysis of PET into monomeric forms *viz.* TPA, EG, mono-2-hydroxyethyl terephthalate (MHET), and *bis*(2-hydroxyethyl) terephthalate (BHET) (Wang et al., 2008; Ribitsch et al., 2011; Ma et al., 2012; Bollinger et al., 2020). **Figure 1** represents a schematic diagram showing various recycling methods for PET with associated processes and their outcomes.

In this review, a brief overview of enzymatic recycling of PET wastes and different methods used to enhance the recycling performance are discussed. Various factors affecting the hydrolysis rate and constraints arising during the hydrolysis process are also highlighted in the review.

BIOLOGICAL APPROACH FOR MANAGEMENT OF PET WASTES

The high recalcitrant nature of plastic waste including PET waste is a major bottleneck; however, biological recycling involving enzymatic-hydrolysis of plastic may be used to tackle the menace of plastic/PET pollution in an eco-friendly and efficient way. As mentioned above, PET is linked by ester bonds, which can be hydrolyzed by various hydrolytic enzymes into its monomers TPA, EG, MHET, and BHET (Ronkvist et al., 2009; Ribitsch et al., 2011). **Figure 2** shows the enzymatic hydrolysis of PET. But ester linkages of PET usually have low accessibility and thus become difficult for depolymerization (Austin et al., 2018). However, enzymes possessing esterase/hydrolytic activity have been used widely for the hydrolysis of PET. **Table 1** shows the list of various enzymes (PET hydrolases) reported to hydrolyze PET.

Although various enzymes are reported to degrade PET naturally, the extent of degradation is found to be quite low. Some esterases, for example, PETase (Austin et al., 2018), cutinase (Sulaiman et al., 2012), and lipase (Macedo and Pio, 2005), have been used to hydrolyze BHET to yield MHET. MHET can further enzymatically degrade using MHETase into TPA and EG (Koshti et al., 2018). Factors such as the crystallinity of PET, hydrophobicity, and structure usually limit enzyme function (Koshti et al., 2018). These and other factors are discussed later in the review.

Interest in PET hydrolases gains momentum with the work of Yoshida et al. (2016). Yoshida and coworkers reported a novel bacterium *Ideonella sakaiensis* 201-F6 capable of using low-crystallinity (1.9%) PET film as a major carbon and energy source. *I. sakaiensis*, when grown on the PET, secretes PET-hydrolyzing enzymes (PETase, MHETase), which synergistically hydrolyze PET polymers into monomeric forms. PETase is a key enzyme for PET degradation, and cutinase has shown maximum similarity with PETase (Liu et al., 2018). But PETase has a broader active site compared to cutinase, making high accommodative region for PET (Chen et al., 2018). As cutinases are found in eukaryotes and prokaryotes, it is widely available for biodegradation. In this review, cutinase is covered to a larger extent because of its wide coverage in literature toward PET degradation.

Enzymes Used for PET Hydrolysis

Cutinase

Cutinase (E.C. 3.1.1.74) is majorly produced by either saprophytic microorganism, which utilizes cutin as a carbon source or by phytopathogenic microorganisms for breaking the cutin barrier to enter into the host plants. Cutinase is a serine esterase that has the catalytic triad consisting of Ser–His–Asp residues (Egmond and de Vlieg, 2000). It belongs to the α/β hydrolase superfamily (Egmond and de Vlieg, 2000). The active site of cutinase can accommodate high-molecular-weight compounds such as cutin and other related synthetic compounds. Hydrolysis of synthetic polymers such as PET (Dimarogona et al., 2015), polycaprolactone (Adıgüzel and Tunçer, 2017), polystyrene (PS) (Ho et al., 2018), polyethylene furanoate (Weinberger et al., 2017), and polybutylene succinate (Hu et al., 2016) have also been reported using cutinase. Cutinase-mediated hydrolysis of polylactic acid is also demonstrated by several authors (Masaki et al., 2005; Kitadokoro et al., 2019).

Cutinase is widely reported from fungal and bacterial species. **Table 2** enlists some of these cutinases with their respective PET hydrolysis rates. The catalytic efficiency of cutinase has been observed maximum with *p*-nitrophenyl butyrate and *p*-nitrophenyl acetate (Herrero Acero et al., 2011; Yang et al., 2013), and thus, it has more affinity for the substrate with less carbon chain length compared to other substrates.

Owing to the versatility of hydrolyzing a broad range of ester bonds and to catalyze esterification and transesterification reactions, cutinase is viewed as a promising enzyme for various industrial applications. For instance, it is widely used in the field of oil and dairy products, flavor compounds, and phenolic

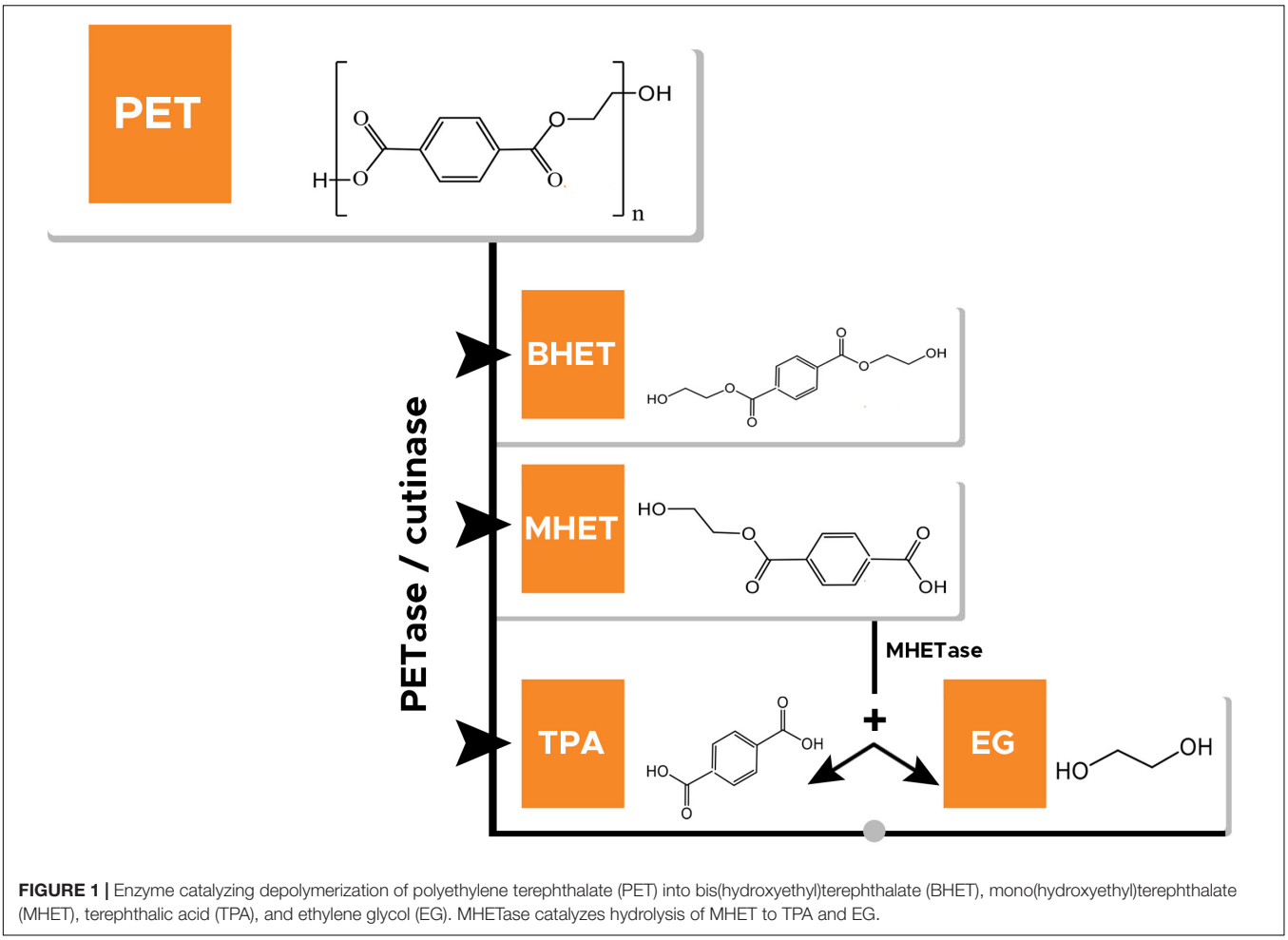

FIGURE 1 | Enzyme catalyzing depolymerization of polyethylene terephthalate (PET) into bis(hydroxyethyl)terephthalate (BHET), mono(hydroxyethyl)terephthalate (MHET), terephthalic acid (TPA), and ethylene glycol (EG). MHETase catalyzes hydrolysis of MHET to TPA and EG.

compounds production (Dutta et al., 2009; Chen et al., 2013). It is also reported in insecticide and pesticide degradation (Dutta et al., 2009; Chen et al., 2013). As an enzyme, it has not only been studied for the degradation of polyesters but has also been used in fiber modification (Alisch et al., 2004). Cutinase possesses valuable properties particularly required for PET degradation, and thus, it has caught the eye of many researchers in recent years. It is a well-studied substitute for harsh chemicals usually practiced during chemical-based hydrolysis/recycling of plastics (Macedo and Pio, 2005; Tournier et al., 2020). Cutinases are also known to synthesize polyesters under non-aqueous media using polycondensation reaction with various diacids and alcohols. In this aspect, cutinase from *Humicola insolens* (HiC) immobilized on Lewatit beads was used by Hunsen et al. (2007) for the synthesis of polyester through the condensation reaction. Similarly, Pellis et al. (2016) used cutinase 1 from *Thermobifida cellulosilytica* for polycondensation of dimethyl adipate with various polyols for the synthesis of high-molecular-weight polyesters.

Lipase

Lipase has also been used by several researchers for the hydrolysis of PET. Effective degradation of PET nanoparticles using lipase from *Candida cylindracea* and *Pseudomonas* sp. has been reported by Ma et al. (2012). Similarly, Wang et al. (2008) employ BHET/TPA-induced lipase from *Aspergillus oryzae* for hydrolysis of PET. Moreover, Carniel et al. (2017) and de Castro et al. (2017) used the combination of lipase from *Candida antarctica* (*C. antarctica* lipase lB CALB) and HiC for efficient PET hydrolysis to TPA. Although HiC showed better performance with PET hydrolysis, the enzyme has limited competence to convert MHET (one of the intermediates of PET hydrolysis) into TPA. On the other hand, CALB can easily convert MHET into TPA but has lower efficiency toward initial PET hydrolysis when used singly. However, the combination of both enzymes synergistically improves the overall PET hydrolysis. However, complete studies on the effect of enzyme dosages, temperature, and pH are lacking. Lipase and cutinase have a common feature of surface hydrophobicity (de Castro et al., 2017). Unlike other lipases, lipase B has a superficial catalytic site; hence, in the absence of the hydrophobic interface, it is still accessible to the substrate (Stauch et al., 2015).

Esterase

Monomers of PET are linked by ester linkage, and these can be cleaved using esterase found in almost all living organisms

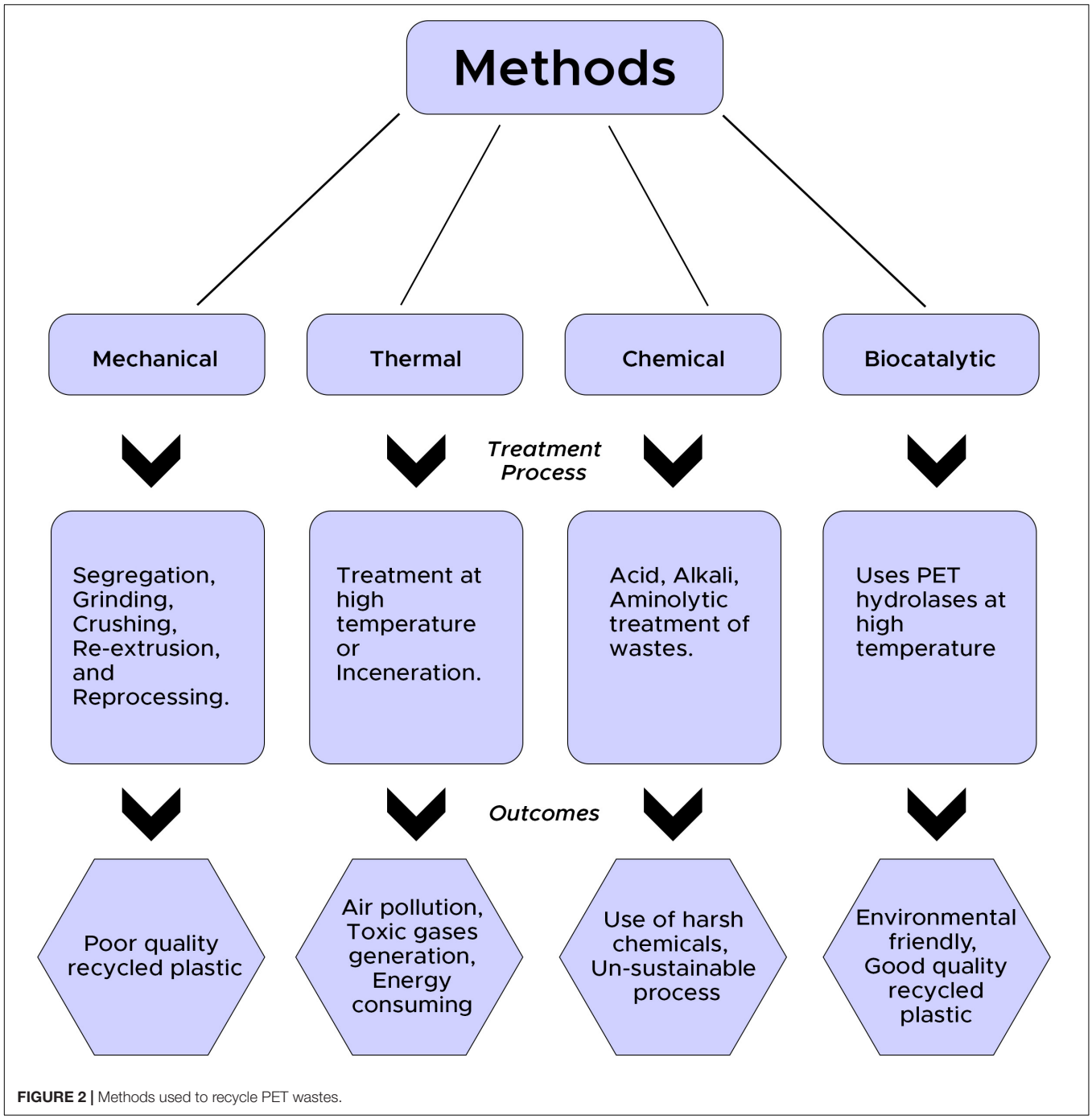

FIGURE 2 | Methods used to recycle PET wastes.

(Koshti et al., 2018). Ribitsch et al. (2011) used *Bacillus subtilis* nitrobenzylesterase (BsEstB) and applied it to hydrolyze PET into TPA and MHET [mono(2-hydroxyethyl)] TPA. Kawai et al. (2014) made use of recombinant thermostabilized polyesterase from *Saccharomonospora viridis* AHK190 capable of hydrolyzing PET and the PET-hydrolyzing activity was observed to increase in presence of Ca ions. Recombinant esterase from *Thermobifida halotolerans* (Thh_Est) was reported by Ribitsch et al. (2012) to degrade PET into TA and MHET.

PETase

PETase (3.1.1.101) was discovered from the bacterium *I. sakaiensis* 201-F6 by Yoshida et al. (2016). PETase and cutinases share high sequence identity, indicating the existence of critical structural features responsible for substrate binding (Fecker et al., 2018; Kawai et al., 2019). Even small differences between these enzymes are crucial and define their specific activities (Chen et al., 2018). High-resolution crystal structure study of PETase highlights the active site, which seems to be wider than the other cutinases, and

TABLE 1 | Microbial enzymes known to hydrolyze PET.

Enzymes	Microorganisms	References
1. PETase	*Ideonella sakaiensis*	Yoshida et al., 2016
2. Cutinase	*Thermobifida fusca*	Müller et al., 2005
	Humicola insolens	Ronkvist et al., 2009
	Thermobifida cellulosilytica	Herrero Acero et al., 2011
	Thermobifida alba	Ribitsch et al., 2012
	Fusarium solani pisi	Sulaiman et al., 2012
	Saccharomonospora viridis	Kawai et al., 2014
	Fusarium oxysporum	Dimarogona et al., 2015
	Aspergillus fumigatus	Ping et al., 2017
3. Lipase	*Triticum aestivum*	Nechwatal et al., 2006
	Thermomyces lanuginosus	Eberl et al., 2009
4. Carboxylesterases	*Thermobifida fusca*	Billig et al., 2010
5. Polyester hydrolase	*Thermomonospora curvata*	Wei et al., 2014
	Pseudomonas aestusnigri	Bollinger et al., 2020

thus this could be a factor of the high specificity of the enzyme toward heavy substrate PET (Chen et al., 2018; Kawai et al., 2020).

Overall, PET hydrolases (PET-hydrolyzing enzymes) are generally limited to cutinases; structurally, they are homologous to lipase, but lack a lid covering the active site (Kawai et al., 2019). This shallow open active site with hydrophobic amino acid residues aids in PET binding and hydrolysis (Kawai et al., 2020). The lid is present in the active site of lipase and is known for interfacial activation in lipases. Lipases are not much active in PET hydrolysis, but like esterases and cutinases, they are known for surface modification of PET fibers. Esterase activity is limited to short-chain acyl esters and thus is also not much reported to hydrolyze hydrophobic PET. Comparative X-ray crystallography data of actinomycetes cutinases and PETase (from *I. sakaiensis*) showed the presence of a broader active site and extra disulfide bond in the latter (Kawai et al., 2020). Also, the active form of cutinases is in the form of a Ca^{2+}-bound state. There is no Ca^{2+}-binding site in the case of PETase. Moreover, serine residue in the catalytic triad of actinomycetes cutinases is replaced with alanine in PETase (Kawai et al., 2020). However, compared to actinomycetes cutinases, PETase is heat liable and act only on lcPET. Considering this, presently researchers are trying to increase the thermostability of PETase and its catalytic efficiency using various protein engineering techniques (Kawai et al., 2020).

The textile or clothing industry is also one of the major producers of PET waste, as it uses polyester as a major raw material. However, the heterogeneous nature of textile waste creates a major hurdle in recycling, as it comprised different types of natural or synthetic plastic wastes. Chemical and mechanical recycling, though, is practiced, but segregation is the first and utmost important step in the recycling of textile wastes. Biocatalytic recycling of textile waste though has potential, but there are limited reports on this aspect. As enzymes are highly specific and thus may target the suitable substrate (PET) in a heterogeneous kind of waste, in this regard, sequential chemical treatment under neutral condition followed by enzymatic treatment for efficient hydrolysis of polyester composed textile waste is reported by Quartinello et al. (2017). Chemical treatment under neutral condition resulted in the production of 85% TA and small oligomers (Quartinello et al., 2017). The oligomers were further hydrolyzed using enzymatic treatment utilizing HiC, yielding 97% of pure TA, available for further recycling. The mixture of PET hydrolases (as mentioned above) could also be used for the biocatalytic conversion of textile polymers into monomers for further recycling. Moreover, compared to other cutinases, actinomycetes cutinases are known to have broad substrate specificity and thus could be used for hydrolysis of a range of polyesters fibers (Kawai et al., 2019).

TABLE 2 | Effect of temperature on enzymatic PET hydrolysis.

Cutinase/hydrolase from microorganism	Optimum temperature for PET degradation	PET degradation rate	References
Humicola insolens	80°C	97% with lcPET with 7% crystallinity	Ronkvist et al., 2009
Pseudomonas mendocina	50°C	5% with lcPET with 7% crystallinity	Ronkvist et al., 2009
Fusarium solani	40°C	5% with lcPET with 7% crystallinity	Ronkvist et al., 2009
Thermobifida cellulosilytica	50°C	24% with semicrystalline PET and 12% with lcPET	Herrero Acero et al., 2011 Gamerith et al., 2017a Gamerith et al., 2017b
Thielavia terrestris	50°C	–	Yang et al., 2013
Thermobifida alba (Tha_cut1)	50°C	–	Ribitsch et al., 2012
Candida antarctica lipase B (CalB)	37–50°C	–(hydrolyze BHET to TPA)	de Castro et al., 2017 Carniel et al., 2017
Leaf and Branch compost cutinase	75°C	25% in 24 h (LCC -NG 95% in 48 h (LCC -G)	Shirke et al., 2018
Thermobifida fusca T. fusca KW3	55°C–60°C 60°C–65°C	50% with lcPET crystallinity of 9% 15.9% ± 1.8% with amorphous PET	Müller et al., 2005 Eberl et al., 2009 Roth et al., 2014 Wei and Zimmermann, 2017
(TfCut2) Tfu_0883/Tfu_0882	40°C or 60°C	–	Silva et al., 2011 Chen et al., 2008

IMPORTANT FACTORS AFFECTING ENZYMATIC PET HYDROLYSIS

Factors such as crystallinity of PET, temperature, pH of the hydrolysis reactions, buffer strength, and nature of substituent/additives present in plastics (as plasticizers) are some of the factors affecting the enzymatic degradation of PET. These factors can affect the enzymatic PET hydrolysis either by altering the enzyme activity or by inhibiting the accessibility to the ester linkage of the PET.

Crystallinity(CrI) of PET

Low-CrI PET (lcPET) is easily degradable compared to those with high CrI. High-CrI PET shows high tensile strength and more compactness, and thus, it is difficult for the enzyme to access the ester linkage. Hence, the degradation is hampered. The different enzyme also shows specificity toward particular CrI, for instance, *Pseudomonas mendocina* cutinase (PmC) showed high affinity toward lcPET of 7% CrI compared to *Fusarium solani* cutinase (FsC) (Ronkvist et al., 2009). Cutinase isolated from *T. cellulosilytica* hydrolyzes high-CrI PET at a slower rate compared with lcPET (Herrero Acero et al., 2011). Aliphatic polyesters have low CrI and low T_g in comparison to semiaromatic polyesters like PET; hence, the latter has limited access to ester linkage (Austin et al., 2018).

Temperature

Usually high temperature results in better degradation of PET, as it creates flexibility and easy accessibility to ester bonds. Moreover, if the enzyme remains stable up to the T_g of PET (70–80°C), and then hydrolysis will further be increased. Thus, there is a need for thermostable enzyme, which can optimally work at high temperature or T_g of PET (70°C). Ronkvist et al. (2009) used *Humicola insolens* cutinase (HiC) to degrade lcPET and observed complete degradation within 94 h of treatment at 70°C, whereas PmC and FsC could only degrade 5% of PET at 50 and 40°C, respectively. Both the enzymes were found to be ineffective with respect to PET degradation at 70°C. Müller et al. (2005) observed only 50% loss in weight of PET at 55°C within 3 weeks of enzymatic treatment using a high concentration of cutinase from *Thermobifida fusca* (*TfC*). On the other hand, 97% PET degradation at 80°C within 96 h was detected using HiC with lesser enzyme content (0.13 mg mL^{-1}) (Ronkvist et al., 2009), indicating superiority of HiC over TfC considering PET hydrolysis. Indeed, HiC can degrade close to T_g of PET, indicating a better option for PET degradation.

pH

Cutinase from most of the organisms are highly active at alkaline pH. However, during continuous enzymatic PET hydrolysis, the media become acidic due to the formation of monomer TPA. This acidic condition affects the enzymatic hydrolysis rate (Ronkvist et al., 2009). Thus, for effective PET hydrolysis, besides thermostability, a broad range of pH stability (toward both alkaline and acidic range) of the enzyme is also one of the prerequisites. Unlike other cutinases, the enzyme from *Thielavia terrestris* was observed to be active at acidic pH in addition to high temperature tolerance, and thus, it is used by Yang et al. (2013) for effective PET degradation.

As TPA-induced acidification of the reaction media is known to inhibit enzymatic activity, increase in buffer strength/concentration lowers this enzyme inhibition (Gamerith et al., 2017a; Furukawa et al., 2019). According to Schmidt et al. (2016), ionic strength and choice of buffer (Tris–HCl, phosphate, MOPS, HEPES) thus affect the enzymatic hydrolysis of PET. Thus, along with pH, the ionic strength of the buffer also affects the PET hydrolysis rate.

Specificity of the Enzyme

Mostly PET is characterized by an amorphous fraction, which is easily accessed by the enzyme and catalytically converted into oligomers or monomers, whereas the crystalline fraction is more rigid and has limited access by the enzyme. Thus, hcPET has lower degradation rates with hydrolases. Generally, PET hydrolases catalyze endo-type hydrolysis activity by cleaving internal ester bonds of PET, resulting in the formation of oligomers (Kawai et al., 2019). However, Wei et al. (2019) using nuclear magnetic resonance spectroscopy detected both endo- and exo-type hydrolysis activity toward the amorphous region of PET (food-packing container) using recombinant PET hydrolase (thermophilic TfCut2 from *T. fusca*) expressed in *B. subtilis*. With the combination of endo and exo activity, an amorphous region is rapidly hydrolyzed by the recombinant enzyme, resulting in >50% weight loss after 96 h of enzymatic treatment at 70°C. The remaining crystalline structure is hydrolyzed slowly with only endo-chain scission activity, resulting in no detectable weight loss (Wei et al., 2019).

METHODS TO ENHANCE THERMOSTABILITY OF PET HYDROLASES

As mentioned previously, enzymatic treatment at high temperature favors the hydrolysis of PET. High temperature above PET T_g (70°C) increases PET/organic polymers mobility, thereby allowing more accessibility to ester links between monomeric units (Shirke et al., 2018). However, high temperature (>70°C) results in kinetic instability and loss of activity. Various methods, *viz.* use of ionic liquids (Jia et al., 2013) phthalic anhydride and glucosamine hydrochloride (Liu et al., 2002) or through other suitable modifiers like Ca^{2+} (DeSantis and Jones, 1999; Miyakawa et al., 2015) and immobilization on suitable matrices (Kumar et al., 2006; Singh et al., 2013), have been used to increase the thermostability of PET hydrolases. The next section discusses some of these approaches.

Screening and Sourcing of Heat-Stable Enzymes From Hyperthermophilic Microbes

Thermozymes from thermophilic organisms are one of the important sources of heat-stable enzymes. These enzymes possess

various adaptive strategies that help them to function in high temperature. These adaptive strategies are very well covered in the review of Han et al. (2019) and Kumar et al. (2019). These types of natural thermophilic PET hydrolases thus could efficiently be used for the management of PET wastes.

Immobilization

To increase the catalytic efficiency, stability, specificity, and selectivity of enzymes, immobilization of the enzyme system in suitable matrices is usually carried out (Samak et al., 2020). This enhancement in enzymatic properties is due to favorable structural changes in enzyme as a result of immobilization (Samak et al., 2020). PET hydrolases/cutinases have been immobilized on different matrixes for increasing thermostability. However, these immobilized systems were majorly used for synthesis reactions instead of PET hydrolysis. To date, there are limited reports on PET hydrolysis using immobilized PET hydrolases.

Cutinases from HiC, immobilized on Lewatit VP OC 1600, not only enhance the thermostability at 90°C but also confer the enzyme conformational rigidity in the organic surrounding having low water content (Su et al., 2018). This immobilized HiC was later used for the synthesis of butyl laureate using butanol and lauric acid in organic solvent–rich media. Generally, solubility/rigidity of PET polymers increases/lowers in organic solvents, thereby allowing easy accessibility of enzyme to ester bonds of PET for efficient hydrolysis. Hence, this immobilized HiC could also be used for the hydrolysis of PET. Similarly, Nikolaivits et al. (2017) used cross-linked enzyme aggregates (CLEAs) for the immobilization of cutinases from Fusarium oxysporum. The immobilized cutinases showed enhanced thermostability and the system was efficiently used for the synthesis of short-chain butyrate ester as a flavoring compound. On the other hand, Barth et al. (2016) applied cutinase from T. fusca KW3 (TfCut2) immobilized on SulfoLink coupling resin and free LC-cutinase for enhanced and complete hydrolysis of amorphous PET film at 60°C. This dual enzymatic system resulted in a 2.4 fold high degradation rate at 60°compared to free TfCut2 at 24 h of incubation. **Table 3** enlists various immobilization matrixes used for the immobilization of cutinases for increasing thermostability and their applications in synthesis as well as hydrolysis reactions.

Glycosylation of Enzyme

Attachment of polysaccharide chains "glycans" to proteins is termed as glycosylation. Modification of protease through glycosylation resulted in a noticeable effect on kinetics, structure, folding, and stability of enzyme (Goettig, 2016). Glycosylation-based modification has been performed on cellulose (Greene et al., 2015), lipase (Pinholt et al., 2010), and α-amylase (Hu et al., 2019) to achieve enhance enzymatic thermostability. In *Thielavia terrestris*, cutinase glycosylation was introduced that inhibited thermal aggregation (Shirke et al., 2016b). Similarly, glycosylation site engineering was introduced in the cutinase of *A. oryzae* showing the same effect (Shirke et al., 2016a). Leaf and branch compost originated cutinase (LCC) was glycosylated (LCC-G) and compared with non-glycosylated (LCC-NG) (Shirke et al., 2018). Glycosylation helps the protein to reach T_g temperature of PET without adversely affecting the protein. Aggregation of LCC-NG was observed at 70°C due to hydrophobic interaction, whereas LCC-G showed 10°C higher temperature to achieve the same level of aggregation.

Use of Surfactants and Additives

The activity of enzymes can be influenced by the application of surfactants. Through binding with the enzymes, surfactants can alter the secondary and tertiary structures or flexibility of enzyme, thereby affecting the enzyme kinetic properties (Rubingh, 1996). In addition to that, the use of surfactants can also improve the dispersibility of PET particles and thus may increase the accessibility of the substrate to enzymes. Chen et al. (2010) in their study recorded increased degradation of PET film (73.65%) using cutinase from *F. solani pisi* in presence of surfactant sodium taurodeoxycholate in the reaction mixture. On the same note, the addition of surfactant alkyl trimethyl ammonium chloride in the reaction media resulted in complete degradation of PET at varied temperature using *Thermobifida fusca* double-mutant cutinase (TfCut2) (Furukawa et al., 2019). Similarly, the use of additives, *viz*. Ca^{2+}, Mg^{2+}, and hydrophobins (cysteine-rich proteins), has also been used to increase the enzymatic PET hydrolysis (Espino-Rammer et al., 2013; Then et al., 2015; Fukuoka et al., 2016; Joo et al., 2018).

Hydrophobins are cysteine-rich fungal proteins laden with high surface-active substances; it helps in creating interfaces between hydrophobic and hydrophilic phases through their natural tendency of absorption to hydrophobic surfaces (Ribitsch et al., 2015). In this way, they assist in establishing proper interaction of hydrophobic substrates with the active site of enzymes. Several researchers fused hydrophobins with PET hydrolases for increasing PET-hydrolyzing activity (Ribitsch et al., 2015; Puspitasari et al., 2020). As far as Ca^{2+} is concerned, cutinases possess three Ca^{2+}-binding sites, and this binding results in activation of the enzyme. Furthermore, Ca^{2+}-bound enzyme is prone to less fluctuation compared to the unbound form of an enzyme (Kawai et al., 2020). This stability due to the binding of Ca^{2+} results in higher temperature tolerance and thus helps in PET hydrolysis (Kawai et al., 2020). Furthermore, the fusion of PET hydrolases with the substrate-binding module (SBM) also has been reported to increases PET hydrolysis. Enzymes acting on an insoluble substrate contain SBM for proper interaction with substrates. Altering the surface properties of cutinases through fusion with SBM from other enzymes facilitates the absorption of PET and *vis-à-vis* PET hydrolysis (Kawai et al., 2020). In this regard, Ribitsch et al. (2013) fused cutinase from *Thermomyces cellullosylitica* (Thc_Cut1) separately with the carbohydrate-binding module of cellobiohydrolase I from *Trichoderma reesei* and polyhydroxyalkanoate (PHA)–binding module of polyhydroxyalkanoate depolymerase from *Alcaligenes faecalis*. The catalytic domain of cutinase was linked with SBM through

TABLE 3 | Immobilization of cutinases on various matrixes for increasing thermostability and their applications in synthesis and hydrolytic reactions.

Microorganism and enzyme	Matrix interaction	Increase in thermal stability	Hydrolysis/synthesis reactions	References
Fusarium solani pisi recombinant cutinase	Silica–covalent bonding	Optimum temperature from 40 to 50∘C	Tricaprylin hydrolysis	Gonçalves et al., 1996
*Fusarium oxysporum*cutinase	Nanoporous gold–polyethyleneimine combination of covalent, electrostatic, and physical adsorption	Maximum activity at 40∘C compared to free enzyme	As adsorbent for removal of contaminants	Zhang et al., 2014
Thermobifida fusca KW3 cutinase (TfCut2)	SulfoLink coupling resin–covalent Interaction	Increased stability at 60∘C	Amorphous PET film hydrolysis in combination with LC cutinase	Barth et al., 2016
Humicola insolens cutinase (HiC)	EC-EP Sepabeads–covalent interaction	Polycondensation at 70∘C	Synthesis of polyesters from diacids/diesters and linear diols	Ferrario et al., 2016
Thermobifida cellulosilytica (Thc_Cut1)	EC-EP Sepabeads– covalent interaction	Polycondensation at 70∘C	Synthesis of polyesters from diacids/diesters and linear diols	Ferrario et al., 2016
Aspergillus sp. RL2Ct, cutinase	Biopolymer graft copolymerization– adsorption	Increase by ∼20% at 35∘C	*p*-nitrophenylbutyrate hydrolysis	Kumari et al., 2017
Fusarium oxysporum cutinase	Cross-linked enzyme aggregates (CLEA)–non-covalent interaction	Thermostability increased by 10% (50∘C)	Synthesis of short-chain butyrate esters	Nikolaivits et al., 2017
Thermobifida Cellulosilytica (Thc_Cut1)	Opal, coral, amber beads chelated with Fe ions via His-tag binding–covalent interaction	57%–78% monomer conversion at 21∘C	Synthesis of aliphatic polyesters	Pellis et al., 2017
Aspergillus oryzae cutinase (AoC)	Lewatit VP OC 1600–hydrophobic interaction	AoC activity increased 43% from 40∘C to 70∘C and decreased ∼30% at 80∘C and 90∘C	Butyl laurate synthesis in organic solvent (nonane)	Su et al., 2018
Humicola insolens cutinase (HiC)	Lewatit VP OC 1600–hydrophobic interaction	HiC activity increased from 40∘C to 75∘C and decreased ∼15% at 80∘C and 90∘C	Butyl laurate synthesis in organic solvent (nonane)	Su et al., 2018
Thielavia terrestris cutinase (TtC)	Lewatit VP OC 1600–hydrophobic interaction	TtC activity increased 60% from 40∘C to 60∘C and showed no significant change at 70∘C, 80∘C, and 90∘C	Butyl laurate synthesis in organic solvent (nonane).	Su et al., 2018

**Application in removal of contaminants, not hydrolysis and synthesis reactions.*

the spacer and thereafter cloned and expressed in *Escherichia coli*. Both fused enzymes showed enhanced hydrophobic interactions with PET and catalytic degradation compared to native cutinase.

Use of Ultrasonic Waves

Ultrasonic waves are high-frequency waves exceeding 20 kHz and are used to increase the enzymatic PET hydrolysis. Pellis et al. (2016) visualized the effect of ultrasonic waves on enzymatic reaction and observed enhanced PET hydrolysis of various CrI. Sonication of amorphous PET (8% CrI) with Thc_Cut1 (cutinase) during hydrolysis reaction showed an increase of 5.2-fold in TA concentration and 6.6-fold in MHET concentration compared to control. The same reaction with PET of 28% CrI resulted in 2.9-fold increase in TA level. For amorphous PET, better enzymatic hydrolysis was observed with 10 min of sonication, whereas for high-CrI PET, it was observed to be 30 min. According to Pellis et al. (2016), sonication causes changes in the secondary structure of an enzyme, and this unfolding/changes with exposed hydrophobic groups lead to increased enzyme–substrate affinity and *vis-à-vis* enhanced hydrolysis rate. Also, the lesser hydrolytic rate observed in the case of film substrate (PET) compared to amorphous PET is due to less accessibility to the substrate surface in a film.

Use of Mixture of Enzymes

Use of consortia/mixture of enzymes *viz.* lipases, esterases, and cutinases has been reported by various authors for increasing PET hydrolysis. Carniel et al. (2017) investigated the synergistic study with HiC and CALB. HiC acts as PETase and converts PET to MHET, whereas CALB acts as MHETase converting MHET to TPA or BHET to TPA. The mixture of enzymes causes a 7.7-fold increase in TPA as compared to HiC alone. With synergistic action of enzymes, degradation is found to be effective with hcPET bottles also.

Engineered Enzymes

In the recent past few years, the most thrilling development is the application of genetic engineering to enzyme technology. A number of enzymatic properties can be altered or improved by genetic engineering to achieve better kinetics of the enzyme and easy downstream processing. This process/technique includes changing amino acid sequences through recombinant DNA mutation. In this regard, double mutation of PETase caused increased PET degradation as a result of more interaction and

high binding affinity with the PET (Ma et al., 2018). Similarly, Furukawa et al. (2019) worked with *T. fusca* double-mutant cutinase and observed complete PET degradation.

Site-directed mutagenesis was performed on *T. fusca* cutinase (Tfu_0883), thereby enhancing its activity on PET through substituting bulky amino acid on active site by smaller residue such as alanine (Silva et al., 2011). This provides a less restrained active site to Tfu_0883 and allows better accommodation of PET. Cutinase (Cut190*) from *S. viridis* AHK190 is the only cutinase exhibiting inactive or active state based on binding of Ca^{2+} in its binding site, where it (Ca^{2+}) plays activation and thermal stabilization functions (Oda et al., 2018). Cut190* has three Ca^{2+}-binding sites having different amino acids at each site. A mutant at these sites has shown the highest T_m values and highest PET degradation of (0.25-mm-thick film of amorphous PET, CrI of 6.3%) more than 30% at 70°C (Oda et al., 2018). Recently, Tournier et al. (2020) modified earlier known as leaf-branch compost cutinase (LCC) using site-specific saturation mutagenesis through altering 11 amino acid residues in the enzyme active site. The resulting modified enzyme showed more than 90% depolymerization of PET into monomers within 10 h of treatment. According to the authors, this is the fastest PET-hydrolyzing enzyme reported so far.

CONSTRAINTS WITH ENZYMATIC PET HYDROLYSIS

Although PET hydrolases, *viz.* cutinase, PETase, lipase, and esterase, have been reported to hydrolyze PET in various forms, but the following constraints usually lower the efficacy of the process:

(1) Low catalytic turnover due to limited accessibility to active sites as a result of high CrI of the substrate.
(2) Inhibition by MHET or intermediate metabolites.
(3) Kinetic instability and loss of activity at the temperature above PETs T_g (70°C) (Shirke et al., 2018).
(4) During biodegradation/hydrolysis, solution gets acidic due to end products leading to inactivation of enzyme and hence slower reaction rate.

CONCLUSION AND PROSPECTS

Among various available methods known for recycling PET, enzymatic methods are considered an environmentally safer and efficient method for managing the PET wastes. Enzyme prominently cutinases are proved to be quite effective in PET hydrolysis. Enzymatic hydrolysis of PET is favored at high temperature, but limited PET hydrolases are known to be active at high temperature. Thus, kinetic instability of enzymes at high temperature is one of the major constraints toward PET hydrolysis. In addition to that, high CrI and low solubility of the substrate and acidic pH of the media during hydrolysis are some of the major factors limiting the hydrolysis rate. However, various modifications such as glycosylation and immobilization of enzymes could be used to enhance thermostability and enzyme activity toward PET. High-CrI PET has higher T_g, and for hydrolysis of these PET, there is a need for enzymes that are stable at such high temperature. Most industries use high CrI plastics, which have high tensile strength and stiffness and are less affected by solvents. Recycling such plastics has become a tedious problem. Additionally, enzymatic treatment of mixed wastes arising from the textile industry too is a problem, as this enzyme needs to have broad substrate specificity. If the search for a novel thermostable PET-hydrolyzable enzyme capable of hydrolyzing hcPET with broad substrate specificity is fulfilled, then this will ultimately help in the overall curbing of plastic pollution in a sustainable pattern.

AUTHOR CONTRIBUTIONS

AM and AB designed and wrote the manuscript, while SK conceived the idea and critically review the manuscript with valuable inputs. All authors contributed to the article and approved the submitted version.

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Biodegradation of Unpretreated Low-Density Polyethylene (LDPE) by *Stenotrophomonas* sp. and *Achromobacter* sp., Isolated From Waste Dumpsite and Drilling Fluid

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Exploring the catabolic repertoire of natural bacteria for biodegradation of plastics is one of the priority areas of biotechnology research. Low Density Polyethylene (LDPE) is recalcitrant and poses serious threats to our environment. The present study explored the LDPE biodegradation potential of aerobic bacteria enriched from municipal waste dumpsite and bentonite based drilling fluids from a deep subsurface drilling operation. Considerable bacterial growth coupled with significant weight loss of the LDPE beads (∼8%), change in pH to acidic condition and biofilm cell growth around the beads (CFU count 10^5–10^6/cm^2) were noted for two samples (P and DF2). The enriched microbial consortia thus obtained displayed high (65–90%) cell surface hydrophobicity, confirming their potential toward LDPE adhesion as well as biofilm formation. Two LDPE degrading bacterial strains affiliated to *Stenotrophomonas* sp. and *Achromobacter* sp. were isolated as pure culture from P and DF2 enrichments. 16S rRNA gene sequences of these isolates indicated their taxonomic novelty. Further biodegradation studies provided strong evidence toward the LDPE metabolizing ability of these two organisms. Atomic Fore Microscopy (AFM) and Scanning Electron Microscopy (SEM) revealed considerable damage (in terms of formation of cracks, grooves, etc.) on the micrometric surface of the LDPE film. Analysis of the average roughness (Ra), root mean square roughness (Rq), average height (Rz), maximum peak height (Rp), and maximum valley depth (Rv) (nano-roughness parameters) through AFM indicated 2–3 fold increase in nano-roughness of the LDPE film. FTIR analysis suggested incorporation of alkoxy (1000–1090 cm^{-1}), acyl (1220 cm^{-1}), nitro (1500–1600 cm^{-1}), carbonyl (1720 cm^{-1}) groups into the carbon backbone, formation of N-O stretching (1360 cm^{-1}) and chain scission (905 cm^{-1}) in the microbially treated LDPEs. Increase in carbonyl index (15–20 fold), double bond index (1.5–2 fold) and terminal double bond index (30–40 fold) confirmed that biodegraded LDPEs had undergone oxidation, vinylene formation and chain scission. The data suggested that oxidation and dehydrogenation could be the key steps allowing formation of low molecular weight products suitable for their further mineralization by the test bacteria. The study highlighted LDPE degrading ability of natural bacteria and provided the opportunity for their development in plastic remediation process.

Keywords: LDPE, biodegradation, *Stenotrophomonas*, *Achromobacter*, bioremediation

INTRODUCTION

Despite being a severe threat to the environment, plastic has become a very fundamental part of human society (Danso et al., 2019). It is widely used in various fields ranging from industries, agriculture to our day to day life, due to its lightness, durability, inertness and cheapness, etc., (Orr et al., 2004; Sudhakar et al., 2008; Yuan et al., 2020). Plastics are polymers of small aliphatic (for example polyethylene is made of [CH_2–CH_2] monomer) or aromatic molecules (e.g., polystyrene is a polymer of styrene) and their derivatives (such as polyvinyl chloride made from vinyl chloride monomer) (Andrady and Neal, 2009; Yang et al., 2014). Almost 6.3 billion tons of plastics were produced worldwide in 2015 and the number is increasing exponentially each year because of its efficient and versatile use (Yuan et al., 2020). Although plastics bring ease to our daily life, their uncontrolled use and careless disposal have been imposing a constant threat to the ecosystem, since they don't get degraded naturally even after many years and interfere with various natural and engineered processes adversely (Albertsson and Karlsson, 1990; Tokiwa et al., 2009; Raddadi and Fava, 2019). Non/slow biodegradation of plastics has led to their accumulation in the environment, thus causing wide spread pollution and harming marine as well as terrestrial life forms (Raddadi and Fava, 2019; Li et al., 2020). Plastics not only cause flooding by blocking the water draining system, but also get incorporated into the food chain of animals and damage their digestive system (Teuten et al., 2009; Muhonja et al., 2018). Long term accumulation of plastics in soil even changes its microbial community structure (Harshvardhan and Jha, 2013; Huang et al., 2019; Fei et al., 2020). Although, the amount of plastic wastes is reduced by incineration, it leads to secondary pollution due to the production of air pollutants like carbon monoxide, nitrogen oxides, etc., (Ru et al., 2020). The weathering and breaking down of plastic produce microplastics (MP) which migrate toward rivers, ponds, lakes, oceans and agricultural fields and affect them adversely (Yuan et al., 2020). Most of the commercially used plastics like polyethylene (PE) (low density, i.e., LDPE and high density or HDPE), polypropylene, polystyrene, polyvinyl chloride (PVC), polyamide (PA), polyethylene terephthalate (PET) are usually resistant toward biodegradation (Tokiwa et al., 2009; Danso et al., 2019). Their biodegradability is generally hindered due to several factors such as, (1) not being able to enter the microbial cell because of high molecular weight, (2) better stability in chemical structure, (3) absence of functional groups where microbial enzymes can attack and (4) high hydrophobicity and degree of crystallinity due to large carbon backbone (Tokiwa et al., 2009; Yang et al., 2014). Environmental toxicity, large-scale accumulation and persistence of plastics warrant immediate action on development of efficient and ecofriendly methods for their degradation and exploration of microbial catabolic potential toward biodegradation of plastics (Montazer et al., 2018; Danso et al., 2019).

It has been found that the most common plastic, e.g., polyethylene (PE) can be subjected to microbial degradation if it is made with added pro-oxidants (generally transition metals) (Lee et al., 1991; Bonhomme et al., 2003). It has been observed that microbial growth and thus degradation of PE can be facilitated by several pretreatments like photo-oxidation (by exposing under UV irradiation), heat treatment (heating with high temperature) or chemical treatment (using acid). These pretreatments usually reduce the hydrophobicity by incorporating various functional groups (carbonyl, keto, nitro, etc.) into the inert carbon backbone (Albertsson et al., 1987; Orr et al., 2004; Hadad et al., 2005). The complex process of complete biodegradation of polyethylene follows these steps: (a) fragmentation by microbial intervention (adhering to the surface of polyethylene) or environmental components and incorporation of functional groups if applicable; (b) breaking down of polymer into oligomers or monomers as well as fatty acids, ketons, aldehydes, alcohols, etc., by enzymatic attack and free radicals; (c) uptake of these small products inside the microbial cells; (d) utilization of those molecules in cellular metabolism and finally production of CO_2, N_2, CH_4, H_2O, etc., (Yang et al., 2014; Wilkes and Aristilde, 2017; Muhonja et al., 2018; Delacuvellerie et al., 2019; Park and Kim, 2019). Both culture-based and culture-independent metagenomic studies have highlighted the PE (LDPE or HDPE) biodegradation abilities of several bacterial taxa *viz. Enterobacter, Bacillus* (Yang et al., 2014); *Brevibacillus* (Hadad et al., 2005); *Pseudomonas* (Muhonja et al., 2018); *Alcanivorax, Ideonella, Marinobacter, Arenibacter* (Delacuvellerie et al., 2019); *Aneurinibacillus* (Raddadi and Fava, 2019); *Chelatococcus* (Yoon et al., 2012); *Achromobacter* (Kowalczyk et al., 2016); *Comamonas, Stenotrophomonas* and *Delftia* (Peixoto et al., 2017). Besides, several members of soil-inhabiting *Actinobacteria* (*Rhodococcus* sp., *Streptomyces coelicoflavus*, *Streptomyces* KU1, KU5, KU6, KU8, *Streptomyces werraensis*, *Streptomyces humidus*, *Streptomyces parvullus*, *Streptomyces aburaviensis*, *Amycolatopsis* sp. HT-32, *Nocardia* sp. *Saccharothrix wayandensis*, *etc.*) have shown either weight reduction or partial degradation of PE films (Sivan et al., 2006; Abraham et al., 2017; Huang et al., 2019; Soulenthone et al., 2020). Among the fungal species, members of *Aspergillus* (Muhonja et al., 2018); *Fusarium* (Sivan, 2011); *Penicillium, Zalerion* (Raddadi and Fava, 2019); *Chaetomium* and *Pullularia* (Sudhakar et al., 2008) are well known for biodegradation of LDPEs and HDPEs. Microbial enzymes playing important role in biodegradation of PE are identified to be proteases, lipases, cutinases, laccases, manganese peroxidases, lignin peroxidases, alkane hydroxylases, etc., (Tokiwa et al., 2009; Bhardwaj et al., 2013; Wei and Zimmermann, 2017; Ahmed et al., 2018). Most of the prior studies have been done to assess the biodegradation of pretreated PEs. It is also observed that LDPE biodegradation takes place in a very slow manner even after the pretreatment (Sivan, 2011). In comparison to these studies, biodegradation of un-pretreated LDPE is not yet well studied. Identification and characterization of microbial enzymes and their molecular mechanisms have been considered to be critical in order to develop biotechnological process for plastic remediation (Danso et al., 2019).

The present study was conducted as a first step toward development of microbial plastic bioremediation through evaluating the potential of natural microbial communities and

isolating suitable bacterial strains with desirable biocatalytic abilities. LDPE biodegradation potential of naturally occurring aerobic microorganisms (present in municipal waste dump site soil and bentonite based drilling fluids used during deep drilling of igneous crust of Deccan Traps, India) was evaluated by using enrichment culture technique. Degradation of LDPE by the enriched microorganisms was evaluated through a number of analytical techniques including examining the weight loss (of LDPE), change in its surface morphology (SEM and AFM) and chemical modification (FTIR). Two selected bacterial strains isolated from these enrichments were further studied for their individual ability of LDPE biodegradation. These strains were identified through 16S rRNA gene sequencing.

MATERIALS AND METHODS

PE Materials, Samples for Microbiological Isolation, Media and Growth Conditions

Two low-density polyethylene (PE) materials were used in this study as the carbon source: (a) Low Density Polyethylene beads (LDPE beads) (Sigma-Aldrich, United States) (b) Low Density Polyethylene plastic films (LDPE plastic films) (Ranco Poly Bags, Haryana, India). Before use, LDPE beads and films were surface sterilized by washing with 70% ethanol and followed by exposing them to UV rays for 15 min.

Two types of environmental samples were used as the source of LDPE degrading microorganisms: (1) waste plastic sample from landfill soil and (2) drilling fluid. Waste plastic samples were collected from the local landfill site (designated as P) inside the IIT Kharagpur campus (22° 18' 45.3" N 87° 19' 28.1" E). The drilling fluid (designated as DF2, DF4, DF7) samples were collected during a 3 km deep pilot borehole (KFD-1) drilling in the Koyna region (17° 24' 6" N 73°45'8" E) of the Deccan Traps, Maharashtra under the pilot phase of the Koyna scientific drilling project (Bose et al., 2020; Roy and Bansal, 2020). The various depths were as follows: DF2: 1901.255 mbs; DF4:2335.62 mbs; DF7: 2908.52 mbs (mbs: meters below surface). Samples were collected in sterile autoclaved bags with the help of sterile equipment. The DF samples were kept at 4 °C during transportation to lab and thereafter they were transferred to −80 °C and stored until further processing. Dumpsite plastic samples were collected from five sites and stored at 4 °C. They were mixed to use as an inoculum for setting the enrichment within 48 h of sample collection. Drilling fluid (1 g each) samples were incubated with 10 mL sodium pyrophosphate (0.1% w/v) overnight under shaking condition to dislodge the cells, and one mL of the supernatant was used as final inoculum.

The carbon free basal medium used for the enrichment of LDPE degrading bacteria had the following composition: 12.5 g/L K_2HPO_4; 3.8 g/L KH_2PO_4; 1.0 g/L $(NH_4)_2SO_4$; 0.1 g/L $MgSO_4 \cdot 7H_2O$ (pH 7.0) and 5 mL trace element solution was mixed in 1 L of the medium. The trace element solution consisted of the following: 0.232 g/L H_3BO_3; 0.174 g/L $ZnSO_4 \cdot 7H_2O$; 0.116 g/L $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$; 0.096 g/L $CoSO_4 \cdot 7H_2O$; 0.022 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; 8.0 g/L $CuSO_4 \cdot 5H_2O$; 8.0 g/L $MnSO_4 \cdot 4H_2O$ (Kyaw et al., 2012). Reasoner's 2A agar (modified to reduce the carbon content by 10 fold) was used for isolation of bacterial strains containing the following components: 0.05 g/L Yeast extract, 0.05 g/L Peptone, 0.5 g/L Casamino acid, 0.05 g/L Glucose, 0.05 g/L Starch, 0.3 g/L K_2HPO_4, 0.05 g/L $MgSO_4$, 0.3 g/L Sodium Pyruvate; 15 g/L Agar. The composition of modified Luria Bertani (LB) agar medium (M/5) used to determine the biofilm cell growth was as follows: Casein enzyme hydrolyzate 2 g/L, Yeast extract 1 g/L, Sodium chloride 2 g/L, Agar 15 g/L (pH $= 7.5 \pm 0.2$). All the enrichment setups were incubated aerobically at 30 °C in an incubator shaker at 150 rpm for 100 days.

Enrichment (Setup) and Isolation of PE Degrading Bacteria

P (approximately 10 pieces) and DF (DF2, DF4, DF7) (1 mL) samples were inoculated in 100 mL of basal medium with LDPE as C source. Ten sterilized LDPE beads of around 0.3 g were used as the sole carbon source. Along with that, separate negative control (without inoculum) was prepared. The samples along with negative control (without bacterial inoculum) were kept at 30 °C for 100 days in an incubator shaker (150 rpm). All experiments were set up in duplicates.

After incubation, biofilm or the bacteria present on the surface of the plastic beads were collected according to Kyaw et al. (2012). The beads were immersed in 2 mL of 0.9% NaCl. They were incubated overnight at room temperature. After incubation, they were vortexed properly for few minutes in order to prepare a cell suspension. Hundred μL liquid cell suspension from each sample was inoculated on the modified R2A agar and kept for incubation at 30 °C for 48 h. The colonies obtained were sub-cultured on the same medium for further studies.

Measurement of Microbial Cell Growth and Change in Medium pH

Following microbial growth, biofilm formation on the surface of the LDPE beads was determined according to the method described by Manijeh et al. (2008). LDPE beads from enrichment cultures were suspended into 0.9% NaCl and vortexed after overnight incubation as described earlier. The resulting suspension was serially diluted within 0.9% NaCl up to 10^5 fold and 0.1 mL of the diluted sample was plated on modified Luria Bertani Agar plates. The CFU was counted after overnight incubation at 30 °C and the following formula was used to determine the biofilm cell growth.

Biofilm cell growth = log [(average CFU/drop volume) × (dilution counted) × (volume scraped into/surface area)].

In order to measure the microbial growth after incubation, 1 mL of liquid suspension was taken and absorbance (OD_{600}) was determined using a UV-VIS spectrophotometer. Similarly, change in pH was also monitored using a pH meter.

PE Weight Reduction and Hydrophobicity

LDPE beads recovered from the enrichment cultures after 100 days of incubation were washed properly to remove the microbial cells to get the accurate measurement of the weight. These beads were incubated with 2% (w/v) aqueous sodium dodecyl sulfate solution for 4 h in shaking condition, followed by washing with distilled water and 70% ethanol in order to remove the bacterial cells and other cell debris (Orr et al., 2004). The beads were dried at 70 °C. The weight reduction percentage was calculated using the formula mentioned below (Kyaw et al., 2012).

Percentage of Weight Reduction = (Initial Weight – Final Weight) × 100/Initial Weight

Bacterial adhesion to hydrocarbon (BATH) test was carried out to calculate the bacterial cell surface hydrophobicity (Rosenberg et al., 1980). Microorganisms enriched in the different setups were re-inoculated in R2A agar medium and were allowed to grow until they reached mid-logarithmic phase. The microbial cell pellets were obtained by centrifugation and washed with PBS buffer (pH 7.1). The washed cell pellets were re-suspended in fresh PBS buffer. Aliquots of 4 mL each were transferred from the resuspension to fresh test tubes and 1.5 mL of hexadecane was added to them. Solution was vortexed for 2 min and kept still for 15 min to obtain the phase separation. The optical density of the separated phase was measured at 600 nm (OD_{600}). Cell-free buffer served as blank. Adherence percentage to hexadecane or BATH was determined using the following formula (Tribedi and Sil, 2013).

Cell surface hydrophobicity (%) = 100 × {(initial OD – final OD)/initial OD}

Isolation and Identification of LDPE Degrading Bacterial Strains

Following 100 days of incubation, microbial cells present on the LDPE surface of the enrichments, which displayed better plastic degradation, were dislodged in 0.9% NaCl. Hundred μL of cell suspension was plated on modified R2A agar supplemented medium and incubated for 48 h to obtain morphologically distinct colonies. The obtained isolates were routinely sub-cultured on same medium at 30 °C and preserved with glycerol (25%, v/v) at −80 °C. The genomic DNA of isolates (P2, DF22) was extracted using DNA minikit protocol following SDS-Lysozyme lysis and subsequent purification using phenol:chloroform:isoamyl alcohol and ethanol (Mohapatra et al., 2018). The 16S rRNA gene was amplified by PCR with bacterial universal primers (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-TACCTTGTTACGACTT-3′). The PCR cycle composed of an initial denaturation step at 95 °C for 5 min, 35 cycles of 95 °C for 45 s, 58 °C for 45 s, 72 °C for 1.5 min, and with a final extension of 72 °C for 7 min. The PCR products were gel purified using a Qia-quick gel extraction kit (QIAGEN), cloned into pTZ57R/T vector (InsTA clone kit, Thermo Fisher Scientific, Waltham, MA, United States), and sequenced using 27F and 1492R, followed by extraction of true nucleotide positions using BioEdit version 7.2.5. Assembly of the whole stretch was done using CAP contig assembly program of BioEdit to obtain ∼1000 bp sequence of 16S rRNA gene. Homology search for maximum similarity of the 16S rRNA gene sequences was carried out using identity tool of EzTaxon-e server[1] and BlastN of NCBI database considering validly published and effectively described type species. Whereas 16S rRNA gene sequences of other non-type species were obtained from NCBI database. Multiple alignments were performed with CLUSTALW package of MEGA X (Kumar et al., 2018), where all ambiguous positions were removed in the final dataset using pairwise deletion option. Phylogenetic reconstruction and validation were performed using neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) with 1000 bootstrap re-iterations using Jukes-Cantor distance model (Jukes and Cantor, 1969). Both maximum-likelihood (ML; Felsenstein, 1981) and minimum-evolution (ME; Takahashi and Nei, 2000) methods were employed to test the robustness of the trees. The sequences were submitted to NCBI GenBank under the accession numbers: MT929273 (DF22) and MT929274 (P2).

PE Biodegradation by Isolated Bacterial Strains

The isolated bacterial strains were checked for their plastic degradation potential. The freshly grown cells (1%) were added to 100 mL of basal medium, where 10 pieces of sterile plastic films of around 2 cm^2 each (total 0.24 g) were used in each sample as sole carbon source. They were incubated aerobically at 30 °C in shaking condition at 150 rpm. After 45 days, the set ups were examined to check the biodegradation of the plastic films. A control which had no inoculum was incubated along with the samples.

Morphological Analysis of PE films by Atomic Force Microscopy (AFM)

Changes on the surface of PE films due to the treatment of bacteria were visualized under Atomic Force Microscope (AFM) (Agilent Technologies Inc., United States). The polythene films were taken out from the basal medium and they were thoroughly washed with 2% sodium dodecyl sulfate to clean the surface. Thereafter, the films were dried overnight at 50 °C and the dried PE films were used for examination under AFM (Gajendiran et al., 2016). The LDPE samples were analyzed with a scan speed of 1.0 Hz and a resolution of 256 × 256 pixels.

Surface Morphology Analysis of PE Films by Scanning Electron Microscopy (SEM)

PE films were also visualized under Scanning Electron Microscope (SEM), in order to examine the surface morphology changes due to microbial action. The PE films were treated with 2% SDS and dried at 50 °C as described above in order to remove the microbial cells and associated derbies. Finally, they were gold coated, and viewed under SEM (SEM, ZEISS and Focused Ion Beam SEM, Germany) using a Cu grid at 2500X magnification (Das and Kumar, 2015).

[1] https://www.ezbiocloud.net/identify

Fourier Transform Infrared Spectroscopy (FTIR) of PE Films

The modification of polyethylene in terms of chemical bonds due to the action of the isolated bacterial strains was assessed by Fourier Transform Infrared Spectroscopy (Thermo Scientific IR Spectrophotometer, United States). Microbially treated and untreated polyethylene films were taken out after 45 days and washed with 2% SDS and dried at 50 °C as described earlier. Finally, control and treated PEs were analyzed under the IR spectrophotometer using transmission mode (Kowalczyk et al., 2016).

RESULTS

Enrichment of PE Degrading Bacterial Cells and Indication of Degradation

Enrichment of PE degrading bacterial cells was attempted by incubating LDPE beads with four different samples (namely, P, DF2, DF4, and DF7). Following 100 days' incubation, various parameters related to cell growth and change in culture pH were measured. Cell growth was measured in terms of culture OD followed by measurement of primary indication of plastic biodegradation by monitoring weight reduction of the LDPE beads, cell surface hydrophobicity and biofilm cell growth (**Figure 1**).

The OD_{600} of the medium showed significant change in turbidity, thus indicating possible cell growth following 100 days' incubation. Highest absorbance was shown by DF7, followed by *P* > DF4 and DF2 displayed the least (**Figure 1A**). Control set showed very low OD. Biofilm growth measured by recovering the LDPE beads from the incubation flasks showed considerable cell growth (**Figure 1B**). Compared to control beads with negligible CFU counts, beads from P setup yielded highest CFU of $10^6/cm^2$, followed by the beads from DF4 culture ($6 \times 10^5/cm^2$) > DF2 ($3 \times 10^5/cm^2$) and DF7 ($9 \times 10^4/cm^2$). It was noticed that the sample treated with landfill plastic associated soil (P) showed higher biofilm cell growth compared to the samples inoculated with drilling fluids (DF).

Compared to nearly unaltered pH of the control set, a drastic change in solution pH tending toward acidic pH from neutral pH was noted for all the biotic samples (**Figure 1C**). Among these, P showed a maximum decrease in pH (from pH 7.0 to pH 5.6) followed by DF2 (pH 6.0 from pH 7.0). Significant weight loss of the LDPE beads was observed in this study following their incubation (**Figure 1E**). Drilling fluid sample DF2 showed maximum reduction (7.54%) followed by P (7.45%), much higher than the untreated sample (no change in weight). This was a preliminary estimation of degradation since the bacteria present in the samples would utilize polyethylene, which was the only carbon source, thus leading to weight reduction of the plastic (Kyaw et al., 2012). The cell surface hydrophobicity was determined by BATH assay, which revealed that all the treated samples had more than 80% hydrophobicity except drilling fluid sample DF2 (having 66.42%) but the untreated sample (C) had only 11.11% hydrophobicity (**Figure 1D**). This assay showed the affinity of the bacterial cells toward the organic hydrocarbon thus highlighting the capability of enriched microbes in adhering to the polyethylene surface. This might result in enhancing the biofilm formation and degradation process (Sarker et al., 2020).

Depending on the abovementioned parameters, it was conferred that the samples P and DF2 showed better response toward biodegradation and they were selected for further analyses as well as isolation of bacterial strains. Two morphologically distinct LDPE degrading bacterial isolates were obtained as pure culture from P and DF2 enrichments. These two isolates (designated as P2 and DF22) were identified and further characterized for their LDPE degrading ability.

16S rRNA Gene Phylogenetic Analysis of PE Degrading Isolates

EzTaxon and BLAST based comparative homology analysis of 16S rRNA gene sequences (∼1000 bp) of PE degrading isolates, P2 and DF22 showed belongingness to members of *Stenotrophomonas* and *Achromobacter*, respectively. Isolate P2 displayed maximum identities of 99.4% to type strain of *Stenotrophomonas pavanii* DSM 25135^T, followed by other *Stenotrophomonas* type species (95.48–98.56%). Whereas isolate DF22 showed maximum of 97.6% identity to type strain of *Achromobacter xylosoxidans* NBRC 15126^T, followed by <97.0% similarity with all other type members. Neighbor-joining phylogenetic reconstruction showed that P2 formed coherent cluster with the type strain *S. pavanii* DSM 25135^T, while DF22 showed its maximum evolutionary closeness with several members (*A. xylosoxidans* NBRC 15126, EC3, A. insolitus DSM 23807, *etc.*) (**Figure 2**). Bootstrap re-sampling analysis together with combined ML and ME based phylogeny displayed similar tree topology demonstrating a strong association of *Stenotrophomonas* P2 with its closest relative *S. pavanii* DSM 25135^T. Whereas, *Achromobacter* sp. DF22 was denoted to be phylogenetically distinct among *Achromobacter* spp.

Phylogenetic analysis involving various plastics (LDPE, HDPE)/polymer degrading type- and non-type members of *Stenotrophomonas* and *Achromobacter* from different habitats showed that isolate P2 was taxonomically closest to the *S. pavanii* PDS-5, which has been characterized to be a polyethylene degrading bacterium from contaminated habitat. Thus, it denoted species level affiliation of P2 to the *S. pavanii* members (**Figure 2**). Isolate DF22 showed distant relatedness with plastic/polymer degrading *Achromobacter* species members (**Figure 2**). Isolates P2 and DF22 were designated as *Stenotrophomonas* sp. P2 and *Achromobacter* sp. DF22.

Atomic Force Microscopy and Scanning Electron Microscopy

Changes in surface morphology of the LDPE films were examined by Atomic Force Microscopy (AFM). The topography images of the treated films showed formation of grooves, corrosions, cracks and pits, etc., (**Figures 3B,C**). These changes were not observed in the untreated samples (**Figure 3A**). Modification

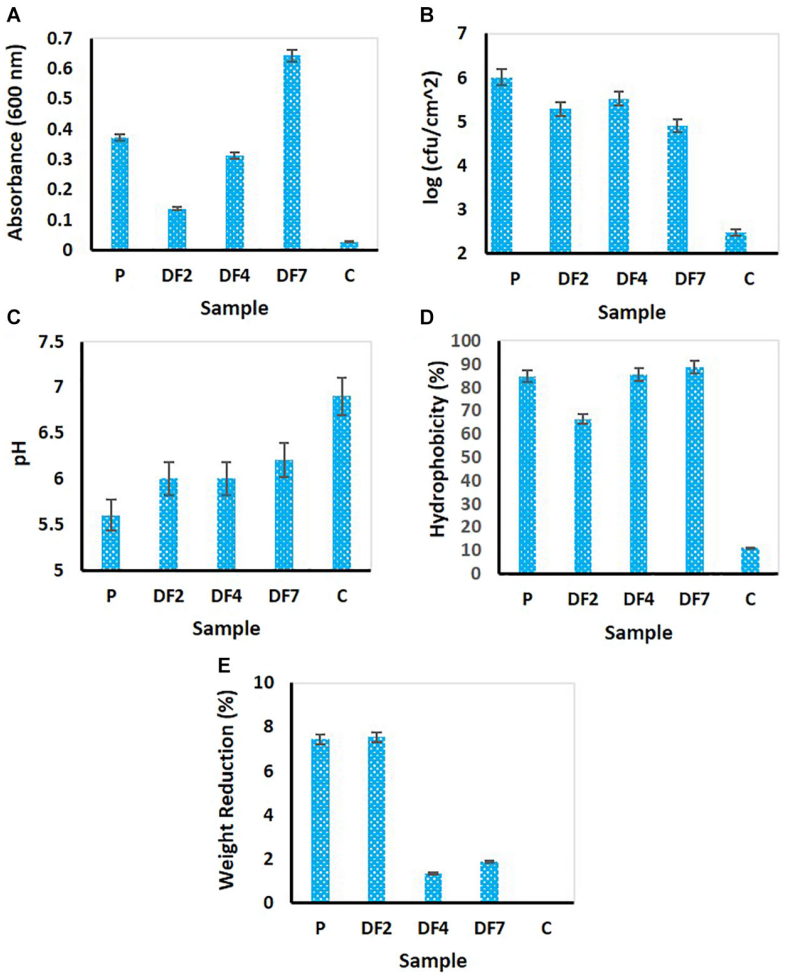

FIGURE 1 | Estimation of cellular activities of microorganisms enriched with LDPE following 100 days' incubation. **(A)** culture turbidity as measured through OD_{600}; **(B)** measurement of biofilm cell growth; **(C)** change in medium pH; **(D)** measurement of cell surface hydrophobicity and **(E)** measurement of weight reduction of the LDPE beads.P: Enrichment derived from waste dumpsite; DF2, DF4, and DF7: Enrichments using drilling fluid as the source of microbes. The analyses were performed in multiple (n = 3–5 replicates). The mean data were used for preparation of the figure.

in the surface morphology might be facilitated due to the action of microorganisms in the treated samples. AFM also indicated toward changes in the roughness of the surface, thus giving an overall idea on surface modification of the treated samples. The incubation for 45 days with the isolated strains caused significant increase in nano-roughness parameters, namely Ra (mean deviation of roughness), Rq (root mean square deviation of roughness), Rz (maximum height of roughness), Rv (maximum valley depth of roughness), Rp (maximum peak height of roughness) (**Figure 3D**), when compared to the untreated plastic. This indicated toward the formation of grooves as the result of enzymatic degradation of the LDPE (Peixoto et al., 2017). *Stenotrophomonas* sp. P2 displayed more maximum height (154 nm) than *Achromobacter* sp. DF22 (132 nm) and untreated films had the lowest (80 nm) for the same. Maximum height was the mathematical visualization of the topographs. It was noticed that *Stenotrophomonas* sp. P2 displayed more differences in maximum height, Rv, Rq, Ra and *Achromobacter* sp. DF22 had more changes in Rz, Rp (**Figures 3A–D**). Post incubation, roughness parameters were increased as much as two to three fold due to the action of both the strains.

Scanning Electron Microscopy (SEM) was performed next to further validate the LDPE surface modification due to the enzymatic action of microbes. Significant alterations on the surface of treated (with microorganisms) films were clearly observed, indicating toward the primary release of debris due to microbial action. Presence of cracks, erosions and grooves was significantly noted in the treated samples (**Figures 4B,C**); whereas the surface of the control or the untreated LDPE was mostly smooth (**Figure 4A**).

Assay of Biodegradation by Spectroscopy

The FTIR spectroscopic analysis of the LDPE films after 45 days of incubation gave several insights into the process of chemical bond modification. When compared with the untreated plastic

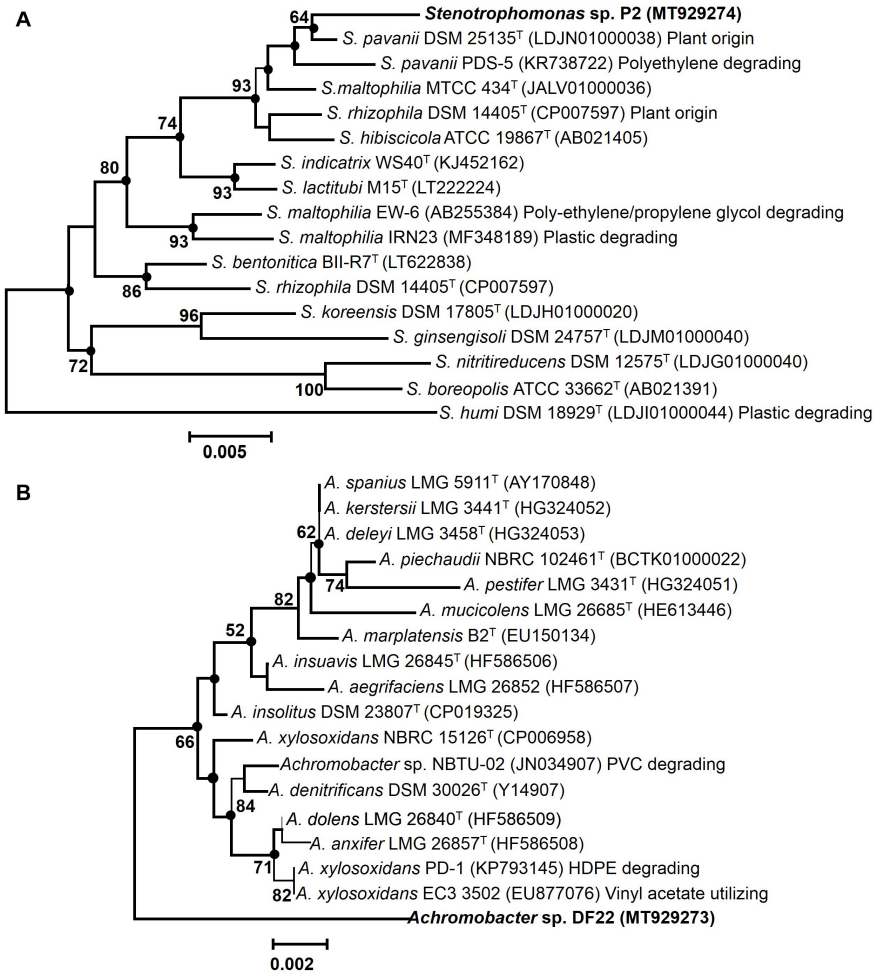

FIGURE 2 | Maximum Composite Likelihood based neighbor-joining (NJ) phylogenetic reconstruction of 16S rRNA gene sequences of LDPE degrading isolates [P2 **(A)**, DF22 **(B)**, in bold] with validly described type species (T) and non-type members with various plastic degrading abilities of respective taxa. The percentage of replicate trees (>50%) based on bootstrap iterations (1000 replicates) are shown next to the branches. The filled circles indicate the corresponding branches and nodes obtained from both Maximum-Likelihood (ML) and Minimum Evolution (ME) trees. Bars, 0.005 and 0.002 represent 0.5% and 0.2% nucleotide substitutions.

film, changes in FTIR spectra in few unique wavelengths in the treated LDPE films were found which confirmed the modification of bonds and generation of new bonds or functional groups in the polyethylene backbone due to the biotic treatment. Majorly altered and the significant characteristic bands were found between 600 cm^{-1} and 2000 cm^{-1}. Treated and untreated samples had few common peaks that belonged to polyethylene. Peaks at 720 cm^{-1} and near 1450 cm^{-1} were formed due to the long chain of C-H (bending vibration) (Yang et al., 2014). The peaks that were different from the control represented the main changes in chemical bonds and incorporation of functional groups. The formation of alkoxy groups, C-O (1000 cm^{-1} to 1090 cm^{-1}); nitro groups, N-O (1500 cm^{-1} to 1600 cm^{-1}); acyl groups, C-O (peak at 1220 cm^{-1}) and carbonyl groups, C = O (peak near 1720 cm^{-1}) was mainly found. In addition, some other modifications were observed, i.e., chain scission, H_2C = C- (905 cm^{-1}) and N-O stretching (peak at 1360 cm^{-1}) (**Figure 5A**). The bacterial enzymes could easily utilize these groups as their function site. Also, the double bond of carbon would be more accessible by bacteria compared to C-C. Chemical bond formation and modification obtained through FTIR spectroscopy helped us to understand the mechanism of enzymatic degradation of LDPE. Apart from the graph, some indices like Carbonyl Index (CI), Double Bond Index (DBI) and Terminal Double Bond Index (TDBI) were calculated to evaluate functional bond formation and LDPE chain modifications such as oxidation, vinylene formation and chain scissioning (**Figures 5B,C**). Treated LDPE showed significant increase in CI as much as 15–20 fold (**Figure 5B**) along with 1.5–2 times increase in DBI and 30–40 fold rise in TBDI (**Figure 5C**). The significant increase of the indices in case of the treated samples was quite promising as a result of biodegradation. The bacteria used LDPE as their source of carbon and made the necessary changes incorporating the functional groups to modify them into simpler products like alcohol, ketone, fatty acids, etc., (Peixoto et al., 2017).

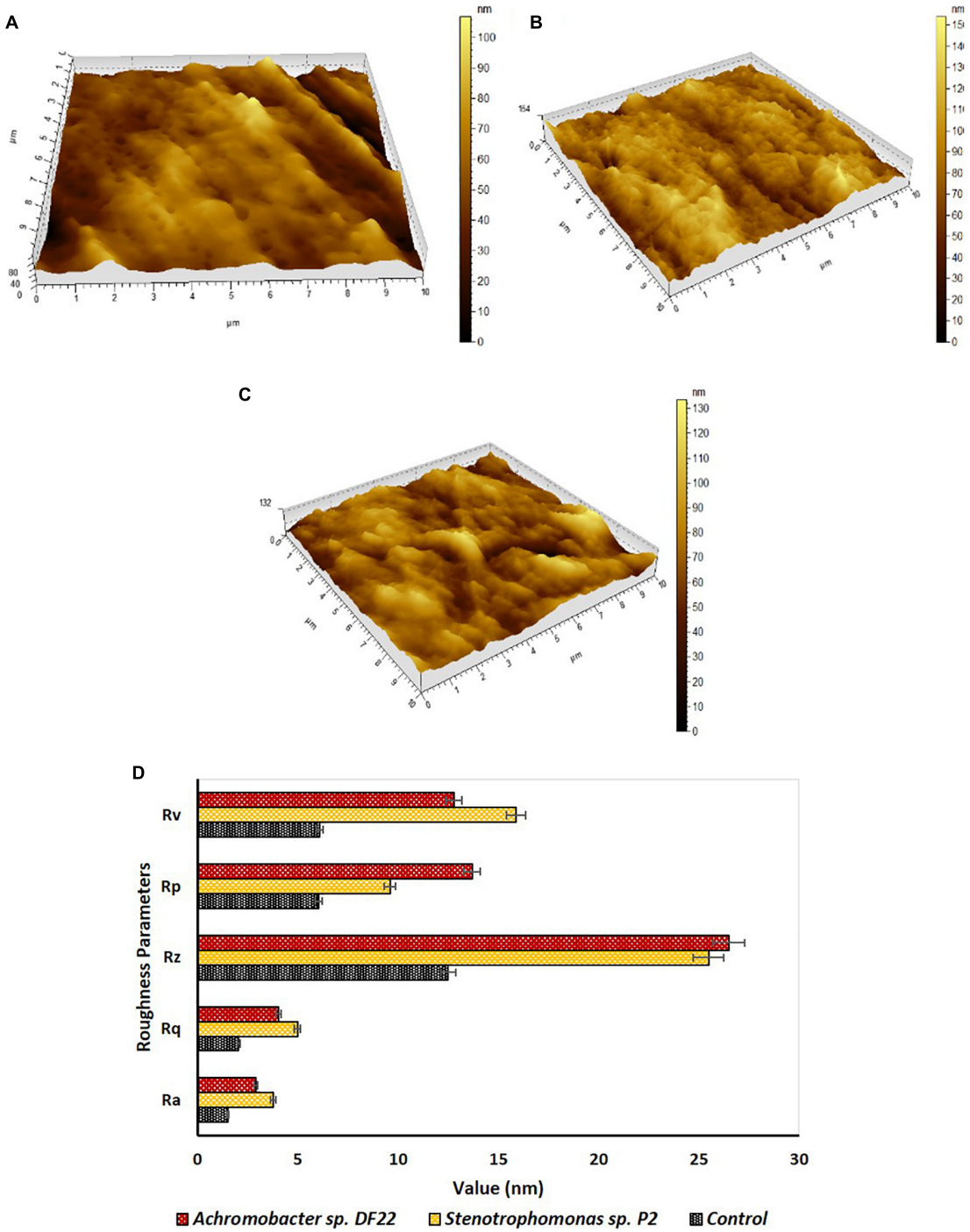

FIGURE 3 | Investigation of surface topography and roughness parameters by AFM. Topography of plastic samples **(A)** control (untreated) and treated with **(B)** *Stenotrophomonas* sp. P2 and **(C)** *Achromobacter* sp DF22 after 45 days of incubation. **(D)** In comparison to Control (Black), increase in roughness parameters [average roughness (Ra), root mean square roughness (Rq), average height (Rz), maximum peak height (Rp), and maximum valley depth (Rv)] was observed for the LDPE films treated with *Stenotrophomonas* sp. P2 (orange) and *Achromobacter* sp. DF22 (brown).

DISCUSSION

Samples from municipal waste dumpsite soil and bentonite based drilling fluid from a deep subsurface drilling operation were collected and used to enrich indigenous microorganisms capable of LDPE biodegradation. Microbial populations inhabiting the landfills have been already explored by several researchers for their polyethylene degrading potential (Gajendiran et al., 2016;

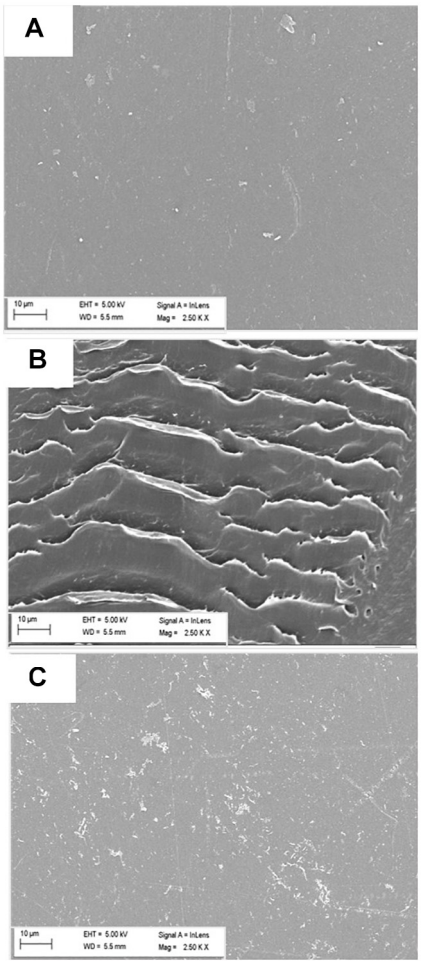

FIGURE 4 | Modification of surface morphology of LDPE films **(A)** untreated, and treated with **(B)** *Stenotrophomonas* sp. P2 and **(C)** *Achromobacter* sp. DF22 observed under SEM at 2500 × amplification. Changes on the surface in terms of corrosion, crack formation, etc., were visible only for the treated samples after the incubation period.

Kowalczyk et al., 2016; Muhonja et al., 2018; Park and Kim, 2019). Regarding the microorganisms of drilling fluids used in deep subsurface drilling operations, various studies have reported their taxonomic versatility and biodegradation potential (Alekhina et al., 2007; Ozyurek and Bilkay, 2017, 2018; Bose et al., 2020). Plastic metabolizing ability of such bacteria, however, has not been explored in detail.

All the test samples were incubated aerobically in a carbon free basal medium with LDPE beads as sole carbon source for 100 days. Visible growth and turbidity in the medium post incubation confirmed the successful enrichment of LDPE degrading microorganisms. Decrease of medium pH to an acidic range suggested the metabolic activity of the enriched cells (Duddu et al., 2015). The fragmentation and depolymerization of LDPE would lead to the formation of smaller molecular weight products such as fatty acids, organic acids, etc., which might cause the decrease in pH (Das and Kumar, 2015; Karamanlioglu et al., 2017). Decrease in medium pH and increase in absorbance of the medium for all the samples established that the enriched microorganisms could utilize LDPE as sole carbon source. This increase in absorbance could also reflect the possibility of the presence of fragmented LDPEs as a result of microbial action (Chatterjee et al., 2010). These enrichment cultures and the LDPE beads were subjected to series of tests for further evaluation of their LDPE degradation potential. High biofilm cell growth and effective colonization on the surface of the treated LDPE beads in all the test samples indicated toward the enrichment of LDPE degrading microorganisms. Biofilm formation on the surface of the hydrophobic and high molecular weight plastic polymers, such as LDPE is considered as one of the most important steps of microbial degradation of these polymers (Sivan, 2011). Furthermore, it has been already reported that the cells in biofilms are known to secrete exopolysaccharides that aid in their attachment to the surface of hydrophobic plastic polymers (Wilkes and Aristilde, 2017 and reference therein). Biofilm formation enabled the microorganisms to attach and efficiently utilize the non-soluble substrate *via* various enzymatic reactions (Orr et al., 2004; Peixoto et al., 2017). High cell surface hydrophobicity in all the test samples further confirmed the biofilm formation, as the hydrophobicity of any organism has a direct correlation with its potential to bind to non-polar hydrocarbons such as polyethylene (Sanin et al., 2003; Tribedi and Sil, 2013; Sarker et al., 2020). Highest weight loss of treated LDPE beads recovered from P and DF2 samples indicated that microbial consortia enriched from these two samples were more efficient in LDPE degradation. This weight reduction must have taken place because of the enzymatic action of the enriched microorganisms attached to the LDPE surface (Kyaw et al., 2012; Gajendiran et al., 2016). These two microbial consortia also displayed quite high metabolic activity. Overall, microbial consortia enriched from both landfill and drilling fluid samples showed promising capability of LDPE degradation. Microbial consortia enriched from P and DF2 samples were found to be more efficient toward LDPE degradation. They were further used for isolation of LDPE degrading bacteria.

Two LDPE degrading bacterial isolates, P2 and DF22 were obtained as pure culture from these enrichments. The isolated strains P2 and DF22 showed closest affiliation with microorganisms belonging to *Stenotrophomonas* and *Achromobacter* respectively. Phylogenetic analysis of the isolates denoted species level affiliation of *Stenotrophomonas* sp. P2 to the *S. pavanii* members. Based on the published criteria of < 98.65% (cut-off) of 16S rDNA similarity for differentiating two species members and description of new species (Kim et al., 2014), *Achromobacter* sp. DF22 might represent a new (novel) species within the genus *Achromobacter*. S. *pavani* PDS-5 (taxonomically closest to *Stenotrophomonas* sp. P2) has been already identified as a potent polyethylene degrader (Mehmood et al., 2016). Earlier studies have also reported the ability of other microbial members (**Table 1**) belonging to this genus toward plastic degradation (Abe et al., 2010; Peixoto et al., 2017; Montazer et al., 2018). Besides, *Stenotrophomonas* spp. have also been used for the bioremediation of organic pollutants

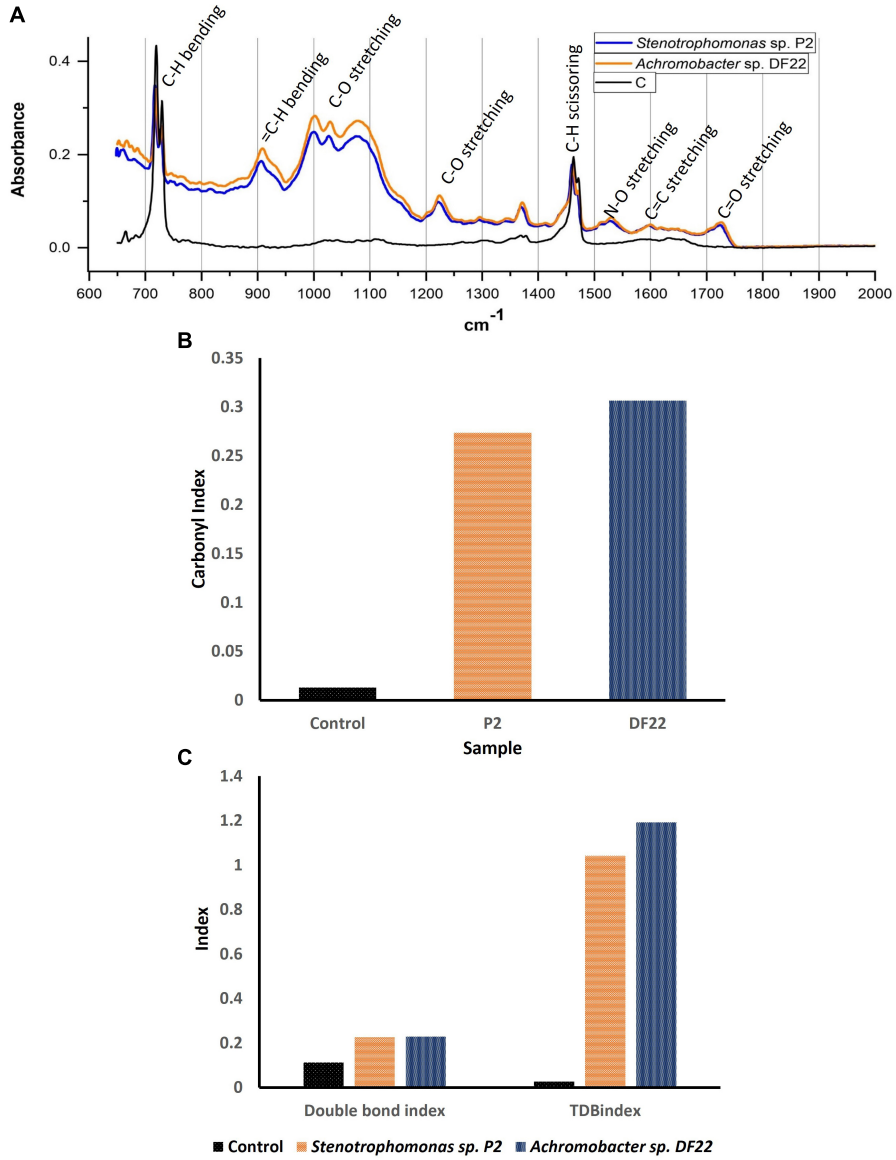

FIGURE 5 | (A) FTIR spectra of plastic films after treating with *Stenotrophomonas* sp. P2 (Blue) and *Achromobacter* sp. DF22 (Orange) showing the clear differences in the chemical bonds compared to control (Black) due to the bacterial treatment. **(B)** Change in Carbonyl Index (CI) for LDPE treated with *Stenotrophomonas* sp. P2 (P2: Orange) and *Achromobacter* sp. DF22 (DF22: Deep blue). **(C)** Changes in Double Bond Index and Terminal Double Bond Index (TDBindex) for the LDPE films incubated with *Stenotrophomonas* sp. P2 (Orange) and *Achromobacter* sp. DF22 (Deep blue) compared to Control (Black).

like fenvalerate, 3-phenoxybenzoic acid, dibenzothiophene, etc., (Chen et al., 2011; Papizadeh et al., 2011). These members were found to be distantly related to *Stenotrophomonas* sp. P2. Such phylogenetic clustering could be the result of similar evolutionary changes and shared metabolic tendency as isolate P2 showed its ability to degrade/impact the LDPE in the medium and isolated as LDPE enriched culture. Microbial members belonging to the genus *Achromobacter* have also displayed their ability toward degradation of plastic based polymers. Kowalczyk et al., 2016 has successfully tested HDPE biodegradation ability of *Achromobacter xylosoxidans*, which was isolated from landfill soil. *Achromobacter* sp. NBTU-02 has shown its potential toward biodegradation of plastics (Das et al., 2012). Along with these strains, some other strains having polyethylene degrading capabilities had been isolated from various environments and their potential toward PE biodegradation was examined by determining weight reduction, cell surface hydrophobicity, chemical modification, surface morphology changes, etc., (**Table 1**). *Stenotrophomonas* sp. P2 and *Achromobacter* sp. DF22 were further tested for their ability toward LDPE degradation.

Both the strains were incubated aerobically for 45 days in a carbon free basal medium, using LDPE films as sole C source. Following incubation, the films were examined for

TABLE 1 | Bacterial members used for biodegradation of various polyethylene (PE) materials from diverse contaminated habitats.

Organisms	Isolation source	Plastic polymer	Incubation duration (days)	Biodegradation observations	References
Stenotrophomonas sp. P2	Waste dump site	LDPE	100	∼8% weight reduction, biofilm, structural deformity, surface hydrophobicity, Chemical stability change	This Study
Achromobacter sp. DF22	Waste dump site	LDPE	100	∼8% weight reduction, biofilm, surface hydrophobicity, structural deformity, Chemical stability change	This Study
Stenotrophomonas pavanii	Solid waste dump site	Modified LDPE	56	Chemical alteration	Mehmood et al., 2016
Stenotrophomonas sp.	Soil with plastic debris	LDPE	90	Chemical stability change	Peixoto et al., 2017
Achromobacter xylosoxidans	Landfill soil	HDPE	150	9% weight reduction and chemical alteration	Kowalczyk et al., 2016
Acinetobacter baumannii	Municipal landfill	PE	30	Biomass increase	Pramila and Ramesh, 2015
Comamonas sp.	Soil with plastic debris	LDPE	90	Chemical stability change	Peixoto et al., 2017
Delftia sp.	Soil with plastic debris	LDPE	90	Chemical stability change	Peixoto et al., 2017
Kocuria palustris M16	Pelagic waters	PE bags	30	1% weight reduction	Harshvardhan and Jha, 2013
Microbacterium paraoxydans	Clinical sample	Pretreated LDPE	60	61% weight reduction	Rajandas et al., 2012
Pseudomonas sp.	Mangrove soil	PE	30	20.5% weight reduction	Kathiresan, 2003
Pseudomonas aeruginosa	Petroleum soil	Low mol. Wt. PE	80	41% weight reduction	Jeon and Kim, 2015
Pseudomonas sp.	Garbage soil	PE bags	180	37% weight reduction	Usha et al., 2011
Pseudomonas citronellolis	Municipal landfill	LDPE	4	17.8% weight reduction	Bhatia et al., 2014
Pseudomonas putida	Soil with plastic debris	PE	120	9–20% weight reduction	Kyaw et al., 2012
Rhodococcus ruber	PE waste soil	LDPE	20–28	0.8–8% weight reduction	Orr et al., 2004
Rhodococcus rhorocuros	Soil	PE	180	60% mineralization	Bonhomme et al., 2003
Rhodococcus sp.	Waste disposal site	Pretreated PE	21	33% weight reduction	Koutny et al., 2009
Streptomyces sp.	River delta	Pretreated PE bags	30	slight weight reduction	El-Shafei et al., 1998
Staphylococcus arlettae	Soil	PE	30	13.6% weight reduction	Divyalakshmi and Subhashini, 2016
Bacillus sp.	Waste coal	PE	225	98% weight reduction	Nowak et al., 2011
Bacillus sphaericus	Shallow ocean water	HDPE/LDPE	365	3.5–10%	Sudhakar et al., 2008
Bacillus megaterium	Soil	Pretreated PE	90	7–10% mineralization	Abrusci et al., 2011
Bacillus amyloliquefaciens	Solid waste dump site	LDPE	60	11–16% weight reduction	Das and Kumar, 2015
Bacillus subtilis H1584	Pelagic waters	PE	30	1.5–1.75% weight reduction	Harshvardhan and Jha, 2013
Chryseobacterium gleum	Activated sludge	UV irradiated LPDPE	30	Chemical alteration	Jeon and Kim, 2014

the evidences of changes in surface morphology and chemical modifications by AFM, SEM and FTIR. Both, AFM and SEM analyses confirmed significant modifications on the surface of LDPE films and weakening of their physical integrity due to microbial action by both the strains (Li et al., 2020). Apart from the visible modifications, almost 2–3 fold increase in nano-roughness parameters namely Ra, Rv, Rp, Rq, and Rz added stronger evidence to the alteration of roughness of the films because of microbial action. Higher values of Rz, which is the maximum height, suggested toward the presence of big grooves and pits (Peixoto et al., 2017). All the changes also indicated toward the penetration of the bacterial cells into the LDPE film surface (Esmaeili et al., 2013). These results indicated that the LDPE degradation had been initiated by the plastic degrading strains due to their enzymatic action, where laccase, esterase, mono-oxygenase, peroxidases might play significant role (Ahmed et al., 2018; Ru et al., 2020). In order to support these results, chemical modifications of the LDPE films were determined by FTIR. Chemical modification reflected the change in carbon backbone and helped to understand the

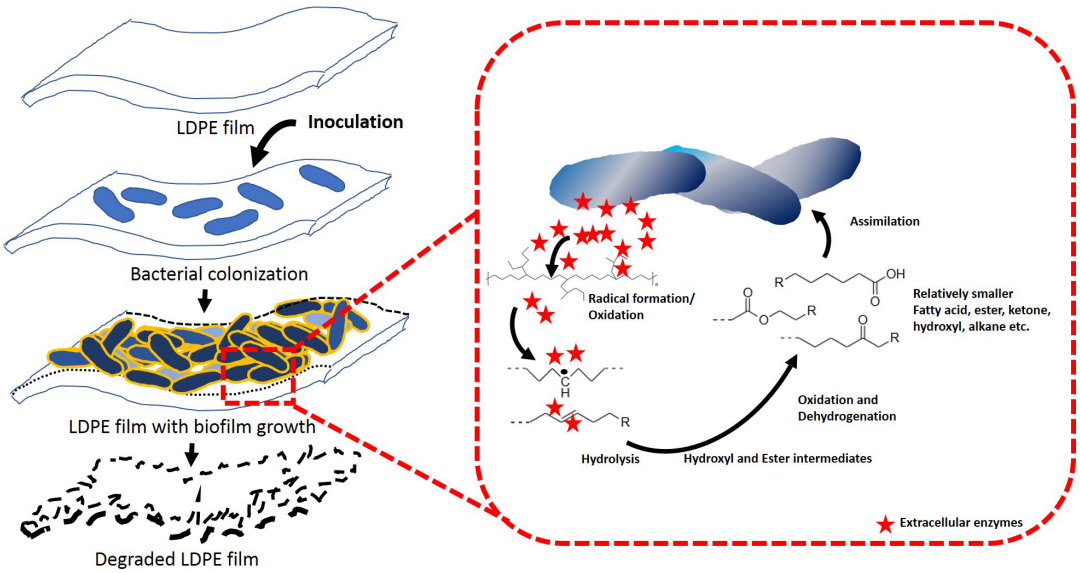

FIGURE 6 | Proposed LDPE degradation mechanism by *Stenotrophomonas* sp. P2 and *Achromobacter* sp. DF22.

formation of functional groups on LDPE or any molecule formed during the degradation process. FTIR spectroscopy is widely used as an efficient analytical technique to identify organic, polymeric and inorganic material and their chemical conformation. This technique had been utilized in earlier studies to display the chemical changes during plastic biodegradation (Peixoto et al., 2017; Montazer et al., 2018; Li et al., 2020). New functional groups (such as alkoxy, acyl, carbonyl, and nitro) were found and modifications in terms of chain scissioning, nitro stretching and double bond formation took place. Thus, the hydrophobicity of inert LDPE was reduced due to the incorporation of functional bonds to facilitate the microbial action. Although the exact enzymatic reactions are yet to be understood, the overall process of biodegradation and enzymatic mechanism could be conceptualized from the FTIR analysis (**Figure 6**). Together from the increased indices (CI, DBI, TDBI) and the spectral analysis it could be inferred that the degradation process might have been initiated by the formation of radicals that helped in enhancing oxidation with the help of oxygen and water. These oxidation products were then transformed to the functional groups and vinylene group probably by Norrish type I and Norrish type II mechanisms or stabilization. Thus, the hydrophobicity of the plastic surface was reduced so that the subsequent bacterial enzymes could enhance the degradation process. Presence of alkoxy and acyl groups in the treated samples indicated ester hydrolysis and alcohol formation due to the action of microbial hydrolytic enzymes such as cutinase, lipase, esterase and alkane monooxygenase. Formation of carbonyl bonds could have occurred due to the action of laccase. These ester, alcohol, etc., could be subsequently converted to smaller fatty acids through an intermediate step of aldehyde production by dehydrogenase enzymes. Thus, smaller molecular products were formed and could be taken up by the bacteria for metabolism, leading to mineralization via β-oxidation (Ogihara, 1963; Albertsson et al., 1987; Orr et al., 2004; Szép et al., 2004; Chatterjee et al., 2010; Peixoto et al., 2017; Ru et al., 2020). These processes resulted in the breaking down of LDPE films causing the surface changes on the films.

CONCLUSION

This study displayed the LDPE biodegradation potential of microbial consortium enriched (using LDPE as sole carbon source) from dumpsite and drilling fluids and the bacterial strains isolated from these enrichments. Significant microbial growth, high cell surface hydrophobicity of the enriched consortia and considerable weight reduction of treated LDPEs gave a primary indication of the biodegradation capacity of the enriched consortia. Two LDPE degrading bacterial strains affiliated to *Stenotrophomonas* sp. and *Achromobacter* sp. were isolated as pure culture from P and DF2 enrichments. SEM and AFM analyses confirmed that both the strains were successful in altering the cell surface morphology of the LDPE beads. The overview of possible mechanism of LDPE biodegradation by these two strains was also established in this study. FTIR analysis suggested that series of chemical changes starting from oxidation followed by dehydrogenation led to the breaking down of LDPE into smaller molecules, which could be subsequently utilized by these two strains for their metabolism. Complete microbial composition of the enrichments can be analyzed through next generation sequencing based approach and would help us to identify the complex network of biological systems involved in LDPE degradation. This would enable us to develop a stable microbial consortium capable of LDPE degradation, which could be utilized for large-scale biodegradation of plastic. Further investigation on the metabolic pathways, enzymatic reactions and metabolites would help us understanding the exact mechanism of biodegradation by these bacterial strains to develop *in situ* process for LDPE biodegradation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

AD performed the experiments, analyzed the data, and drafted the manuscript. HB helped with the phylogenetic analysis and manuscript preparation. BM helped in analytical methods and data analysis. PS conceived the idea, arranged funds, supervised all the experiments, data analysis, and manuscript writing. All authors contributed to the article and approved the submitted version.

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Allochthonous and Autochthonous Halothermotolerant Bioanodes From Hypersaline Sediment and Textile Wastewater: A Promising Microbial Electrochemical Process for Energy Recovery Coupled With Real Textile Wastewater Treatment

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The textile and clothing industry is the first manufacture sector in Tunisia in terms of employment and number of enterprises. It generates large volumes of textile dyeing wastewater (TDWW) containing high concentrations of saline, alkaline, and recalcitrant pollutants that could fuel tenacious and resilient electrochemically active microorganisms in bioanodes of bioelectrochemical systems. In this study, a designed hybrid bacterial halothermotolerant bioanode incorporating indigenous and exogenous bacteria from both hypersaline sediment of Chott El Djerid (HSCE) and TDWW is proposed for simultaneous treatment of real TDWW and anodic current generation under high salinity. For the proposed halothermotolerant bioanodes, electrical current production, chemical oxygen demand (COD) removal efficiency, and bacterial community dynamics were monitored. All the experiments of halothermotolerant bioanode formation have been conducted on 6 cm^2 carbon felt electrodes polarized at −0.1 V/SCE and inoculated with 80% of TDWW and 20% of HSCE for 17 days at 45°C. A reproducible current production of about 12.5 ± 0.2 A/m^2 and a total of 91 ± 3% of COD removal efficiency were experimentally validated. Metagenomic analysis demonstrated significant differences in bacterial diversity mainly at species level between anodic biofilms incorporating allochthonous and autochthonous bacteria and anodic biofilm containing only autochthonous bacteria as a control. Therefore, we concluded that these results provide for the first time a new noteworthy alternative for achieving treatment and recover energy, in the form of a high electric current, from real saline TDWW.

Keywords: halothermotolerant bioanodes, hypersaline sediment, textile wastewater, COD removal, energy recovery, autochthonous bacteria, allochthonous bacteria

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INTRODUCTION

The textile and clothing industry has become one of the most important sectors of activity. Despite the use of high-tech equipment and modern technologies, it remains among the highest water consuming industries. As reported by the United States Environmental Protection Agency (USEPA), the production of 9,072 kg of finished textile per day requires about 36,000 L of water only for wet processing (Ghaly et al., 2014; Berkessa et al., 2020). Moreover, almost the water consumed generates large volumes of textile dyeing wastewater (TDWW). The TDWW contains more than 1.5 g/L of waste dye as the uptake of these dyes by textile fabrics is very poor, high salinity of about 5–6% NaCl and 5% of Na_2SO_4 and other chemicals, such as various acids, alkalis, sulfur, naphthol, surfactant-dispersing agents, formaldehyde-based dye fixing agents, hydrocarbon-based softeners, and heavy metals (Verma et al., 2012; Lin et al., 2015; Pazdzior et al., 2018). Most of these chemicals and products of their degradation, i.e., metabolites, are recalcitrant in nature and severely affect both aquatic and terrestrial life (Ben Mansour et al., 2012; Kant, 2012; Chandrakant et al., 2016). It is therefore well-established that these hazardous pollutants should be removed from TDWW by appropriate and effective methods prior to their release into the environment.

Different TDWW treatment methods and techniques have been implemented and evaluated over the past two decades. These methods involve (i) physical methods (coagulation–flocculation, adsorption, and filtration techniques), (ii) oxidation methods categorized as advanced oxidation processes (cavitation, photocatalytic oxidation, Fenton chemistry) and chemical oxidation using oxidizing agents (O_3 and H_2O_2), and (iii) bioremediation methods (fungi, algae, bacteria) (Gosavi and Sharma, 2014; Yeap et al., 2014; Chandrakant et al., 2016; Chouchane et al., 2018; Jiang et al., 2018). For several reasons, such as eco-friendly, cost competitive, less sludge production, and giving non-hazardous metabolites or full mineralization (Hayat et al., 2015), the biological methods are qualified as the most sustainable method for wastewater treatment. However, biological methods cannot guarantee the achievement of required results of the TDWW, as some of the dye molecules or other chemical components are hazardous and/or recalcitrant to microorganism-driven degradation. The most appropriate method should be cost-effective, valuable in the degradation of resistant compounds, and produce safe and good quality effluent. There is therefore a great environmental and economical need to develop a processing technology that addresses these severe challenges. Tenacious, resistant, and exoelectrogenic microorganisms used in bioelectrochemical systems (BES) can perform the dual function of degrading pollutants and recovering in the form of electrical energy, the energy resulting from the oxidation of these pollutants. Indeed, previous studies reported that exoelectrogenic populations have favorably demonstrated an added value for the treatment of recalcitrant pollutants, such as azo dyes, petroleum hydrocarbon, and heavy metals (Adelaja et al., 2015; Choudhury et al., 2017; Monzon et al., 2017; Grattieri and Minteer, 2018; Vijay et al., 2018; Askri et al., 2019; Elabed et al., 2019a,b).

As already reported by Xie et al. (2011), organic and inorganic compounds in TDWW contain almost five times more energy than that consumed to treat it. Thus, it is hypothetically possible to extract electrical energy from the TDWW by applying well-adapted electrochemically active microorganisms capable of degrading recalcitrant pollutants, transmuting dangerous metabolites, and capturing an electronic flow that can be converted into electrical energy under high salinity. In this context, an earlier study by Askri et al. (2019) demonstrated the enrichment of efficient exoelectrogenic microorganisms from hypersaline sediment of Chott El Djerid (HSCE) able to produce a current density in the range of 7 A/m^2 under combined high temperature and hypersaline conditions (temperature 45°C, salinity 165 g/L) using lactate as carbon source, i.e., anodic fuel. Halothermophilic microorganisms are thus suitable candidates for the treatment of the high saline wastewaters generated, for example, in the textile dyeing (2–10 g/L), seafood processing (8–20 g/L), tannery (40–80 g/L), and petroleum industries (few g/L to 300 g/L) (Shehab et al., 2017; Cherif et al., 2018; Askri et al., 2019).

The aim of this work was therefore to demonstrate, for the first time, proof of the feasibility of designating an efficient microbial halothermotolerant bioanodes from both hypersaline sediment (HSCE) and saline TDWW microbium able to treat textile wastewater and generating an electrical current collected *via* an electrode. At this stage of progress, the exploitation of the anodic current flow generated is not investigated at all. It is only quantified in terms of bioelectrochemical kinetics, from the acquisition of the $J = f(E)$ curves. It is thus not at all a question here of developing a microbial fuel cell (MFC), a microbial electrolysis cell (MEC), or any other BES, judged too fluctuating and random to focus attention on the precisely targeted phenomenon, i.e., the formation of a bioanode without limitation caused by a limiting step of a larger and more complete BES process. On the contrary, bioanode formation was studied here in three-electrode electrochemical bioreactor installations to ensure well-controlled electroanalysis conditions, as explained in the review by Rimboud et al. (2014), who point out the fundamental basic principles and advantages of the three-electrode arrangement compared with MFC or other BES devices.

Once the potential for electrical current generation and TDWW treatment of the halothermotolerant bioanodes had been proven *via* electrochemical and analytical tools, contributions of autochthonous microorganisms from TDWW and allochthonous from HSCE were investigated through comparative metagenomic analyses of biofilms, HSCE, and TDWW.

MATERIALS AND METHODS

Collection of Hypersaline Sediment and Textile Wastewater Samples

Hypersaline sediments used as inoculums were sampled from an extreme environment Chott El Djerid, located in the south of Tunisia (N 33°59′965″ and E 08°25′332″). Samples were a

mixture of saturated water and sediment (2:1 vol.:vol.) collected from the surface with a conductivity higher than 200 mS cm^{-1} (Askri et al., 2019). The TDWW was collected from "Sitex" textile industry located in Monastir, Tunisia (N 35°39′06″ and E 10°53′03″). All samples were conserved in closed plastic bags and bottles at +4°C until experiments were started.

General Condition of Three-Electrode Bioelectrochemical Cell Configuration and Operation

All experiments were conducted in a 500 ml glass three-electrode reactor containing 80% of real textile effluents and 20% of saline sediments. After the homogenization step, reactors were hermetically closed without any gas flow. A conventional three-electrode system [working electrode (WE), auxiliary electrode, and reference electrode] was implemented with a VSP multichannel potentiostat (Biologic SAS) equipped with EC lab software. The WE made of a porous carbon felt of 6 cm^2 projected surface area was electrically connected to a platinum wire (1 mm diameter and 15 cm long) and polarized at −0.1 V/SCE. A platinum grid was used as the counter electrode (CE), and a saturated calomel reference electrode (+0.24 V/SHE) was located between the counter and the WEs. Cyclic voltammetry (CV) was performed *in situ* between −0.6 and 0.3 V/SCE at a scan rate of 1 $mV.S^{-1}$.

Electrochemical Data Processing

Chronoamperometric (CA) maximum current densities of the halothermotolerant biofilms were carried out as indicated in the previous study by Askri et al. (2019). CV was used to (i) validate the formation of an electroactive biofilm by comparing a control CV of the WE immersed in the TDWW with HSCE before starting the CA and a turnover CV at the end of the experiments and (ii) get access to I = f(E) of the bioanode that was shaped.

COD Measurement

The chemical oxygen demand (COD) removal rate (%) corresponds to the percentage of the total organic matter eliminated in the wastewater. Therefore, COD change monitoring is a relevant parameter to evaluate the performance of the halothermotolerant bioanode. The monitoring was carried out according to the following equation:

$$\text{COD removal rate} = (\text{COD}_{\text{influent}} - \text{COD}_{\text{effluent}})/\text{COD}_{\text{influent}} * 100$$

Samples were recuperated from anolytes of each reactor, and the COD was measured using LCK 514 (100–2,000 mg/O_2) after a 1/6 dilution and filtration through the syringe of the chloride elimination kit LCW925 (Hach Lange) to remove any analytical interference from the chloride ions in the media.

Epifluorescence Microscopy

To study global biofilm structure at the end of experiments, WEs covered by HSCE and/or TDWW microorganisms were removed from the reactors and immediately stained with acridine orange 0.01% (A6014 Sigma) for 10 min. Then, bioanode coupons were washed carefully with sterile physiological water after incubation and dried at ambient temperature overnight. Biofilms were then imaged with epifluorescence microscopy as described by Blanchet et al. (2016), Rousseau et al. (2016), and Askri et al. (2019).

Microbial Diversity Analysis of HSCE and TDWW Samples and Biofilms

Genomic DNA extractions from HSCE, TDWW, and biofilms were performed using a NucleoSpin® Soil kit according to the manufacturer's instructions. The purity [absorbance ratio (A_{260}/A_{280})] and DNA concentration measurements (ng μl^{-1}) were checked by Nanodrop. Then, Illumina Miseq 16S rRNA sequencing was performed in order to analyze the composition of the microbial communities in HSCE and TDWW samples and in different biofilms. The 16S rRNA gene V4 variable region PCR primers 515/806 were used in single-step 30 cycles PCR using the HotStarTaqPlus Master Mix Kit (Qiagen, USA). Sequencing was performed at MR DNA (www.mrdnalab.com; Shallowater, TX, USA).

Statistical Analyses

All experiments were performed in triplicates under the same conditions to prove the reproducibility of experimental results. The mentioned results are the average values with a standard deviation.

RESULTS AND DISCUSSION

Bioanode Growth and Electrochemical Characterization

Four microbial bioanodes were independently grown by chronoamperometry with a VSP multichannel potentiostat (Biologic SAS), setting the potential value of the WEs at −0.1 V/SCE. This electrode potential has particularly shown its relevance to efficiently lead to the formation of particularly robust and efficient bioanodes from wastewater (Blanchet et al., 2015) and also from sediments (Erable et al., 2017).

The four bioanodes grown at this polarization potential are referred to as TDWW, TDWWS1, TDWWS2, and TDWWS3 all through the figures and text. The constant electric polarization was maintained for 17 days, which is a reasonable amount of time to obtain matured bioanode on carbon based electrodes colonized by halo-exoelectrogenic bacteria (Erable et al., 2009; Rousseau et al., 2014; González-Muñoz et al., 2018; Askri et al., 2019). Current densities vs. time plots for −0.1 V/SCE polarization potential are shown in **Figure 1**. The start of current production was observed almost after 2 days of polarization for all three bioanodes co-inoculated with HSCE, in comparison with the TDWW bioanode, where current production was only recorded from day 5 with very low densities throughout the experiment. This suggests that the electroactive community developed faster and more efficiently in reactors inoculated with HSCE. Remarkably, the maximal current performance reached during the experiments was almost the same for the three bioanodes TDWWS1, TDWWS2, and TDWWS3. A reproducible current production of about 12.5 ± 0.2 A/m^2 was obtained. However, maximum current density peaks were reached after

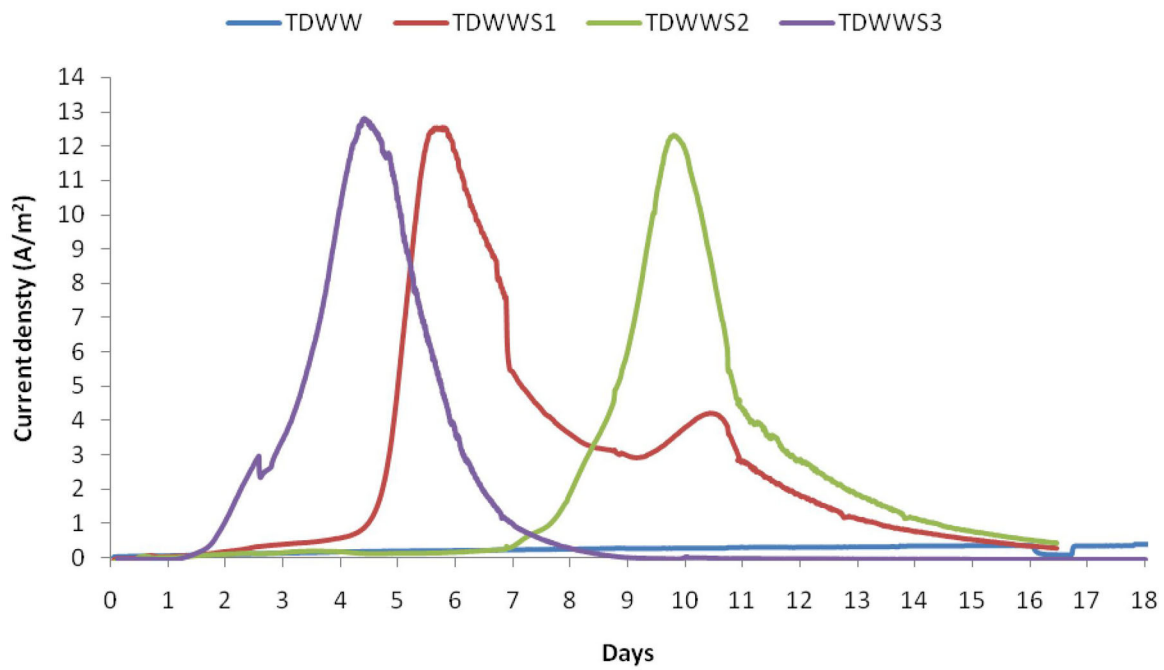

FIGURE 1 | Evolution of the current density (A/m^2) vs. time (days) for experiments on a carbon felt electrode of 6 cm^2 projected surface area polarized at −0.1 V/SCE in a reactor containing textile dyeing wastewater (TDWW) and reactors containing 80% of TDWW and 20% of saline sediments (TDWWS1, TDWWS2, TDWWS3).

different periods of polarization for 4, 6, and 10 days for TDWWS3, TDWWS1, and TDWWS2, respectively. Successive batches of addition of textile wastewater usually make it possible to harmonize the current densities over the long term of several replicates of experiments. Here, the reproducibility of the TDWWS replicates observed from the first batch is to be underlined and certainly comes from the inexistence of competitive reactions due to the inhibition of non-electroactive microorganisms by the high salinity, the toxicity of the pollutants, and the possible presence of oxygen traces.

Previous works have shown that the performance of bioanode systems is basically inversely proportional to the complexity of the substrate as in the case of TDWW (Pant et al., 2010; Pandey et al., 2016; Heidrich et al., 2018). However, it is worth noting that in this study, the oxidation currents obtained are almost doubled compared with those obtained with the same inoculum (HSCE) using lactate (5 g/L) as substrate (Askri et al., 2019), and the initial COD for both sets of experiments was 975 and 1,432 mg/l, respectively. The diversity of the TDWWS biofilm composed jointly of autochthonous and allochthonous bacteria may be critical to its performance.

Overall, wastewater used to feed BES, mainly MECs and MFCs, was either domestic wastewater or industrial wastewater from a wide variety of sources (breweries, dairies, refineries). The highest current densities obtained by BES fed with real industrial effluents were 10.7 and 10.3 A/m^2 where biorefinery (Pannell et al., 2016) and brewery wastewaters (Yu et al., 2015) have been used, respectively. However, for BES fed with domestic wastewater, the highest current densities obtained were 3.8 (Ullery and Logan, 2015) and 3.5 A/m^2 (Blanchet et al., 2016). The average current densities calculated from 48 research papers are 2.6 A/m^2 for industrial wastewater and 0.8 A/m^2 for domestic wastewater. Two main reasons could explain in part this significant difference in average current densities: (i) industrial wastewater is generally more conductive (7 mS/cm) than domestic wastewater (1.5 mS/cm) (Yen et al., 2016) and (ii) the total organic matter concentration in industrial wastewater is between 5,000 and 12,000 mg/L (Rajeshwari et al., 2000), whereas that in domestic wastewater is between 320 and 740 mg/L (Almeida et al., 1999).

The obtained results demonstrated that the TDWW, although rather recalcitrant to biological treatment, is found as the most suitable effluent to generate electric current using hypersaline sediment as a source of tenacious and exoelectrogen biocatalysts.

CV was used as a tool to confirm the presence of electroactive biofilm on the electrode surface. Results from CV tests for the four different bioanodes are shown in **Figure 2**. The initial CVs of the TDWW (negative control) and TDWWS1, S2, and S3 on the porous carbon felt electrode clearly showed the absence of an electroactive biofilm due to the fate shape of the voltammogram from 0.0 to +0.3 V. Interestingly from 0.0 to −0.6, a sharp decrease of the current was observed very likely due to the electrochemical reduction of some compounds in the medium. Another CV cycles were performed when the maximum values of the current density were reached for all reactors (turnover CVs). For the TDWW, no noticeable change from its initial state is visible, confirming the total absence of development of an electroactive biofilm on the corresponding carbon felt electrode.

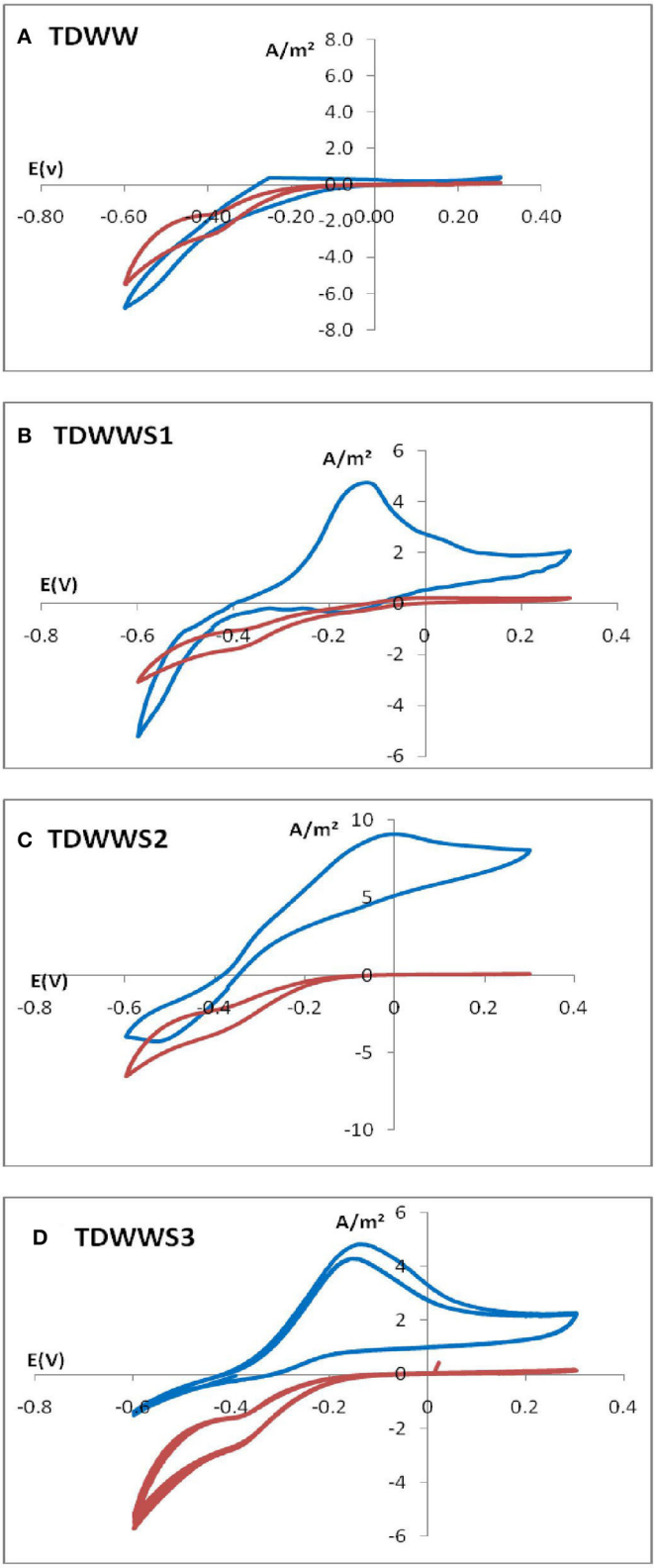

FIGURE 2 | Cyclic voltammetry performed in different conditions: **(A)** CV of porous carbon felt electrode immersed in TDWW; **(B–D)** CV of porous carbon felt electrode immersed in TDWW and HSCE sediment obtained at two different times: CV_I (–): at the beginning of the experiment; CV_M (–) at maximum current density.

On the other hand, for the other samples, TDWWS1, S2, and S3, a remarkable difference in the general shape of the turnover CVs is identified compared with their initial states and with the TDWW.

All three TDWWs turnover CVs have a zero current potential very close to −0.4 V/SCE. Below this potential, a reduction current is detected that increases to the lower potential limit of −0.6 V/SCE. This reduction current is a less pronounced residual of the same reduction phenomenon(s) observed on all electrodes at t = 0.

Concerning the visible oxidation current for potentials higher than −0.4 V/SCE, it is constantly increasing from −0.4 to −0.1 V or even 0.0 V/SCE. Beyond these potential values, i.e., for potentials more positive than −0.1 V or 0.0 V/SCE, the maximum speed of the exchange current is reached, and a current plateau is therefore observable between −0.1 V and +0.3 V/SCE. The current density even tends to decrease over this anode potential range in the case of **Figures 2B,D** because the scanning speed is certainly too fast to ensure a steady state of bacterial metabolic phenomena limiting the production of the electron flow.

In sum, the CV results clearly indicate the establishment of a biofilm with electrocatalytic properties on the carbon felt electrodes, as well as a successful enrichment of this electroactive biofilm with bacteria capable of using electron acceptor electrodes (Harnisch and Freguia, 2012; Rimboud et al., 2014). The hysteresis identified between the forward and return curves of the CVS (**Figures 2B–D**) is classic of bioanodes formed in saline or hypersaline environments (Erable and Bergel, 2009; Rousseau et al., 2014), where capacitive phenomena related to the high ionic charge of the electrolyte and especially the capacity of the electrode material and electroactive biofilm couple coexist.

COD Measurement

Table 1 indicates that reactors inoculated with HSCE showed satisfactory performance in terms of both current production and COD removal that were about 12.5 ± 0.2 A/m^2 and 91 ± 3%, respectively. Interestingly, these reactors demonstrated COD removal proportional to the production of current generation. However, COD removal in the reactor not inoculated with HSCE was much lower, i.e., 42.3%, than those in the inoculated reactors, and the production of the current also remains very low. For bioremediation of wastewater containing recalcitrant pollutants, these findings revealed that reactors inoculated with HSCE were found to be comparatively higher in COD removal efficiency than other textile wastewater treatments, such as (i) anaerobic internal circulation reactor (COD removal = 87%), (ii) Fenton's process with and without pH adjustment where COD removal was 89 and 33%, respectively (Hayat et al., 2015), and (iii) the combination of homogenization decantation and membrane treatments (COD removal = 66%) (Buscio et al., 2015).

Microscopy Analysis of Biofilms Morphology

Colonization of the carbon felt electrodes was evaluated at the end of the experiments, mainly on the outer surface of the felts but also within the porosity of the felt. Indeed, previous work has shown the difficulty of electroactive biofilms to colonize the internal surfaces of felt electrode structures (Chong et al., 2019), especially when real effluents either viscous, highly charged with suspended solids, or very highly loaded with COD are used (Blanchet et al., 2016). Carbon felt WEs possibly covered with HSCE and/or TDWW microorganisms were removed from the reactors and imaged by epifluorescence microscopy (**Figure 3**). Samples of carbon felt WEs were TDWW that is a bioanode with very low current production (300 mA/m^2) and TDWWS1, TDWWS2, and TDWWS3 that produced similar current densities of 12.5 ± 0.2 A/m^2.

TDWW was the electrode sample with the lowest surface colonization rate. Only a few fibers on the surface of the carbon felt electrode were partially colonized by scattered, non-continuous, sparse groups of bacterial colonies with low bacterial cell density. Since this bioanode generated almost no current, it is not surprising to observe a low rate of colonization. The few isolated clusters of bacterial colonies are probably mostly similar to suspended biological substances, kinds of microbial flocs participating in the non-bioelectrochemical oxidation of the COD of textile effluents.

In comparison, the electrode surfaces of TDWWS1, TDWWS2, and TDWWS3 were much more thickly colonized. In spite of relatively similar bioelectrochemical behaviors, but with a time lag, the microscopic patterns of the biofilms show significant differences. TDWWS1 was colonized by a continuous biofilm settled between the surface fibers. The production of exopolymeric substances was also much more prominent for this biofilm. TDWWS2 showed a non-homogeneous colonization of the carbon fibers. The appearance of the biofilm was like the control not inoculated with sediment from HSCE, but the density and number of microbial clusters were much higher. Finally, TDWWS3 showed an intermediate colonization profile between TDWWS1 and TDWWS2, with a thin continuous biofilm that closely covered the surface fibers of the carbon felt. This type of electroactive biofilm enveloping the carbon fibers is generally a feature of biofilms in hypersaline environments (Rousseau et al., 2014). However, we have also recently revealed that the physical structure of electroactive biofilms formed from sediments from HSCE, i.e., enveloping the fibers or distributed between the fibers, was not systematically a sign of improved or, on the contrary, decreased current production (Askri et al., 2019).

Microscopic inspection of the internal porosity of the carbon felt electrodes revealed a very low degree of internal porosity colonization for the electrodes TDWWS1, TDWWS2, and TDWWS3 (**Figure 4**). Even so, no trace of biofilm could be seen in the core of the TDWW electrode belonging to the electrochemical reactor not inoculated with HSCE. The poor accessibility to the porosity of the carbon felt is usually due to the clogging phenomena of the external pores of the electrode exerted by particles in suspension present in the wastewater (Blanchet et al., 2016). Insofar as the external faces of the carbon felts do not present a colonization obstructing the pores, i.e., the inter-fiber spaces, this hypothesis is not at all conceivable here. The lack of agitation of the reaction medium, the hydrophobicity of the carbon felt, and the low growth rate of microorganisms in the textile wastewater are certainly individual or combined tracks

TABLE 1 | COD removal efficiency of TDWW from different reactors.

Reactors	Contents	Temperature (°C)	Duration (day)	Maximal current density (A/m^2)	COD removal rate (%)
1	20% HSCE + 80% TDWW	45	17	12.5	91.0
2	20% HSCE + 80% TDWW	45	17	12.3	93.6
3	20% HSCE + 80% TDWW	45	17	12.7	88.3
4 (negative control)	100% TDWW	45	17	0.3	42.3

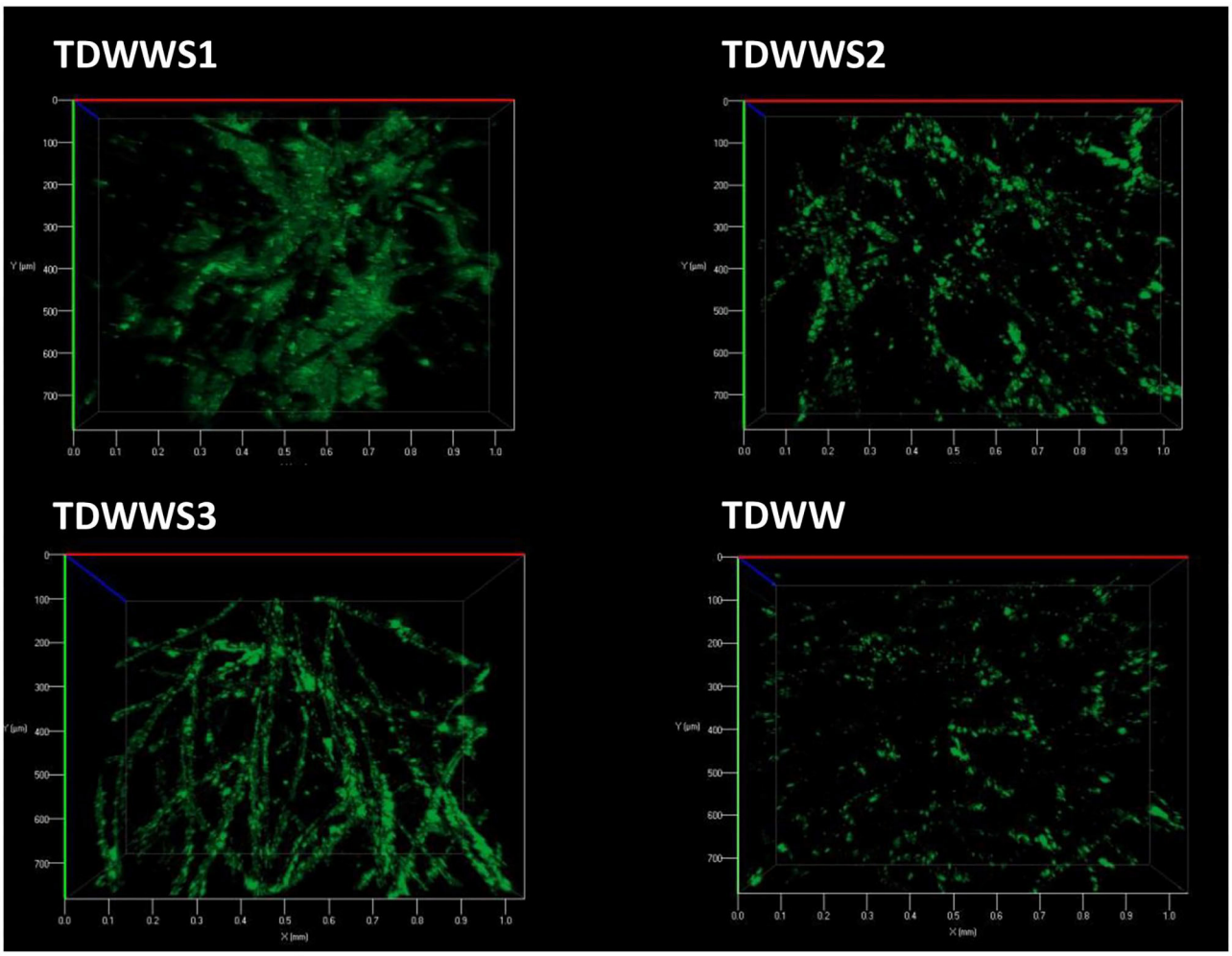

FIGURE 3 | Evaluation of microbial proliferation on the outer surface of carbon felt electrodes by epifluorescence microscopy imaging. Carbon felt electrode surface has been stained with acridine orange to localize living and dead microbial cells.

that can partly justify the low internal colonization rate of the 3D felt electrodes.

Molecular Analysis of the Bacterial Communities

The composition and abundance distribution of each sample (rTDWW and HSCE) and biofilms (TDWW, TDWWS1, TDWWS2, and TDWWS3) at the taxonomic levels of phylum and species are shown in **Figures 5A,B**.

At phylum level (**Figure 5A**), raw textile dyeing wastewater (rTDWW) bacterial community, considered as the autochtone community, was mainly characterized by Bacteroidetes (48.5%), Actinobacteria (14.5%), Proteobacteria (12.5%), Planctomycetes (9.5%), and Chloroflexi (8%). Previous studies have demonstrated that the bacterial phyla Acidobacteria, Planctomycetes, and Chloroflexi were more abundant in samples from textile wastewater (Meerbergen et al., 2016). However, in hypersaline sediment sample of Chott el Djerid (HSCE), source of the allochthonous community, the most

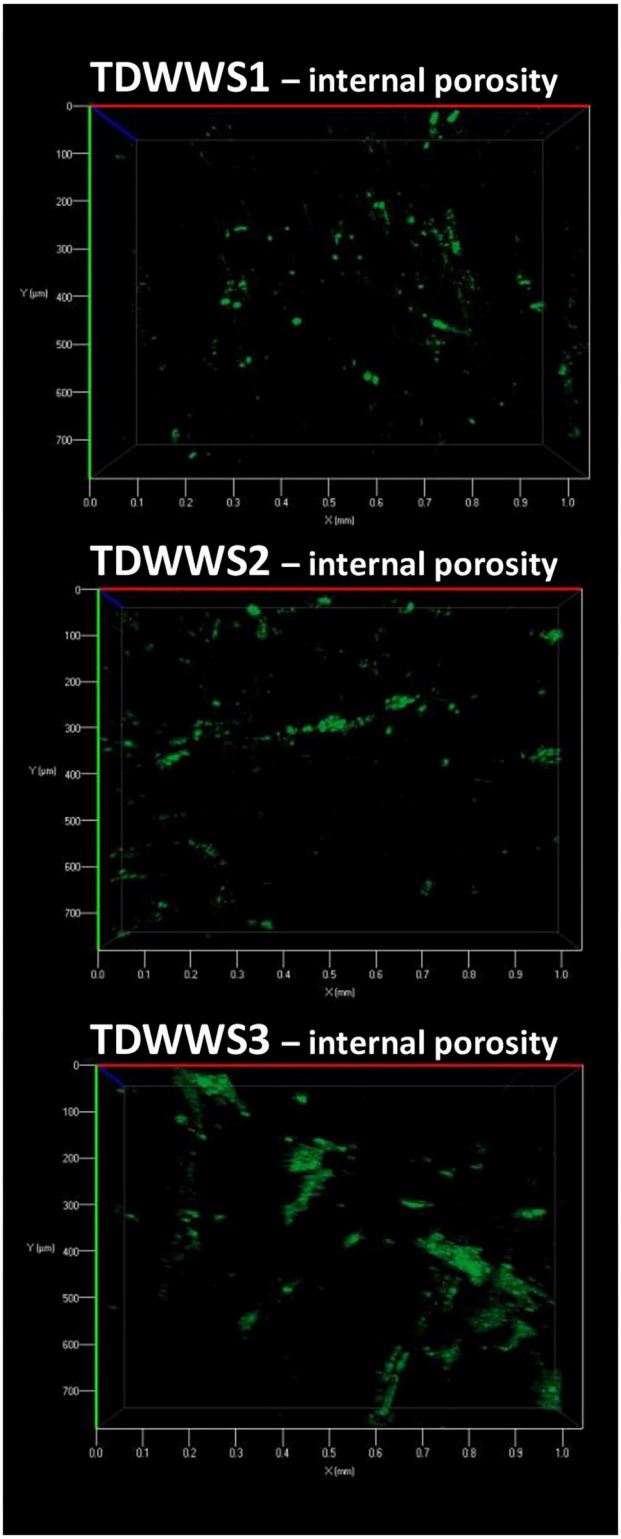

FIGURE 4 | Observation of the internal microbial colonization of the carbon felt electrodes. A 1 cm thick cross-section of the electrodes was prepared, and observations were then performed in the center of the porosity of the electrodes after staining the dead and living microbial cells with acridine orange.

abundant phylum was Proteobacteria (86%) approximately seven times higher than that in rTDWW (12.5%). In addition to Proteobacteria, the HSCE sample was hosted by other low-grade phyla, such as Firmicutes (6.8%), Cyanobacteria (5%), and Bacteroidetes (2%). Earlier studies using different molecular methods (Ben Abdallah et al., 2016, 2018) have demonstrated that the bacterial community in HSCE was dominated by Proteobacteria, followed by Firmicutes, Bacteroidetes, Cyanobacteria, and Actinobacteria. Based on the species assignment results (**Figure 5B**), *Dysgonomonas gadei* and *Filimana aquilariae* were the two dominant species in rTDWW, wherein their relative abundances were 21 and 19%, respectively. From the detected phyla Actinobacteria, *Micrococcus* sp. and *Rhodococcus* sp. were the most representative species at relative abundances of 12.6 and 11.5%, respectively. However, the Proteobacteria phylum shows a bacterial profile with the enrichment of *Pseudomonas* sp. (12.5%), *Comamonas acidovorans* (12%), *Lysobacter* sp. (11%), *Aeromonas hydrophila* (10.2%), *Burkholderia cepacia* (9%), and *Achromobacter* sp. (8.5%). By contrast, sample coming from HSCE was represented by various species of Proteobacteria with a predominance of *Psychrobacter aquaticus* (14.5%), *Marinobacter sediminum* (13.5%), *Psychrobacter alimentarius* (12.5%), *Geobacter sulfurreducens* (9.5%), *Marinobacter hydrocarbonoclasticus* (9%), *Geobacter metallireducens* (9%), and *Halomonas* spp. (7.5%).

Figure 5B indicates that the relative abundances of bacteria at phylum level in TDWWS1, TDWWS2, and TDWWS3 biofilms are quite similar, whereas those in TDWW biofilm show some differences. TDWWS1, TDWWS2, and TDWWS3 biofilms demonstrated the presence of the same phyla and largely at the same pattern of abundance Proteobacteria > Bacteroidetes = Firmicutes > Actinobacteria > Chloroflexi = Planctomycetes > Thermotogae. As example, TDWWS1 biofilm showed Proteobacteria (30.5%) > Bacteroidetes (17.43%) = Firmicutes (17.5%) > Actinobacteria (5.8%) > Chloroflexi (1.5%) = Planctomycetes (1.6%) > Thermotogae (0.35%). It is worth noting that the bacterial phyla identified in the three biofilms show heterogeneous profiles composed of phyla found in rTDWW and in HSCE. In this case, TDWWS1, TDWWS2, and TDWWS3 biofilms harbored autochthonous phyla from rTDWW represented in particular by Bacteroidetes, Actinobacteria, Chloroflexi, and Planctomycetes and allochthonous phyla from HSCE, such as Proteobacteria, Firmicutes, and Thermotogae. The heterogeneous bacterial profile of the different biofilms occurs more clearly at species level. *P. aquaticus* (8.06–16.02%), *P. alimentarius* (3.04–6.5%), *G. sulfurreducens* (8.2–11.8%), *G. metallireducens* (2.5–5.4%), *M. hydrocarbonoclasticus* (6.3–7.4%), and *M. sediminum* (2.3–11.7%) as allochthonous species (and not found in rTDWW sample) were the most abundant species in TDWWS1, TDWWS2, and TDWWS3 biofilms. Furthermore, *B. cepacia* (2.4–7.6%), *Achromobacter* sp. (10.1–16.4%), *C. acidovorans* (8.4–14.2), *D. gadei* (2.6–15.0%), *Rhodococcus* sp. (3.2–9.6%), and *Micrococcus* sp. (5.8–15.7%) as autochthonous species (and not found in HSCE) were also abundant in TDWWS1, TDWWS2, and TDWWS3 biofilms.

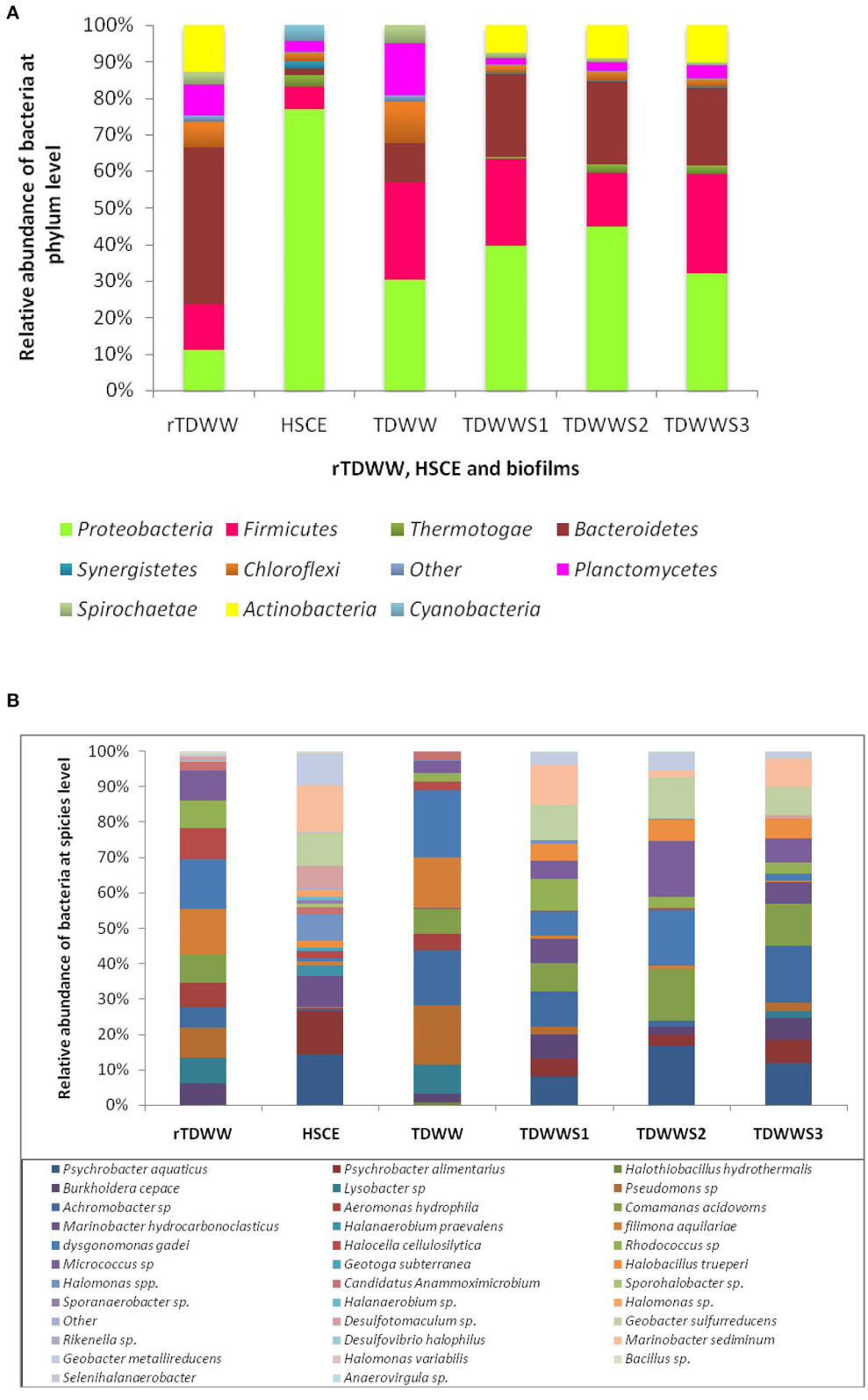

FIGURE 5 | (A) Bacterial distribution at phylum level of samples from raw textile dyeing wastewater (rTDWW), hypersaline sediment of Chott El Djerid (HSCE), and biofilms from a reactor containing textile dyeing wastewater (TDWW) and reactors containing 80% of TDWW and 20% of saline sediments (TDWWS1, TDWWS2, and TDWWS3). **(B)** Bacterial distribution at species level of samples from rTDWW, HSCE, and biofilms from a reactor containing TDWW and reactors containing 80% of TDWW and 20% of saline sediments (TDWWS1, TDWWS2, and TDWWS3).

By comparing the bacterial community of these three biofilms to that hosted TDWW, considered as a control, we found that TDWW harbored less bacteria belonging to the Proteobacteria (9.47%) and Bacteroidetes (3.22%) phyla. However, it hosted more bacteria from the Planctomycetes (4.5%), Chloroflexi (3.6%), and Spirochaeta (1.5%) phyla. At species level, the most abundant bacteria in TDWW biofilm were *D. gadei* (19.04%), *Pseudomonas* sp. (16.66%), *Achromobacter* sp. (15.47%), *F. aquilariae* (14.28%), and *Lysobacter* sp. (8.33%). This biofilm (TDWW) composed only of autochthonous bacteria was not electrochemically effective, as shown in **Figure 1.** Only few hundred milliamperes were obtained as current generation (300 mA). These strains were also noticed in previous studies on microbial communities in textile wastewater (Meerbergen et al., 2016).

Strikingly, high current production was obtained (12.5 ± 0.2 A/m^2) with biofilms (TDWWS1, TDWWS2, and TDWWS3) incorporated by both bacteria from HSCE and TDWW samples. A core bacterial community that was shared by the three biofilms could be highly involved in the current production. This core was composed of three autochthonous strains, *Achromobacter* sp., *C. acidovorans*, *D. gadei*, and four allochthonous strains, *P. aquaticus*, *G. sulfurreducens*, *G. metallireducens*, and *M. sediminum*.

CONCLUSION

This work is the first to demonstrate the potential to develop a novel halothermotolerant bioanode incorporating allochthonous and autochthonous bacteria from both hypersaline sediment and TDWW. A core bacterial community composed of three autochthonous strains, *Achromobacter* sp., *C. acidovorans*, *D. gadei*, and four allochthonous strains, *P. aquaticus*, *G. sulfurreducens*, *G. metallireducens*, and *M. sediminum*, ensures the effectiveness of the bioanode by producing high current density (12.5 A/m^2) and a total of 91% of COD removal efficiency. These findings, achieved under both hypersaline (165 g/L) and thermophilic conditions (45°), could lead to possible applications of BES technology for treatment and energy recovering from high-temperature and high-saline wastewaters.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found in Zenodo: http://doi.org/10.5281/zenodo.4276276.

AUTHOR CONTRIBUTIONS

HC, BE, RA, and AC: conceived and designed the experiments and analyzed the data. RA, SS, MN, and HC: sampling. RA, BE, LE, and HC: performed bioelectrochemical experiments. BE, RA, and HC: performed electrochemical analysis. HC, BE, RA, SS, MN, and AC: performed microbial and genomic analyses. HC, RA, BE, and AC: manuscript preparation and revision. HC, BE, and AC: supervised the entire project. All authors: contributed to the article and approved the submitted version.

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Improving Biological Treatment of Real Bilge Wastewater With Zero Valent Iron and Activated Charcoal Addition

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From the ships engine rooms a recalcitrant wastewater is produced called "bilge" which contains oil, metal working fluids, surfactants, and salinity. This study investigated the treatment of real bilge wastewater in short experiments using the following processes: (i) anaerobic digestion with granular sludge and ZVI addition for enhancement of methane production, (ii) activated charcoal addition to biological treatment (aerobic and anaerobic) for Chemical Oxygen Demand (COD) significant reduction and (iii) combination of ZVI and anaerobic charcoal addition for high performance treatment. The addition of ZVI in anaerobic sludge resulted in higher performance mostly in cumulative CH_4 production. The microbial profile of anaerobic granular sludge exposed to ZVI was determined and *Acetobacterium* and *Arcobacter* were the most dominant bacteria genera. Activated charcoal achieved higher COD removal, compared to biological degradation (aerobic and anaerobic). The combination of the two mechanisms, activated charcoal and biomass, had higher COD removal only for aerobic biomass. The combination of ZVI and activated charcoal to anaerobic digestion resulted in higher CH_4 production and significant COD removal in short contact time.

Keywords: bilge wastewater, anaerobic granular sludge, activated charcoal, methane, *Acetobacterium*, aerobic biomass

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INTRODUCTION

Bilge wastewater is generated in the ships engine room and is stored in the lowest part of the ship called bilge tank (Uma and Gandhimathi, 2019). Bilge is a recalcitrant wastewater that contains diesel oil, soluble oil, metal working fluids, surfactants and has high salinity (15–30 g L^{-1}). McLaughlin et al. (2014) reported that bilge wastewater contains hydrocarbons such as toluene, benzene, butylbenzene, ethylbenzene, acenaphthene, phenanthrene, pyrene, fluorene, metals, and substantial concentrations of detergents and solvents. The high concentration of surfactants causes the solubilization of high concentration of oil compounds in bilge wastewater and this contributes to high chemical oxygen demand (COD) (Vyrides et al., 2018b). In addition, according to International Maritime Organization (IMO) regulations (MARPOL 73/78) and the European directive 2000/59/EC bilge wastewater cannot be discharged to environment and should be properly treated enroute or to be deposited at reception facilities on land (Julian, 2000; Vyrides et al., 2018b). Treatment processes include physicochemical methods, such as oil separation techniques,

coagulation, chemical oxidation, membrane filtration and others, as well as biological treatment methods (Varjani et al., 2020).

Up to date several studies examined the biological treatment of bilge wastewater using aerobic mix consortia. Specifically, Vyrides et al. (2018b) found approximately 60% Chemical Oxygen Demand (COD) removal from real bilge wastewater in a pilot (200 L) Moving Bed Biofilm Reactor (MBBR) system. Nisenbaum et al. (2020) used an enriched aerobic microbial consortium to treat bilge wastewater and found 66.65, 72.33, and 97.76% removal of total petroleum hydrocarbons, aromatics and n-alkanes, respectively. Hwang et al. (2019) used single chamber microbial fuel cells (MFCs) for bilge wastewater biodegradation and concurrent electricity generation. In this system, the COD removal was greatly increased with the addition of anionic surfactant (SDS).

Regarding the anaerobic treatment of bilge water, Emadian et al. (2015) reported 75% COD removal of low strength bilge water whereas Vyrides et al. (2018b) in a batch test pointed out 28% of COD removal from real bilge wastewater. As a strategy to treat recalcitrant wastewater and enhance methane production several studies examined chemicals addition to the process. A recent study examined CaO_2 pretreatment of waste activaded sludge in order to degrade refractory compounds such as humus and lignocelluloses, increasing importantly methane production during anaerobic digestion (Wang et al., 2019). Other researchers added zero valent iron directly in the anaerobic digestion process; Liu et al. (2011) for dye wastewater, Wang et al. (2017) for Fischer-Tropsch (FT) wastewater, Xu et al. (2020) for coking wastewater and Pan et al. (2019) for tetracycline biodegradation. The addition of ZVI in the anaerobic digestion results in the abiotic H_2 production due to anaerobic oxidation of ZVI. The generated H_2 is utilized by hydrogenotrophic methanogens along with CO_2 and therefore increase CH_4 in the system. In addition the ZVI anaerobic oxidation provides alkalinity in the system and due to the enhancement of hydrogen utilizing microorganisms can contribute to propionic acid utilization.

Regarding adsorption method so far only Vlaev et al. (2011) investigated the bilge wastewater adsorption using sing rice husks ash. The results showed that rice husks ash had high adsorption capacity for hydrocarbon removal for bilge water. In addition, the final product was characterized with high calorific value and was proposed to be used as a feedstock in incinerators, industrial ovens or steam generators.

The aim of this study was to improve biological treatment of bilge wastewater using advanced processes. Biological treatment was chosen as a relatively inexpensive and easy to apply method. For this reason the following conditions were examined under batch mode experimental set up and at small laboratory scale.

(A) Firstly, anaerobic digestion of bilge wastewater was examined as an initial biological treatment step for decreasing high organic load and the addition of ZVI was evaluated as a strategy to increase methane production. The microbial profile under these conditions was studied using next generation sequencing.
(B) Secondly, a purification step was tested, combining activated charcoal adsorption and biological activity. Both aerobic and anaerobic conditions were tested in this case in order to determine the more efficient for organic load decrease.
(C) Finally, both treatment steps were combined in order to evaluate treatment performance and reduce treatment time. In this case anaerobic digestion was examined with the simultaneous addition of (i) ZVI; for methane enhancement, and (ii) activated charcoal; for organic load decrease.

To the author's knowledge, this is the first study examining ZVI addition for enhancement of real bilge wastewater anaerobic treatment, and the first to combine this approach with activated charcoal as a second individual treatment step or as a combination of processes.

MATERIALS AND METHODS

Anaerobic Digestion of Bilge Wastewater

For all anaerobic experiments, sealed glass serum bottles were used and were incubated at 37°C. Anaerobic granular sludge was used at a concentration of 4% w/v, collected from a mesophilic upflow anaerobic sludge blanket reactor (UASB) that was treating dairy wastewater (Charalambides Christis Ltd, Limassol, Cyprus), operated at pH 6.8–7.3 (as reported by Samanides et al., 2020). Raw bilge wastewater was used, collected by a company treating this wastewater type (Ecofuel Ltd, Zygi, Cyprus), the wastewater characteristics are described in detail in a previous study by Vyrides et al. (2018b). A mineral medium as described by Angelidaki et al. (2009) was added to create the same initial concentration in all experiments. Raw bilge wastewater was enriched with the following stock solutions (chemicals given are concentrations in g L^{-1} in distilled water) Solution A: NH_4Cl, 100; NaCl, 10; $MgCl_26H_2O$, 10; $CaCl_22H_2O$, 5, Solution B: $K_2HPO_43H_2O$, 200. To 993 ml of raw bilge wastewater the following volume of each stock solution was added: (Solution A) 5 ml, (Solution B) 2 ml as well as 3 g of $NaHCO_3$. As a last step, prior to incubation, the serum bottles were sealed with rubber septa and a screw cap and the headspace was flushed with CO_2 (99.99% purity) for 1 min for the creation of anaerobic conditions.

Preliminary investigation included the test of three dilution rates for the treatment of bilge wastewater of low initial COD with anaerobic digestion. The following dilution factors were applied to the wastewater: 1:1, 1:2, and 1:4, leading to wastewater concentration of 100, 50, and 25% in each case. Serum bottles of 125 ml were used with a working volume of 70 ml. Treatment time was 82 days.

Monitoring of the anaerobic digestion process included the determination of biogas volume production as well as gas composition of the headspace. For this reason a gas chromatograph, coupled with a thermal conductivity detector (GC-TCD, Agilent technologies 7820A GC system, Wilmington, DE) was used, according to the method described by Vyrides et al. (2018b). Furthermore, the Chemical Oxygen Demand (COD) value of the wastewater was measured at

the beginning, during and at the end of the experiments. For COD quantification a modified colorimetric method was used (absorbance quantification with HACH, DR1900 spectrophotometer), according to Freire and Sant'Anna (1998) and Vyrides and Stuckey (2009) in order to take into consideration the oxidation of chloride ions present in bilge wastewater due to high salinity.

ZVI Batch Experiments

For the examination of more realistic industrial conditions, the enhancement of anaerobic digestion of high organic load bilge wastewater with the use of Zero-Valent-Iron (ZVI) was tested as a technique to facilitate microbial activity via H_2 production. Powder ZVI (Merck, Iron for analysis reduced, particle size 10 μm, CAS-No: 7439-89-6) was used at two concentration levels (10, 25 g L^{-1}) in a small scale batch experiment (125 ml glass serum bottles with 70 ml working volume) and in a second experimental cycle the periodic addition of ZVI was tested under semi batch conditions in a larger scale experiment (500 ml glass bottles with 300 ml working volume). Bilge wastewater, granular anaerobic sludge and anaerobic conditions were obtained and handled as described in section "Anaerobic Digestion of Bilge Wastewater." COD and gas composition were monitored over time in order to evaluate digestion performance. The microbial profile (bacteria and archea) of the granular sludge was evaluated after 47 days of operation under semi batch conditions (larger scale experiment). Approximately 180–220 mg of biomass were collected from each bioreactor. Then the total genomic DNA was extracted through the NucleoSpin DNA stool (Macherey-Nagel, Germany) and was sent to DNASense Company (Denmark) for sequencing.

Activated Charcoal Batch Experiments

Activated charcoal was tested conjointly with microorganisms for further reduction of organic load in bilge wastewater. Aerobic and anaerobic experiments were conducted with medium initial COD level and under batch mode in sealed glass serum bottles (125 ml) with 70 ml working volume and 55 ml of headspace. Activated charcoal (AC) (Fluka, CAS-No: 7440-44-0) was used at the concentration level of 3 g L^{-1} whereas optimum performance was observed after preliminary experiments.

For anaerobic treatment, the set up of the experiments was conducted as described in section "Anaerobic Digestion of Bilge Wastewater." For aerobic treatment, a liquid mixed bacteria culture, obtained by enrichment steps (3 months), was used with the addition of 4% v/v in the serum bottle. For the examination of aerobic treatment performance, COD was monitored and UV-VIS spectra of the effluent was examined using a benchtop spectrophotometer (Jenway 7315 UV/Visible spectrophotometer).

Combined ZVI and Activated Charcoal Batch Experiment

The combination of the techniques described in section "ZVI Batch Experiments" and section "Activated Charcoal Batch Experiments" were applied in order to test the parallel reduction of high organic load in combination with high CH_4 production. Sealed glass serum bottles (125 ml) with 70 ml working volume and 55 ml of headspace were used and anaerobic granular sludge was added at a concentration of 4% w/v. Anaerobic digestion of bilge wastewater of high initial COD was examined (i) with granular sludge and with (ii) granular sludge with ZVI (10 g L^{-1}) and activated charcoal (3 g L^{-1}) addition at the beginning of the experiment. Samples were created in triplicates and monitored over 15 days, as described in section "Anaerobic Digestion of Bilge Wastewater."

RESULTS

Treatment of Bilge Wastewater Using Anaerobic Granular Sludge With and Without Zero Valent Iron Addition

Figure 1A points out that the exposure of anaerobic granular sludge to undiluted real bilge wastewater (initial COD 2200 mg L^{-1}) resulted in relatively low methane production over a period of 82 days. The dilution of the wastewater caused higher methane production, probably as a result of toxicity decrease. The COD removal after 82 days varied from 50 to 60% under these low initial COD conditions.

As a strategy to alleviate bilge inhibition to anaerobic granular sludge, zero valent iron was added at 10 and 25 g ZVI L^{-1}. As shown in **Figure 1B**, addition of ZVI generated higher amount of CH_4 compared to anaerobic granular sludge without ZVI. The COD removal under these conditions was low (**Figure 1B**), however, the COD removal in the reactors containing ZVI was slightly higher compared to the reactors with anaerobic granular sludge free of ZVI. Noteworthy, the initial COD was 5150 mg L^{-1} which was more than double than the experiment related with **Figure 1A**. It is likely that the higher COD in the experiment related with **Figure 1B** has contributed to higher toxicity to anaerobic granular sludge and therefore the anaerobic granular sludge (with no ZVI) resulted in negligible COD removal. Part of the higher CH_4 composition (**Figure 1B**) in anaerobic granular sludge with ZVI could be due to H_2 production due to anaerobic aquatic zero valent iron oxidation followed by the H_2 and CO_2 utilization by hydrogenotrophic methanogens (Vyrides et al., 2018a; Menikea et al., 2020).

Based on the positive findings regarding the ZVI addition a larger scale (500 ml glass bottles with 300 ml working volume) experiment took place as descripted in section "Materials and Methods." As shown at **Table 1**, at the beginning of the experiment the anaerobic granular sludge with 10 g L^{-1} ZVI resulted in dramatically higher methane production (55.5 ml CH_4 at day 7) compared to anaerobic granular sludge free of ZVI (8.5 ml CH_4 at day 7). This trend was continued through the experiment and higher performance was found for the bioreactor with 10 g L^{-1}, followed by the bioreactor with 2 g L^{-1} initial ZVI concentration (were ZVI 2 g L^{-1} was periodically added). The lower performance was found in the anaerobic granular sludge free of ZVI (**Table 1**). This difference was also more profound in the CH_4 composition under these conditions.

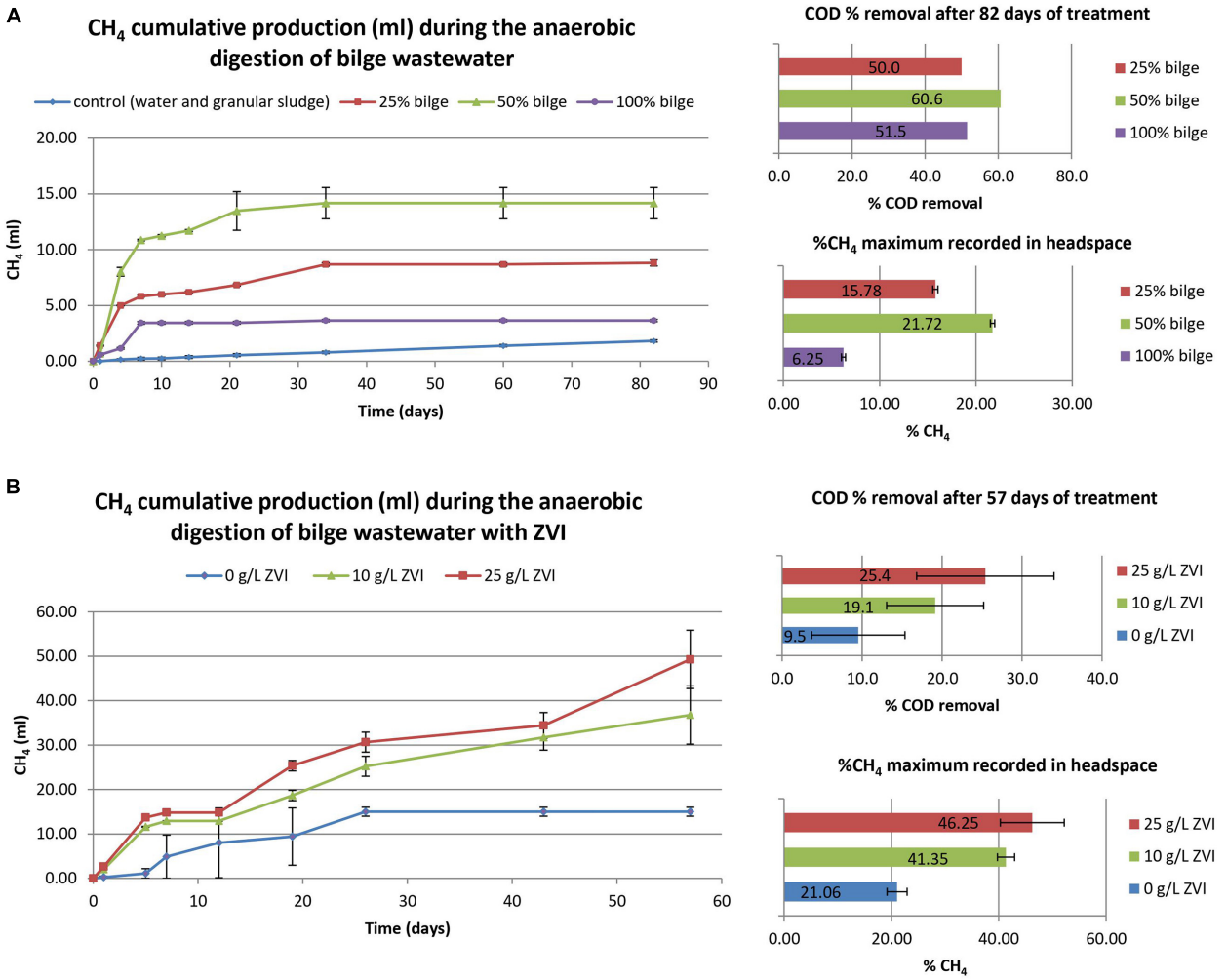

FIGURE 1 | Performance of anaerobic digestion of bilge wastewater with the use of granular sludge **(A)** under different dilution factors (100, 50, and 25% concentration of bilge) and low initial COD (2200, 1100, and 550 mg L^{-1}) and **(B)** with the addition of powder ZVI (25, 10, and 0 g L^{-1}) and with a high initial COD (5150 mg L^{-1}).

The relative abundance of the archaeal 16S rRNA gene at the genus levels is shown in **Figure 2A**. Samples were withdrawn from bioreactors on day 47. The most dominant genus in anaerobic sludge with ZVI were *Acetobacterium* and *Arcobacter*. However, these genera were negligible in anaerobic granular sludge without ZVI (**Figure 2A**). *Acetobacterium* are homoacetogens and can convert H_2 and CO_2 to acetic acid; they were stimulated due to the presence of ZVI and the release of H_2 (Menikea et al., 2020). The *Arcobacter* genus includes a diverse assemblage of species which are obligate and facultative chemolithoautotrophs as well as heterotrophs. *Arcobacter* spp. have been enriched in engineered systems containing high levels of sulfide and high levels of organic matter and benefit from the transfer of organic matter and hydrogen (Callbeck et al., 2019).

Methanosaeta sp. (acetoclastic methanogens) have been proposed as one of the primary microbial groups responsible for methanogenic granule formation and this explains its high abundance in all samples; however, a higher relative abundance was found in anaerobic granular sludge that was exposed to 10 g L^{-1} of ZVI (**Figure 2**). These findings are in line with the study of Vyrides et al. (2018a) which found higher relative abundance in anaerobic granular sludge exposed to ZVI. *Methanolinea*, a hydrogenotrophic methanogen, was the dominant genus (∼50%) in the anaerobic granular sludge where 2 g ZVI L^{-1} was periodically added in the bioreactor (**Table 1** and **Figure 2B**). Finally, another hydrogenotrophic methanogen, *Methanobacterium,* was found at a higher relative abundance in anaerobic granular sludge exposed to ZVI compare to anaerobic granular sludge free of ZVI (**Figure 2B**).

Activated Charcoal

As observed in preliminary and ZVI anaerobic experiments, the COD removal from bilge wastewater was relatively low, requiring considerable amount of time, probably due to the toxicity of the wastewater toward anaerobic granular sludge. As a strategy to overcome this, the treatment of bilge wastewater in a system of activated charcoal and anaerobic granular sludge was examined. At an initial COD level of 2750 mg L^{-1}, with the presence of

TABLE 1 | Performance and operational parameters of ZVI batch experiment (large scale) for bilge wastewater anaerobic digestion.

Large Scale semi batch anaerobic digestion reactor performance (500 ml reactor volume with 300 ml working volume), *T* = 37°C													
Operation day	**1**	**7**	**8**	**14**	**21**	**22**	**28**	**35**	**37**	**43**	**47**	**48**	**77**
CONTROL													
CH_4 % in headspace	0.2	3.8		27.2	15.1		15.0	9.0		0.6	0.9		0.8
CH_4 cumulative production (ml)	0.6	8.5		88.9	136.7		147.2	154.8		156.1	156.7		156.7
pH adjustment			✓			✓			✓			✓	
CO_2 flush			✓			✓			✓			✓	
EXP 1 (10 g/L ZVI)													
CH_4 % in headspace	3.4	27.5		9.1	14.2		34.7	40.1		14.9	22.1		33.2
CH_4 cumulative production (ml)	7.3	55.5		73.6	114.7		157.7	182.9		212.8	227.2		249.3
pH adjustment			✓			✓			✓			✓	
CO_2 flush			✓			✓			✓			✓	
EXP 2 (2 g/L ZVI)													
CH_4 % in headspace	2.3	19.4		9.4	11.3		29.0	41.2		16.6	23.0		32.0
CH_4 cumulative production (ml)	5.4	39.6		60.1	93.4		128.8	153.1		186.2	199.0		217.2
pH adjustment			✓			✓			✓			✓	
CO_2 flush			✓			✓			✓			✓	
ZVI addition (2 g/L)			✓			✓			✓			✓	

activated charcoal in the anaerobic granular sludge reactor, 52 and 60% COD removal rates were achieved after 3 and 63 days of exposure, respectively. Under the same conditions, activated charcoal without anaerobic granular sludge, removed 48 and 84% of the COD load (after 3 and 63 days, respectively). The lower COD removal observed after 63 days of treatment when both activated charcoal and granular sludge were present could be attributed to the decrease of sorption capacity because of VFAs production and adsorption onto activated charcoal after the first days of anaerobic digestion. This trend was observed in a similar study by Jiang et al. (2021) where VFAs where monitored and found to be of lower concentration when activated carbon was present in the anaerobic digestion process. Anaerobic granular sludge without addition of activated charcoal pointed out low COD removal on day 3 and then the removal increased to 62% on day 63. The methane production of anaerobic granular sludge during bilge treatment with and without activated charcoal was similar (**Figure 3B**). It is highly probable that in the system of anaerobic granular sludge where activated charcoal is present, COD is removed through adsorption on activated charcoal during the first days of treatment and then part of the organics are biodegraded by anaerobic granular sludge (**Figure 3A**).

A similar experiment was conducted testing the addition of activated charcoal under aerobic conditions and with the addition of an aerobic mixed culture instead of anaerobic granular sludge (**Figure 3C**). After 3 days of treatment, the COD removal (initial COD: 3470 mg L^{-1}) achieved by aerobic biomass with activated charcoal and activated charcoal exclusively was 59 and 61%, respectively. The COD removal slightly increased over time, reaching 62 and 65% removal after 90 days of treatment (for aerobic biomass with activated charcoal and activated charcoal, respectively) On the other hand, aerobic biomass without any activated charcoal pointed out the lower COD removal (19% on day 3 and 33% on day 90). It is highly probable that the contribution of biomass is not significant due to the rapid adsorption of easily biodegradable compounds (low molecular weight organic compounds) on activated charcoal, creating a hostile environment for microorganisms whereas, compounds hard to biodegrade are left in the liquid phase increasing toxicity. The UV-VIS spectra of treated effluent (after 90 days of treatment) revealed the significant impact of activated charcoal addition (**Figure 3D**). The compounds detected at around 290 nm were substantially removed in the reactors containing activated charcoal and remained present in the reactors free of activated charcoal.

Effect of ZVI and Activated Charcoal Combined Addition

The performance of anaerobic digestion of bilge wastewater with granular sludge was evaluated over the same process with the addition of ZVI and activated charcoal. The addition of both compounds simultaneously had a positive effect on the treatment performance. Over 15 days of treatment the conventional anaerobic digestion reduced less than 5% of the initial COD of the wastewater while only 2.5 ± 0.1 ml of CH_4 were produced. On the other hand, with the addition of ZVI and activated charcoal almost 50% of initial COD was reduced in 15 days, while the CH_4 production reached 44.3 ± 3.4 ml and the CH_4 percentage in the biogas reached 76.3 ± 2.5% (see **Supplementary Material**).

DISCUSSION

The treatment of real bilge wastewater with a high variability in the organic content was examined in short experiments using (i) anaerobic digestion with granular sludge and ZVI

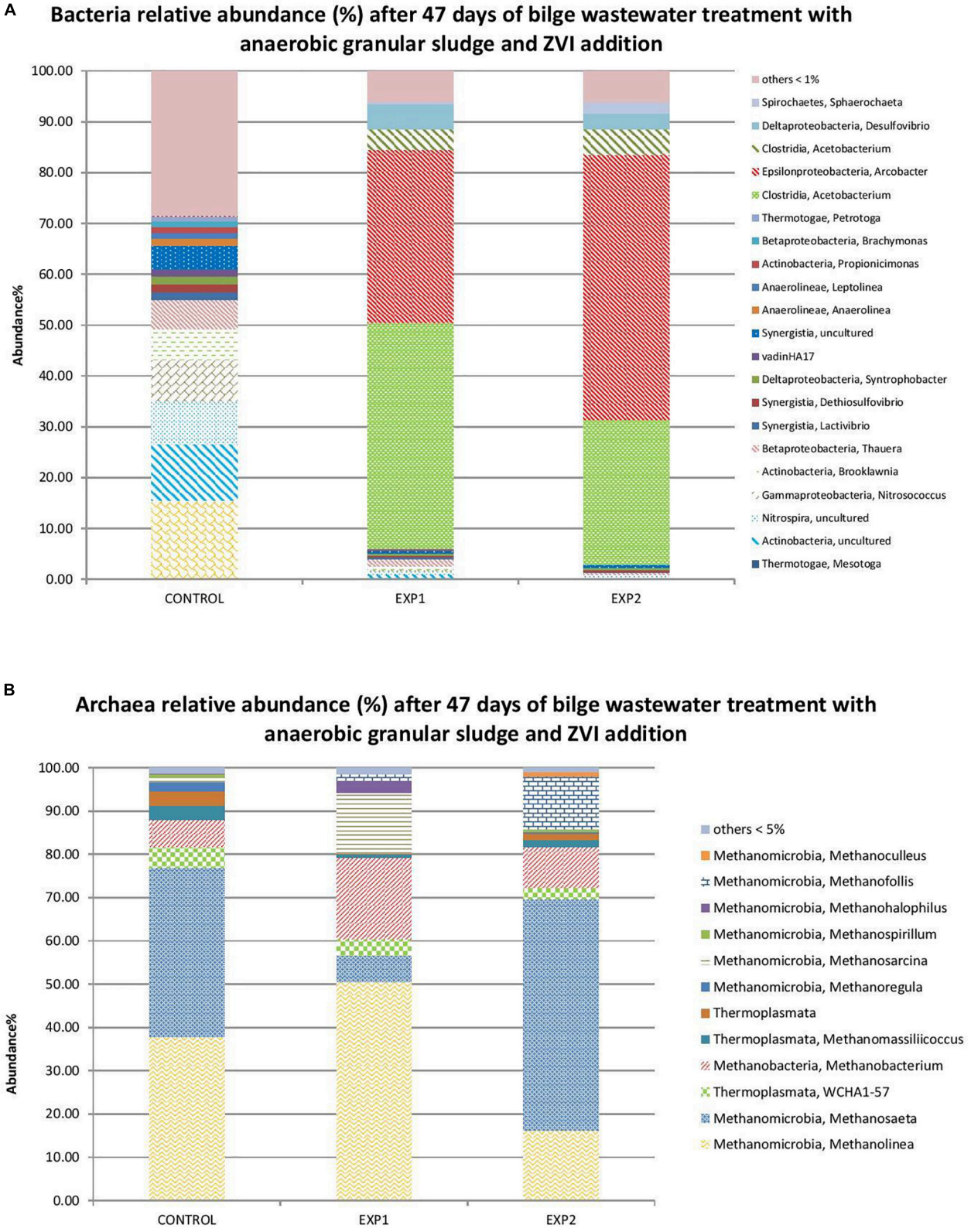

FIGURE 2 | Microbial profile of anaerobic granular sludge after 47 days of exposure to bilge wastewater (CONTROL), with 10 g L^{-1} powder ZVI addition at $t = 0$ (EXP 1) and 2 g L^{-1} powder ZVI addition at $t = 0$ (EXP 2). Relative abundance % is illustrated for populations of **(A)** bacteria and **(B)** archaea.

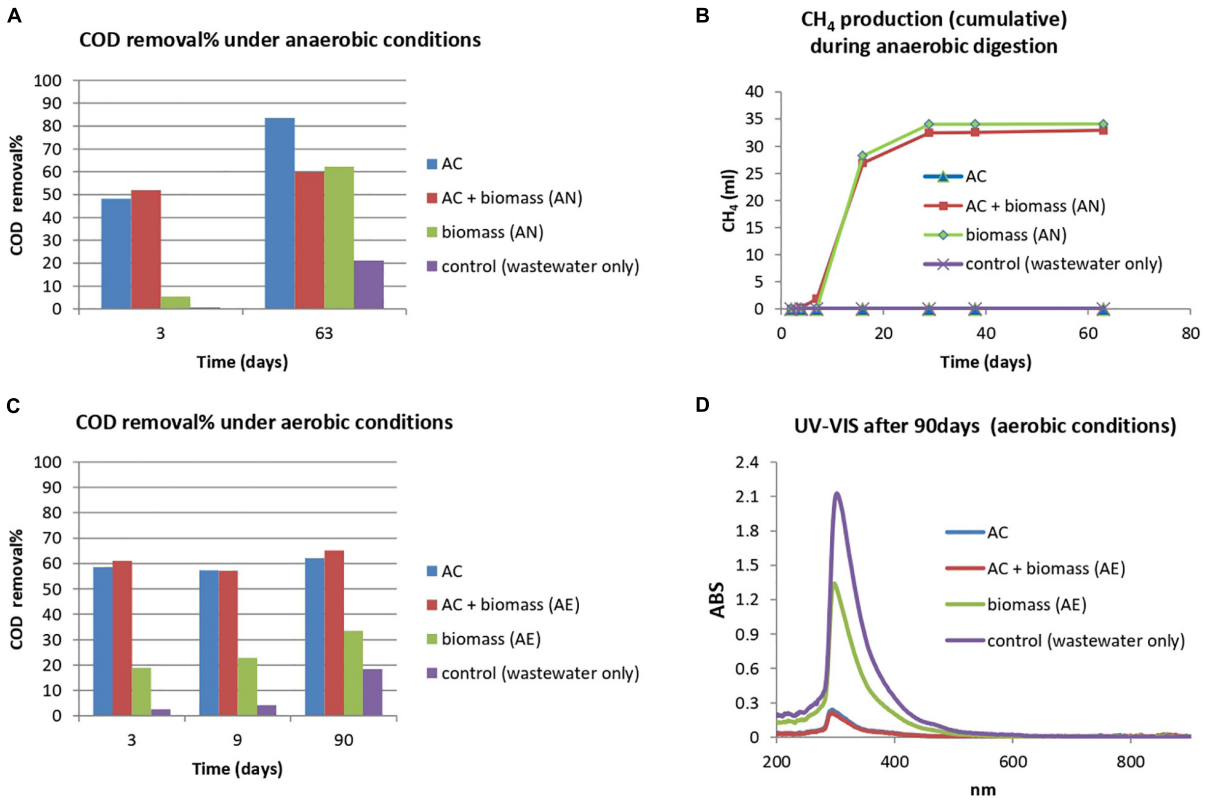

FIGURE 3 | Treatment of bilge wastewater with the use of anaerobic/aerobic biomass and activated charcoal (AC), COD reduction of bilge wastewater (initial COD: 2750 mg L^{-1}) **(A)** with the addition of (i) activated charcoal (AC), (ii) AC and anaerobic biomass (granular sludge), (iii) anaerobic biomass (granular sludge), (iv) control (bilge wastewater only), and **(B)** CH_4 cumulative production (ml) under anaerobic conditions, **(C)** COD reduction of bilge wastewater (initial COD: 3470 mg L^{-1}) with the addition of (i) activated charcoal (AC), (ii) AC and aerobic biomass, (iii) aerobic biomass, (iv) control (bilge wastewater only), **(D)** UV-VIS absorbance values of the treated effluent after 90 days with aerobic conditions.

addition, (ii) biological treatment (aerobic and anaerobic) with activated charcoal addition, and (iii) combination of ZVI and activated charcoal addition in anaerobic digestion. It was found that undiluted bilge wastewater (initial COD 2200 mg COD L^{-1}) showed little cumulative CH_4 production whereas dilution leading to 50 and 25% concentration of the wastewater resulted in higher cumulative CH_4. Under these conditions the COD removal was between 50 to 60% after 82 days of treatment. In another experiment where the initial COD was 5150 mg L^{-1} anaerobic granular sludge removed negligible percentage of COD and this showed that the higher the initial COD the harder for the microorganisms to efficiently degrade the wastewater, probably due to higher toxicity toward anaerobic granular sludge. At initial COD 5150 mg L^{-1} the anaerobic granular sludge with ZVI pointed out higher performance even though the difference with anaerobic granular sludge without ZVI was more profound in CH_4 than in the COD removal. The higher CH_4 could be due to abiotic anaerobic ZVI oxidation and the released H_2 that was then utilized by hydrogenotrophic microorganisms. The higher performance of anaerobic granular sludge with ZVI was also found at a larger scale experiment (500 ml glass bottles with 300 ml working volume) whereas anaerobic granular sludge with ZVI 10 g L^{-1} resulted in the highest performance followed by anaerobic granular sludge where ZVI 2 g L^{-1} was periodically added. As previously described (section "Treatment of Bilge Wastewater Using Anaerobic Granular Sludge With and Without Zero Valent Iron Addition"), the H_2 produced is probably the main mechanism increasing the methane production. Regarding the two addition methods tested (10 g L^{-1} initial addition or progressively addition of 2 g L^{-1} each time) it seems that with a low concentration periodical addition of ZVI, H_2 is released at a lower rate, rendering the process less effective (Vyrides et al., 2018a). The lowest performance was identified for anaerobic granular sludge without any addition of ZVI in terms of total CH_4 production and CH_4 concentration in biogas composition. The bacteria at genus level with the higher relative abundance in anaerobic granular sludge with ZVI were *Acetobacterium* and *Arcobacter*, which can utilize H_2 along with CO_2 or organic matter (Callbeck et al., 2019). *Methanosaeta* sp. (acetoclastic methanogens) found in high abundance in all anaerobic granules probably due to its role in granule formation. On the other hand, Methanobacterium (hydrogenotrophic methanogen), was found at a higher relative abundance in anaerobic granular sludge exposed to ZVI compare to anaerobic granular sludge free of ZVI most likely due to the presence of H_2 due to anaerobic ZVI oxidation.

The addition of activated charcoal in anaerobic granular sludge resulted in fast COD removal after 3 days, however, the COD removal has slightly increased over time. Interestingly, the cumulative CH_4 was the same for both conditions (anaerobic sludge with activated charcoal and without) and this indicate that activate charcoal contributes mostly by organics adsorption and has not enhanced further the organic biodegradation by anaerobic granular sludge. The same trend was also found for aerobic biomass with activated charcoal which shows approximately 60% COD removal after 3 days whereas aerobic biomass only resulted in 19% COD removal during the same contact time.

These results point out that for rapid COD removal a physicochemical process (activated charcoal) is needed and that the biological process (anaerobic and aerobic) is slow due to toxicity of bilge toward biomass and/or due to recalcitrant organics compounds in bilge wastewater. The integration of activated charcoal in biomass (aerobic or anaerobic) resulted in high COD removal in the first days and then the COD removal was stabilize; however, more research can be done to examine its effect over longer time period. It is probable that the adsorption capacity of activated charcoal is high at the beginning of the treatment, leading to fast COD deterioration, but as activated charcoal reaches saturation the removal rate gets stable (Jiang et al., 2021).

The combination of ZVI and activated charcoal addition had a positive effect on the anaerobic digestion process. Both COD decrease and CH_4 production were achieved at a relatively low contact time. Further research could focus on the underlying mechanisms of this treatment scheme, while larger scale experiments could determine the feasibility of the method application.

To summarize, greater bioremediation performance, in terms of COD removal, was observed when activated charcoal was added to the biological process. Additionally, the amount and quality of biogas produced during the anaerobic digestion of bilge wastewater can be increased via ZVI addition to the wastewater. The independent use of the two techniques could be used for efficient treatment but would increase treatment time. The combination of both approaches provided promising results and with further investigation could lead to an applicable method.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AM: investigation, validation, visualization, and writing-original draft preparation. GN: investigation. GS: investigation. IV: conceptualization, methodology, visualization, project administration, supervision, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.614510/full#supplementary-material

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Green Manure Species for Phytoremediation of Soil With Tebuthiuron and Vinasse

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Tebuthiuron is often used to control weed growth in sugarcane cultures. This herbicide is highly toxic and can persist in soil for up to 2 years according to its degradation half-life. Hence, its residual effect is highly hazardous for the environment and local habitants via leaching, surface runoff. Screening out of species of green manure as potential phytoremediators for tebuthiuron in soil, with and with no vinasse, accordingly is the scientific point of this study. Green manure species selected for the trial in greenhouse were jack bean [*Canavalia ensiformis* (L.) DC.], pigeon pea [*Cajanus cajan* (L. Millsp.)], velvet bean [*Mucuna pruriens* (L.) DC.)], and millet [*Pennisetum glaucum* (L.) R.Br.], and *Crotalaria juncea* L. as bioindicator of this herbicide. The determination/quantification of height, stem diameter, and number of leaves in all plants were monitored, as well as other morphological traits for drafting any inference on biomass production. Moreover, ecotoxicity bioassays were performed from soil samples at the beginning and at the end of the experiment. Results showed preliminary evidence of effective phytoremediation capacity by *M. pruriens* and *P. glaucum* in soils with tebuthiuron, as the growth of *C. juncea* was sustained. Both Gompertz approach and principal component analysis predicted that these green manure species could grow healthier and for longer periods in soils containing tebuthiuron and vinasse and, thus, reduce physiological anomalies due to ecotoxicity. The implications of this study may aid in the implementation of cost-effective strategies targeting decontamination of tebuthiuron in sugarcane crops with vinasse application in fertigation.

Keywords: bioremediation, ecotoxicity, fertigation, herbicide, sugarcane

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INTRODUCTION

Sugarcane is affected by weeds, despite its highly efficient photosynthetic pathway (C_4) that promotes adequate development, especially in its early stages. Weeds compete for available soil resources and therefore undermine agricultural yields (Victoria Filho and Christoffoleti, 2004; Sandaniel et al., 2008).

The planting of sugarcane takes place in wide open areas so that high productivity is achieved, aided by technological tools for the proper weed management as herbicides (Kuva et al., 2008; Oliveira and Brighenti, 2011). Such chemical method is the first choice of agricultural producers due to its ease of access, availability, and low operational costs, compared to other control techniques (Kuva et al., 2008).

Among the herbicides commercialized for sugarcane, tebuthiuron is the most used, whose selective pre-emergent action controls main weeds in the crop (Moraes et al., 2016). This molecule [1-(5-tert-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea] has a systemic action and acts in the inhibition of photosystem II (Breitenbach et al., 2001). However, tebuthiuron can cause environmental damage since it is considered dangerous to the environment due to its high persistence and long half-life in the environment, moderate to extreme toxicity (Rodrigues and Almeida, 2011), low sorting capacity in soil (Koskinen et al., 1996), and high solubility in water (Franco-Bernardes et al., 2014). Hence, residual concentration is an extremely important factor, as it results in a greater potential for contamination. Therefore, successive applications without proper management can make its potential for impact even greater on soil and groundwater (Christofoletti et al., 2017).

Vinasse can also be applied to farmlands as growth-inducing agents, as opposed to herbicides. Vinasse is one of the many by-products of sugar production also found in alcohol distilleries in enormous quantities. It is highly applicable as fertilizer during crop production (Andrade, 2007). It is considered a residue from the alcohol production, generated at a 10–14:1 ratio (Assad, 2017). The outstandingly large amount of vinasse generated everyday highlights its expressive polluting potential and, therefore, demands the development of proper disposal protocols. However, vinasse can also be used to enrich soils due to its nutritional value. Still, its environmental effects combined with herbicides are yet to be determined.

The cleaning up process of areas with previous pesticides release is not simple, but fortunately, many solutions have been improved in the past decades. Feasible solutions should follow four requirements, as proposed by Ferro et al. (1994): (i) high decontamination efficiency, (ii) straightforward execution, (iii) fast and reliable protocols, and (iv) cost effectiveness. Bioremediation is as an ecologically viable strategy that meets such requirements during the treatment of impacted areas by organic pollutants. The acceleration of natural biological processes that reduce the concentration and toxic effects of polluting agents is the core of all bioremediation strategies (Fasanella and Cardoso, 2016).

Phytoremediation further expands this definition by using plants to reduce the toxicity of contaminants in the environment (Ali et al., 2013). Research related to this technique seeks to understand the plant–contaminant interactions that may lead to full pollutant removal (Vasconcellos et al., 2012). Therefore, the plants must be capable of absorbing toxic elements in the soil to promote decontamination (Souza et al., 2011).

Pires et al. (2008) reported that millet (*Pennisetum typhoides*), velvet bean (*Stizolobium aterrimum*), Jack bean (*Canavalia ensiformis*), and pigeon pea (*Cajanus cajan*) were highly effective toward tebuthiuron phytoremediation. They used sunn hemp (*Crotalarea juncea*) as the bioindicator plant. Several studies reinforce this approach, as many authors have observed a decrease in pesticides concentration in soils by using phytoremediation (Pires et al., 2003, 2005, 2006; Pires et al., 2008; Madalão et al., 2013; Melo et al., 2017).

However, there is a major drawback in all those studies: the toxicity of these samples has not been quantified before and after the treatments. The degradation of organic compounds could potentially generate intermediate compounds that are often more toxic than the original formulation (Rocha et al., 2018). We argue that it is imperative to evaluate the ecotoxicological potential in a broader time-dependent approach to demonstrate the success of bioremediation strategies (Banks and Schultz, 2005).

In this context, we evaluated the potential of four plant species to remediate soil samples contaminated with tebuthiuron and the effects of vinasse in the process.

MATERIALS AND METHODS

Experiments were set up in a greenhouse located at the College of Agricultural and Technological Sciences, Sao Paulo State University (Unesp), Dracena, São Paulo, Brazil, with geographical coordinates of 21°28′57″ S 51°31′58″ W and 400 m elevation.

According to the Köppen (1948) classification, the regional climate type is Aw (tropical humid). The average local temperature and precipitation are 22.1°C and 1,200 mm, respectively. The meteorological data were provided by our own station (Dracena EMA/FCAT).

The observations occurred between May and July 2019. The average temperature and relative humidity were 22.6°C and 62.9%, respectively, also obtained from the Dracena EMA/FCAT weather station.

Soil, Vinasse, and Tebuthiuron Sampling

The regional soil is a dystrophic red-yellow argisol type according to the classification proposed by Santos et al. (2018). The physical analysis revealed that it is composed of 89.9% sand, 7.1% clay, and 3.0% silt.

The soil has the following chemical characteristics: phosphorus, 5 mg/dm^3; organic matter, 3 mg/dm^3; pH 5.2; potassium, 1.7 mmolc/dm^3; calcium, 15 mmolc/dm^3; magnesium, 4 mmolc/dm^3; H + Al, 13 mmolc/dm^3; CTC, 34 mmolc/dm^3; sum of bases, 21 mmolc/dm^3; and base CTC saturation (V%), 61%. This characterization served as a basis to the optimal fertilizing conditions in our pots for all the proposed species.

Fertilizer dosages per pot were set individually to meet each species needs. We applied 80 g of urea diluted in 1.5 L of water, divided into three applications, in *Pennisetum glaucum* (L.) R.Br. We applied 8 g of urea diluted in 4.5 L of water, applied only once at sowing, in legumes [*C. ensiformis* (L.) DC., *C. cajan* (L. Millsp.), *Mucuna pruriens* (L.) DC.]. For all other pots, we added 125 g of KCl diluted in 6 L of water, divided into three applications, and 445 g of simple super phosphate to 320 L of soil, necessary

TABLE 1 | Morphological aspects of green manure species in soil with tebuthiuron and vinasse.

Species	Test			
	T-V-	T-V+	T+V-	T+V+
			Height, cm	
C. cajan	64.40 Aa	49.20 Bb	ND*	ND*
C. ensiformis	75.00 Aa	64.80 Bb	ND*	ND*
M. pruriens	125.80 Aa	134.60 Aa	115.50 Aab	44.00 Bb
P. glaucum	71.00 Aa	53.60 Ba	60.75 Ba	56.65 Aa
			Diameter, mm	
C. cajan	5.40 Ba	4.69 Bb	ND*	ND*
C. ensiformis	5.65 Ba	5.60 Bb	ND*	ND*
M. pruriens	5.05 Ba	5.05 Ba	4.45 Bab	3.50 Bb
P. glaucum	52.70 Aa	42.30 Aa	35.90 Aa	23.15 Ab
			Leaves	
C. cajan	16.80 Aba	13.00 Bb	ND*	ND*
C. ensiformis	4.80 Ca	4.20 Cb	ND*	ND*
M. pruriens	14.40 Ba	11.00 Ba	11.50 Ba	2.00 Ab
P. glaucum	22.50 Aa	20.80 Aa	20.50 Aa	16.30 Ba

Same letters, whether uppercase in the column and lowercase in the row, describe statistically similar means by post hoc Tukey's HSD test at $p < 0.05$;.
**As the plant dead, the trait became undetected (ND).*

for filling the pot total volume. The volume of each vessel was 4 dm^3.

The vinasse was collected from a sugar-energy plant in the Dracena-SP region using sterile glass bottles. The vinasse was subsequently stored in a refrigerator at 4°C until its use in the experimental units preparation (stored for 3 days).

The herbicide tebuthiuron was provided by Combine® 500SC—Dow AgroSciences Industrial Ltd.

Plant Species

The plant species were chosen according to their capability to remediate pesticide-contaminated soils. Their agricultural potential to improve overall soil quality was another criterion. We narrowed our plants selection to species that are often found as green manure and/or forage in the rotation of the sugarcane cultures. Thus, we used the following species: pigeon pea (*C. cajan*), jack bean (*C. ensiformis*) (Madalão et al., 2013, 2016), velvet bean (*M. pruriens*) (Pires et al., 2005, 2008), and millet (*P. glaucum*) (Pires et al., 2008).

Experimental Setup

We designed the experiments according to a randomized blocks approach at a 2 × 2 × 4 factorial scheme with five repetitions. The parameters were tebuthiuron concentration, vinasse volume, and the four plant species.

The treatments are further referred to in this paper as indicated in the brackets: [T–], absence of tebuthiuron; [T+], presence of tebuthiuron; [V–], absence of vinasse; and [V+] presence of vinasse.

Preparation of Experimental Units

Experimental units (pots 4 dm^3 and their contents) were filled with soil and received the four potentially phytoremediating species. Treatments containing vinasse had to undergo manual compound application to endure homogeneity. The vinasse was added first, at 150 $m^3\ ha^{-1}$ (150 ml dm^{-3}), following the CETESB Technical Standard P4.231/2005 (2005) on the procedures to apply vinasse to agricultural soils.

The Combine® 500 SC was sprayed at the following day, at 2 L ha^{-1} as the recommended rate of this herbicide in sugarcane crops, using a CO_2 pressurized sprayer (Herbicat®) equipped with six XR 8002 flat jet nozzles at a pressure of 2 bar (0.65 L min^{-1} flowrate) from a minimum distance of 0.5 m. The application was carried out 0.75 m above the pots at a constant speed (5 km h^{-1}) until 250 L ha^{-1} had been applied. Environmental conditions such as temperature and relative humidity were monitored at the time of spraying using a portable digital thermo-hygro-anemometer-luximeter (Instrutherm® model THAL-300). The spraying occurred inside our greenhouse to avoid wind interference during the application. An equivalent volume of deionized water was added to treatments without vinasse and/or tebuthiuron.

Finally, 10 seeds per pot were sown the day after the tebuthiuron application. Thinning was performed on the eight DAS to keep only one plant per pot. These were irrigated daily by microsprinklers for 60 min (30 min at 6:00 a.m. and 30 min at 6:00 p.m.) to ensure adequate conditions for plant growth.

The cultivation of species with phytoremediation potential was performed for 50 days. Therefore, the final phytoremediation evaluation time for these plants is t_{50}.

Ten days after harvesting the plants of these species, we sowed 10 seeds of the bioindicator species sunn hemp (*C. juncea* L).

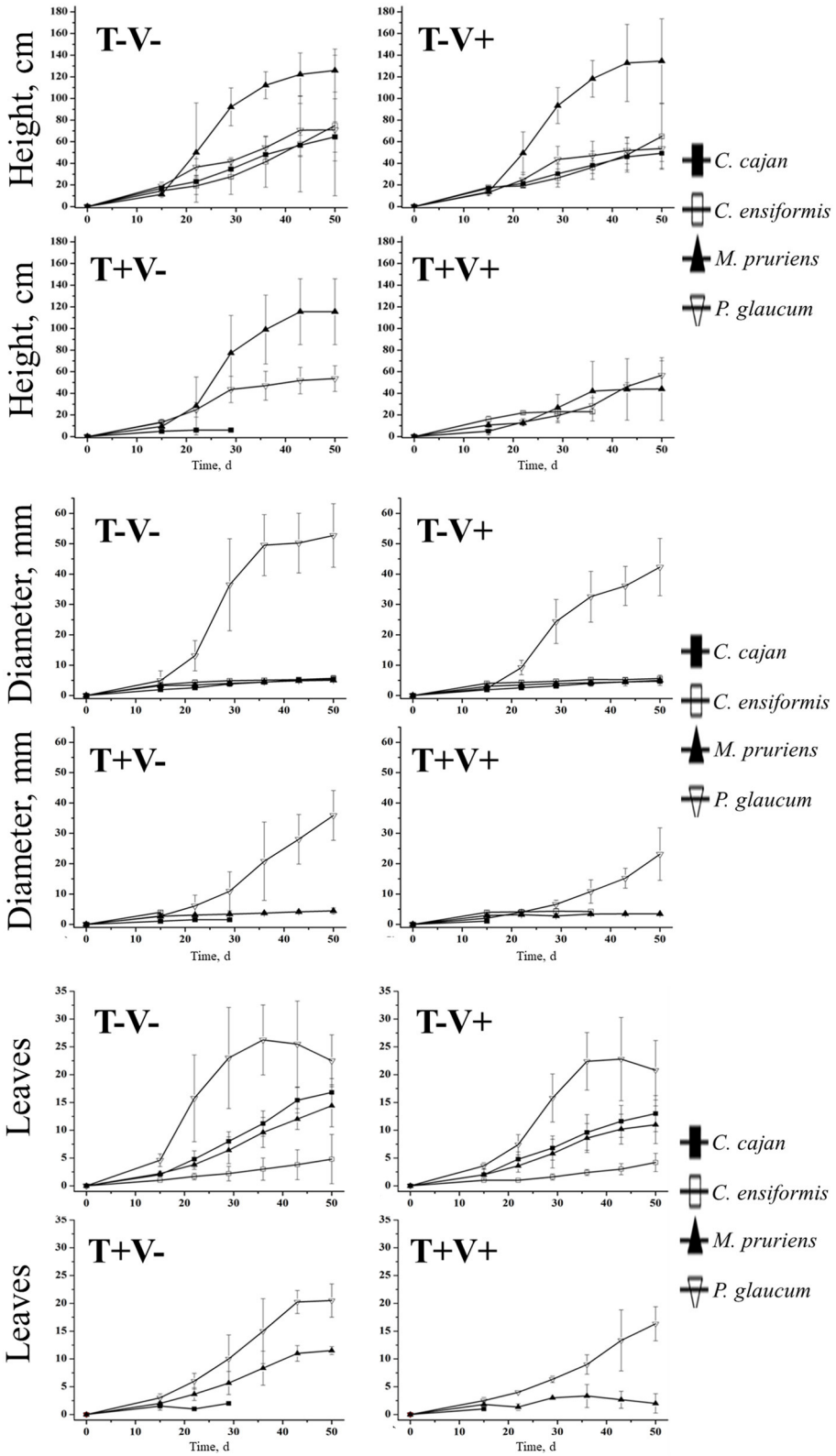

FIGURE 1 | Production of biomass by green manure species in soil with tebuthiuron and vinasse.

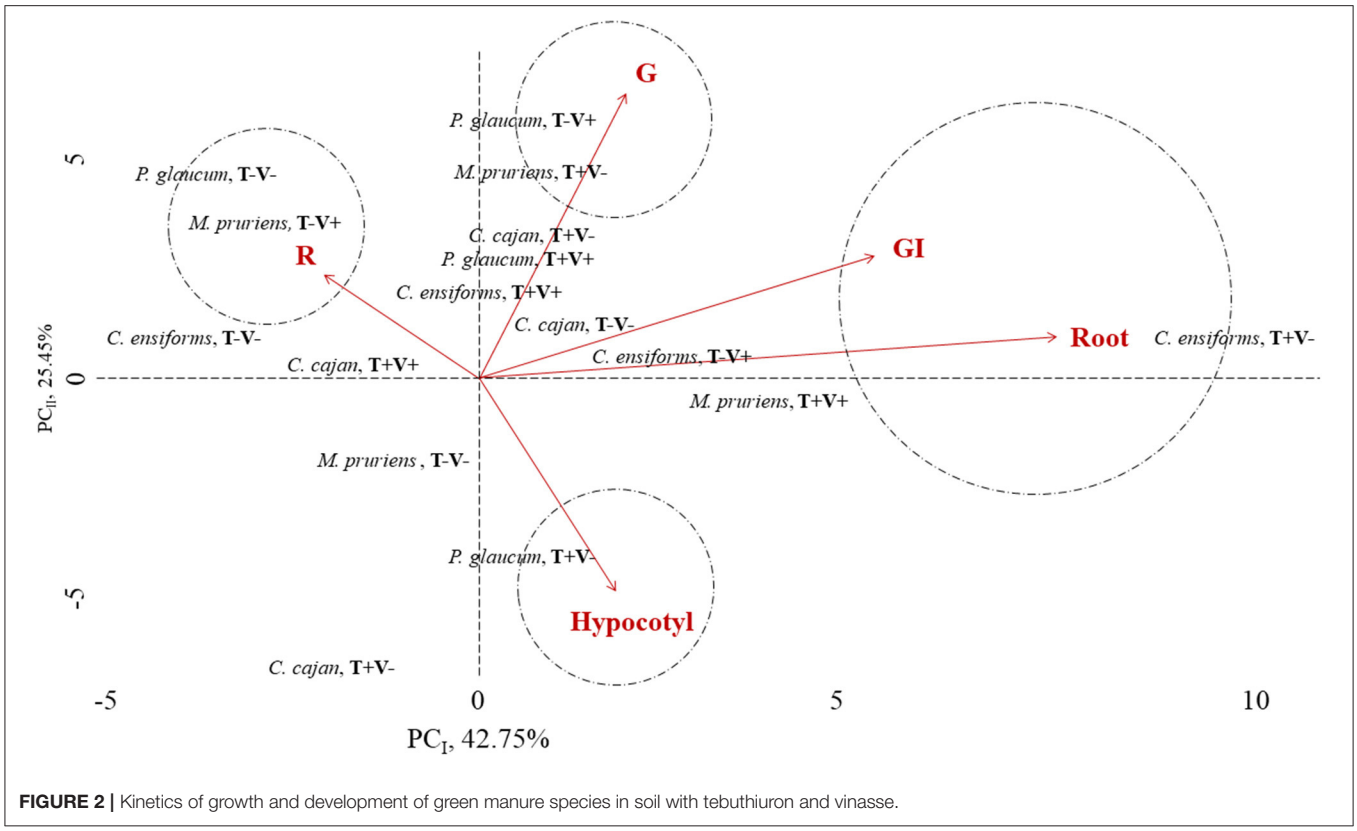

FIGURE 2 | Kinetics of growth and development of green manure species in soil with tebuthiuron and vinasse.

Thinning was performed on the 10th DAS. These were also irrigated daily by microsprinkling at 6 mm/h for 60 min (30 min at 6:00 A.M. and 30 min at 6:00 P.M.) to sustain proper plant development conditions.

Evaluation of Plant Growth

Plant growth was quantified weekly. The monitored parameters were (i) stem diameter in millimeter, (ii) height of shoot in centimeter, and (iii) number of leaves.

For *C. cajan*, *C. ensiformis*, *M. pruriens*, and *P. glaucum*, the periodic monitoring running from the 15th to the 50th DAS yielded six time-point datasets until the end (t_{50}) of cultivation. *C. juncea* was planted in all experimental units, and its morphological parameters were monitored from 17th to the 45th DAS, thus yielding five time-point datasets until the end of experiment (t_{95}).

After cultivation, plants were separated for quantification of biomass: the fresh and dry matter of shoots and roots. The separation of the shoots and roots occurred by cutting the stalks close to the soil between the stem and the root. The roots were thoroughly washed so that all the soil was removed. After separation, each fraction was weighed separately and then packed in a paper bag to dryness in oven at 65°C, over 72 h. The resulting samples were weighted again to obtain the dry mass.

Ecotoxicity Bioassays

Bioassays monitored the ecotoxicological potential of each treatment over the proposed time frame. The phytotoxicity of the soil samples was determined at the initial (t_0) and final (t_{50}) times when it was cultivated the phytoremediation species (*C. cajan*, *C. ensiformis*, *M. pruriens*, and *P. glaucum*), just before the *C. juncea* introduction.

Lettuce seeds (*Lactuca sativa*) were the test organism, according to Sobrero and Ronco (2004). Phytotoxic effect determination of each treatment was performed in six replicates from the solubilized soil extract, according to the NBR 10.006 (ABNT , 2004).

Ecotoxicity tests were prepared in Petri dishes with filter paper supported with 2.0 ml of the solubilized extract and 10 lettuce seeds. Petri dishes were then wrapped with polyvinyl chloride (PVC) film and incubated at 20 ± 2°C for 120 h in the dark.

Positive control was prepared using 0.05 M zinc sulfate to inhibit seed germination and negative control using deionized water to test the base germination and growth values of the seeds (Sobrero and Ronco, 2004).

The following parameters were determined: seed germination, root and hypocotyl elongation (≥0.1 mm), and the Germination Index (GI) that combines seed germination (% G) and root elongation (% R) at the CN. The GI was used to assess the toxicity

TABLE 2 | Kinetic parameters for the growth and development of green manure species in soil with tebuthiuron and vinasse.

Species	Test	Parameter				R^2_{adj}
		α	β	κ	$\alpha\kappa e^{-1}$	
			Height			
C. cajan	T-V-	78.50	4.40	0.40	11.55	0.9895**
	T-V+	61.65	4.50	0.45	10.20	0.9585*
	T+V-	5.10	560.65	4.80	9.00	0.0350
	T+V+	5.05	1.05	1.45	2.70	0.9995**
C. ensiformis	T-V-	217.90	4.70	0.20	16.05	0.9895**
	T-V+	193.05	4.20	0.20	14.20	0.9710*
	T+V-	4.50	$3.20e^{-7}$	−5.20	−8.60	0.2285
	T+V+	16.85	730.90	4.80	29.75	0.9995**
M. pruriens	T-V-	127.95	18.25	1.00	47.05	0.8755*
	T-V+	140.40	14.40	0.90	46.50	0.8495*
	T+V-	121.80	24.95	0.95	42.55	0.7815*
	T+V+	50.85	7.45	0.60	11.20	0.7405*
P. glaucum	T-V-	80.30	4.55	0.50	14.75	0.8190*
	T-V+	54.85	8.40	0.80	16.15	0.8765*
	T+V-	133.10	5.20	0.25	12.25	0.6075
	T+V+	134.55	5.45	0.25	12.35	0.6290
			Diameter, mm			
C. cajan	T-V-	4.85	2.25	0.60	2.90	0.9655*
	T-V+	4.85	2.25	0.60	2.90	0.9655*
	T+V-	1.05	1.25	−6.50	−6.80	0.5235
	T+V+	0.05	$4.80e^{-10}$	−1.10	−0.05	0.3690
C. ensiformis	T-V-	5.20	3.10	1.90	9.90	0.9735*
	T-V+	5.05	3.65	2.70	13.65	0.9570*
	T+V-	1.95	$5.50e^{-10}$	−1.15	−2.25	0.4040
	T+V+	3.35	$1.10e^{-12}$	−5.70	−19.10	0.5220
M. pruriens	T-V-	4.50	2.60	1.70	7.65	0.9280*
	T-V+	4.40	2.65	1.65	7.25	0.9390*
	T+V-	3.90	2.60	1.65	6.40	0.9245*
	T+V+	3.30	4.25	3.15	10.40	0.9780*
P. glaucum	T-V-	53.05	17.55	1.30	68.95	0.9940**
	T-V+	43.20	7.25	0.80	34.55	0.9940**
	T+V-	62.60	4.90	0.35	21.90	0.9970**
	T+V+	243.05	5.55	0.15	36.45	0.9960**

*Significant code: **$p < 0.01$; *$p < 0.05$.*

of soil samples in the test organism, according to Equation 1 (Labouriau and Agudo, 1987):

$$G_I = \frac{G \times R}{100}. \quad (1)$$

Data Analysis

Procedures of Shapiro–Wilk and Bartlett checked the normalcy and homoscedasticity of the dataset, respectively, and the analysis of variance tested the significance of effect of factors on the soil phytoremediation. The tests were separated by *post hoc* Tukey's honest significant difference (HSD) test. The data of temporal variability were fitted for Gompertz (Equation 2). This stochastic model has probability density function enough to predict how long would it take the green manure in soil with tebuthiuron and stillage to reach the maximum of growth and develop and stop. The kinetic parameters, α, β, and κ, will assist in drafting inferences about the potential phytoremediators and figuring out the best chance to deal with how to solve the side effects of these contaminants as much suitably as possible.

$$f = \alpha e^{-\beta e^{-kx}} \quad (2)$$

where:

f(x): height or stem diameter;

x: time of sampling;

α: upper asymptote or the maximum of height or stem diameter;

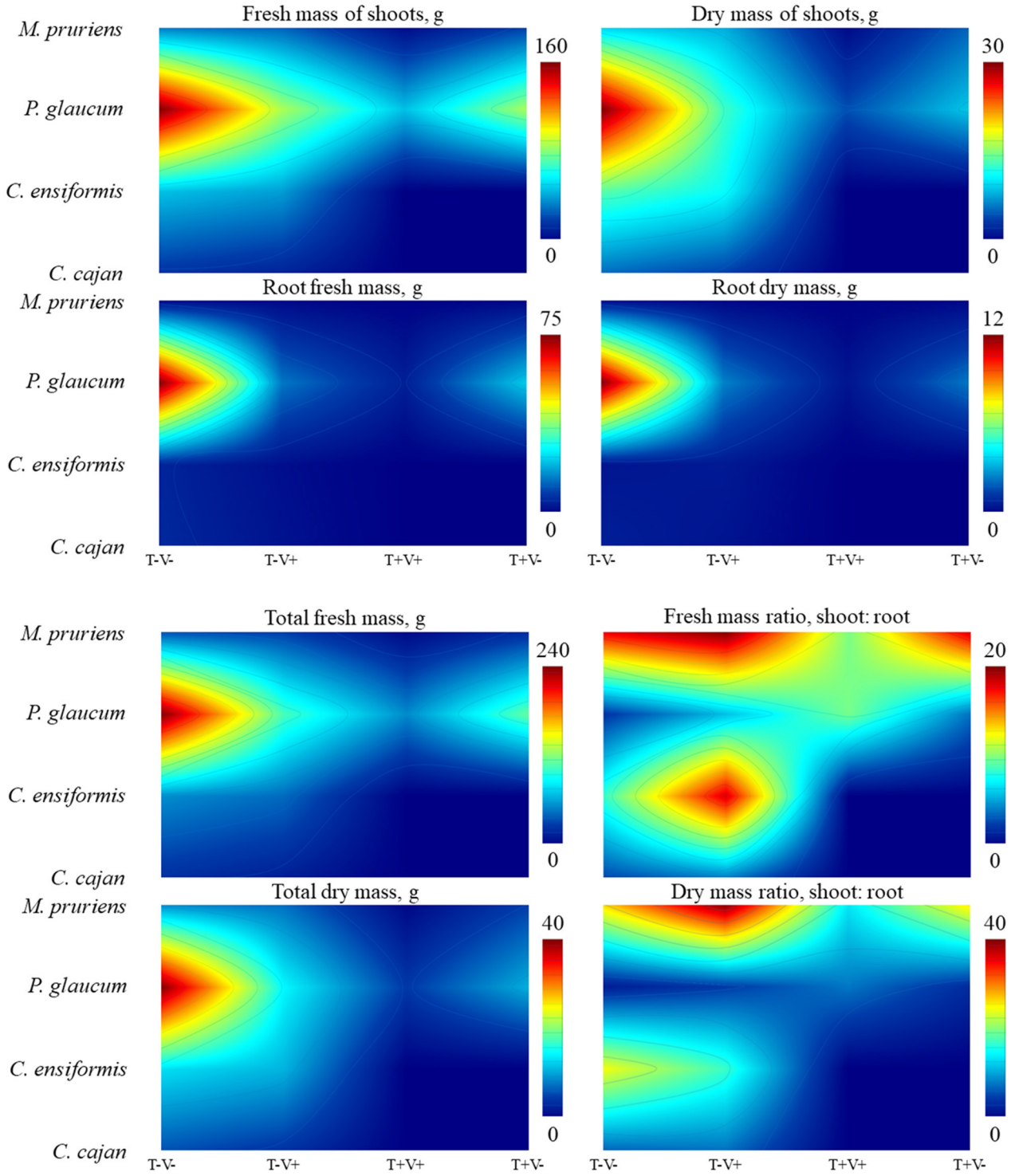

FIGURE 3 | Growth and development of *C. juncea* as bioindicator of phytoremediation of green manure species in soil with tebuthiuron and vinasse.

β: inflection point;
κ: specific-growth rate;
$\alpha\kappa e^{-1}$: absolute-growth rate;
e: Euler number.

An unbiased soft computing technique of contour plotting was performed to chart the spatial production of phytomass by the models for green manure. To an optimization of visualization of non-Boolean patterns in chromatic wireframe by contour

TABLE 3 | Performance of *Crotalaria juncea* after green manure species cultivation in soil with tebuthiuron and vinasse.

Species	Test			
	T-V-	T-V+	T+V-	T+V+
			Height, cm	
C. cajan	74.60 Aa	60.00 Aa	ND*	ND*
C. ensiformis	73.00 Aa	72.20 Aa	ND*	ND*
M. pruriens	78.40 Aa	85.80 Aa	58.50 Aab	18.75 Ab
P. glaucum	72.80 Aa	66.80 Aa	57.00 Aab	11.50 Ab
			Diameter, mm	
C. cajan	4.60 Aa	4.15 Aa	ND*	ND*
C. ensiformis	4.45 Aa	4.25 Aa	ND*	ND*
M. pruriens	4.85 Aa	4.90 Aa	3.40 Aab	1.80 Ab
P. glaucum	4.40 Aa	3.60 Aa	2.80 Aab	1.60 Ab
			Leaves	
C. cajan	32.40 Aa	22.80 Aab	ND*	ND*
C. ensiformis	31.40 Aa	32.80 Aa	ND*	ND*
M. pruriens	32.20 Aa	34.00 Aa	24.50 Aab	10.00 Ab
P. glaucum	34.80 Aa	25.60 Aa	22.00 Aab	7.50 Ab
			Shoot fresh mass, g	
C. cajan	25.45 Aa	21.20 Aa	ND*	ND*
C. ensiformis	23.35 Aa	30.80 Aa	ND*	ND*
M. pruriens	29.75 Aa	34.55 Aa	12.00 Ab	2.35 Ab
P. glaucum	28.95 Aa	21.25 Bab	7.80 Ab	0.25 Ab
			Shoot dry mass, g	
C. cajan	22.80 Aa	13.80 Ab	ND*	ND*
C. ensiformis	23.95 Aa	15.65 Aa	ND*	ND*
M. pruriens	7.90 Aa	6.90 Aa	2.20 Aab	0.35 Ab
P. glaucum	6.05 Aa	3.70 Bab	1.25 Ab	0.10 Ab
			Root fresh mass, g	
C. cajan	5.20 Aa	4.30 Aa	ND*	ND*
C. ensiformis	7.20 Aa	5.65 Aa	ND*	ND*
M. pruriens	24.05 Aa	19.20 Aa	9.55 Aa	2.80 Ab
P. glaucum	19.10 Aab	24.50 Aa	6.70 Aab	0.10 Ab
			Root dry mass, g	
C. cajan	2.15 Aa	1.30 Aa	ND*	ND*
C. ensiformis	3.40 Aa	1.75 Aab	ND*	ND*
M. pruriens	3.00 Aa	2.55 Aa	0.90 Aab	0.15 Ab
P. glaucum	1.95 Aab	2.75 Aa	0.45 Abc	0.05 Ac

Same letters, whether uppercase in the column and lowercase in the row, describe statistically similar means by post hoc Tukey's HSD test at $p < 0.05$;.
**As the plant dead, the trait became undetected (ND).*

plotting approach, fuzzy logic to turn any ambiguity off from the data was implemented. Another method of applying non-traditional mathematics to establish an eventual effect of green manure on the decontamination of the soil included principal component analysis (PCA). The Kaiser–Meyer–Olkin test was applied to determine how many components should be necessary to reduce the high-dimensionality data, while preserving as much attributable variability as possible into orthogonal subsets without collinearities. The software was R-project. This multiparadigm programming open-coding language provides a user-friendly environment for statistical computing and graphs.

RESULTS AND DISCUSSION

Performance of Species of Green Manure

Morphological Traits

The effects of green manure species and the microenvironment toward the phytoremediation potential was determined. Phytoremediators response occurred regardless of their morphological traits. The variations found in each assay allowed us to determine their sources (**Table 1**).

Tebuthiuron and vinasse underwent reactions in the soil, thus collectively influenced the phytotoxicity of the microclimate.

TABLE 4 | Principal components into ecotoxicity bioassays in soil samples with green manure species, tebuthiuron and vinasse.

Index/variable	Bartlett's test of sphericity	
Chi-squared	104.95	
Degree of freedom	10	
p-value	<0.01**	
	Kaiser-Meyer-Olkin test	
	Component	
	PC_I	**PC_{II}**
Eigenvalue	2.05*	1.20*
Percentage of variance	42.75	25.45
Cumulative percentage of variance	42.75	68.20
	Loading	
Hypocotyl	0.45	−0.55*
Root	0.95**	−0.05
%G	0.05	0.90**
%R	−0.35	0.25
GI	0.95**	0.30
	Contribution, %	
Hypocotyl	9.05	24.40*
Root	43.45**	0.05
%G	0.15	63.95**
%R	5.50	4.35
GI	41.85**	7.25
	Physiological vigor	Physiological anomaly

*Significant code: **p < 0.01; *p < 0.05.*

The herbicide alone was more toxic to *C. cajan* and *C. ensiforms*. In contrast, *M. pruriens* and *P. glaucum* resisted longer to chemically stressed microenvironment. Vinasse addition significantly reduced the toxicity. Hence, green manure species produced larger amounts of mass of roots and shoots in these soil samples (**Figure 1**).

Advantages of vinasse on the ecotoxicity were more prominent in *M. pruriens* and *P. glaucum*. These were the most effective strategies of manuring for phytoremediation potential. The primary assumption for vinasse attenuation by on phytotoxicity of soil may be its availability of soluble carbon. Thus, it is likely to power up the microbial metabolism and enhance the subsequent degradation of pesticides (Prata et al., 2000, 2001; Villaverde et al., 2008). Tebuthiuron is highly available and can move smoothly through the structure of agricultural soils with lows levels of organic matter and clay (Chang and Stritzke, 1977). These authors reported 40.00 and 1.00% herbicide adsorbed in particles at 4.8 and 0.30% organic matter, respectively. Bioavailability is one of the keys to an effective and consistent biodegradation. If the pollutant or contaminant is not available from the environment, (micro)organism cannot successfully perform longer. The nature and physicochemical properties of the pesticide (e.g., chemical stability, spatial structure, feedback effect, and intermediate metabolites) and its multiplicity of interactions with the rhizosphere are factors influencing greatly its bioavailability and, of course, kinetics of biodegradation.

Kinetics of Growth and Development

The Gompertz approach predicted accurately how long would it take for the chemical contamination of soil by tebuthiuron to become limiting for the growth and development of green manure species (**Figure 2**). Estimates for the absolute primary growth rate for the *C. cajan* in soil with tebuthiuron and vinasse combined was the lowest (**Table 2**). As long as the target molecule is rather persistent than readily degradable, the more probable the strategy of manuring is to inefficiently decontaminate an area. The highest estimations for both the maximum of size and absolute growth rate for this potential phytoremediator in soil with no tebuthiuron supported the high phytotoxicity of this herbicide to the primary growth.

In contrast, the association of tebuthiuron and vinasse allowed *C. ensiformis* to achieve its highest primary growth rate, whether specific or absolute. The negative estimation for absolute growth rate for this specie in soil with tebuthiuron alone was proof that vinasse was an effective source of nutrients to speed up the plant's growth and development, thus assisting in phytoremediation. The use of this agroindustrial residue also enhanced the height of *M. pruriens* and *P. glaucum* in soil samples with tebuthiuron. The herbicide severely limited the development of *C. cajan* and *C. ensiformis*, according to the lowest estimations for stem diameter. Thus, *M. pruriens* and *P. glaucum* are recommended over *C. cajan* and *C. ensiforms* for the phytoremediation of tebuthiuron in fields of sugarcane, even without the application of vinasse. The growth and development of *C. juncea*, the bioindicator chosen for this contaminant, became healthier and longer when sowing either *M. pruriens* and *P. glaucum*.

Performance of *C. juncea* as Bioindicator of Phytoremediation

The combination of tebuthiuron and vinasse considerably dropped the height, stem diameter, and number of leaves in *C. juncea* over time, compared to the control (**Figure 3**). In contrast, the herbicide alone had no significant effect, whether negative or positive, on the growth and development by the bioindicator species sowed after growing green manure species, consistent with the outcomes of explanatory analysis.

The explanation for the extensive decrease in leaves production (**Table 3**) may be either phytotoxicity by the compounds at high concentrations in soil or natural senescence, as plants become metabolically and physiologically ineffective over time. In contrast, the control soil sample (without tebuthiuron and vinasse) peaked in height, stem diameter, and number of leaves. Therefore, *C. juncea* was highly susceptible to tebuthiuron. Practically, this molecule more severely disabled both *C. cajan* and *C. ensiformis* to grow and develop as healthily as possible prior to sowing *C. juncea* for monitoring the potential phytoremediation of soil with green manure.

The behavior of bioindicator species *C. juncea* supported how persistent should be tebuthiuron in a microenvironment,

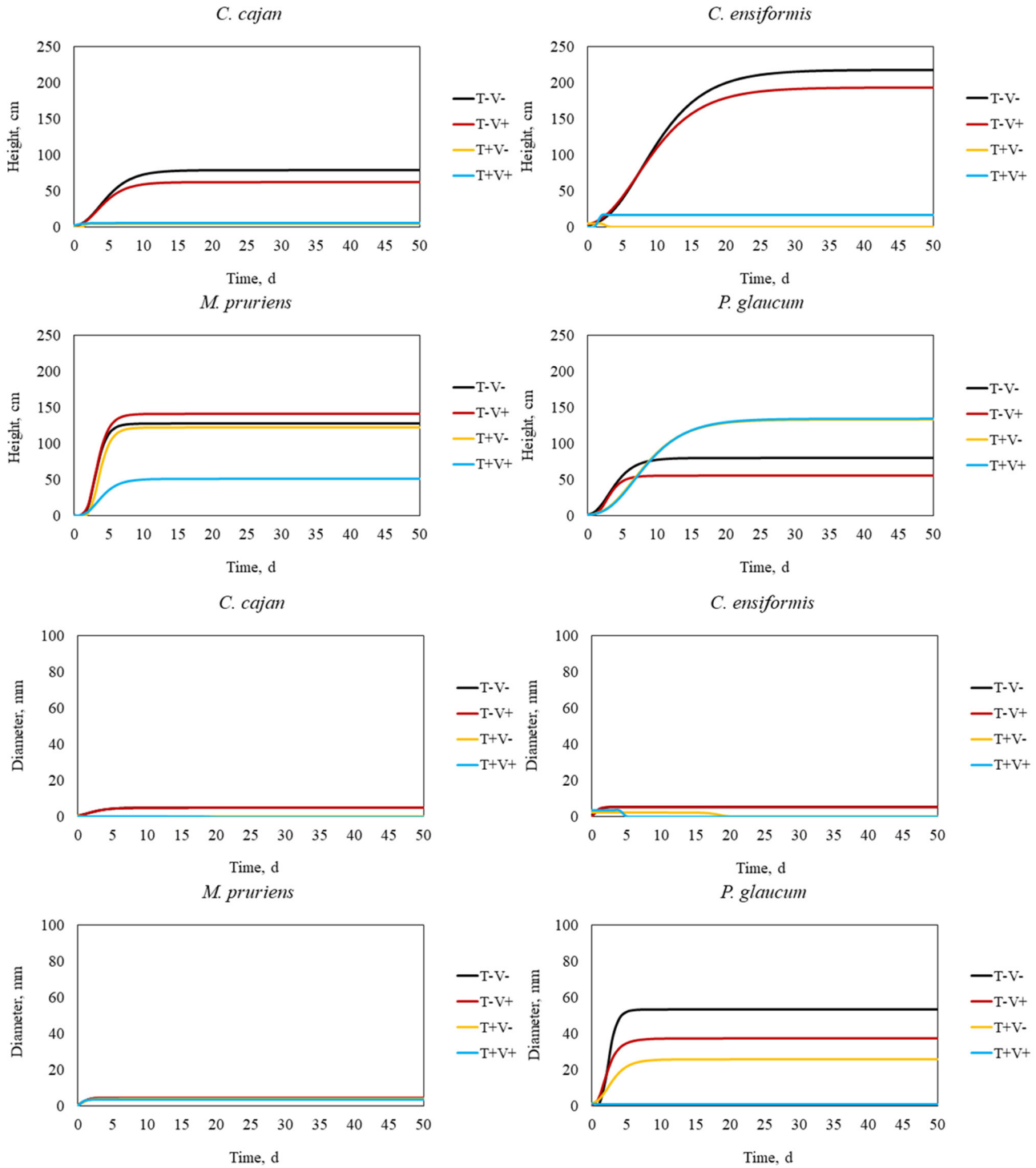

FIGURE 4 | Principal components into the ecotoxicity bioassays after 50 days of cultivation of green manure species in soil with tebuthiuron and vinasse.

regardless of vinasse application as source of nutrients to speed up the growth and development and, hence, assist green manure species in extensively remediating the herbicide. *C. cajan* and *C. ensiformis* ended up much more effectively remediating tebuthiuron and, hence, ensured the soil more suitable for the bioindicator species' growth and development

(Pires et al., 2008), inconsistent with the trends in this study. Some plants are capable of highly remediating contaminants (Ferraço et al., 2017). Cultivation of *C. juncea* slightly reduced sulfentrazone concentration in soil. Bioindicators of this herbicide then grew more consistently over time, regardless of sowing density, but not as consistent as control assay (Ferraço et al., 2019).

Franco et al. (2014) reported phytoremediation benefits by *Phaseolus vulgaris* on the growth and development of *Urochloa brizantha*. Plant heigh, leaf production, and area all increased in *C. juncea* with decreasing concentration of contaminant. Thus, the longer the postcultivation is, the more probable the phytoremediation is to becoming effective.

Beans in soil with picloram at 32.00 g ha^{-1} produced low amount of dry mass due to plants' death at high pesticide concentration. However, this morphological trait increased with phytoremediation by the *Urochloa* sp. (Franco et al., 2015). Belo et al. (2016) reported similar trend for the dry mass of *P. glaucum* after phytoremediation by *C. juncea* and *C. ensiformis*. Data on phytoremediation potential of herbicides, like sulfentrazone, by *P. glaucum* are neither conclusive nor conducive to commercial application yet, and this requires further investigations (Madalão et al., 2012a,b). These references supported the major findings in this study on the negative effect of tebuthiuron on biomass accumulation in roots and shoots of green manures species. Other reliable and executable bioindicators of soil decontamination are finger millet (*Eleusine cocracana)*, for chlorimuron-ethyl and sulfumeturon-methyl (Assis et al., 2010), and cucumber for picloram (Galon et al., 2017).

Ecotoxicity Bioassays

The principal component analysis robustly reduced the dimensionality of dataset and preserved as much interpretable variability as possible into the components, PC_I and PC_{II}. These components, collectively, explained ∼70.00% variance in ecotoxicity of soil samples on germination, growth, and development of the test-organism *L. sativa* (**Table 4**).

The first component, attributable to seed physiological vigor, had positive correlations with hypocotyl length and germination index (GI). Cartesian coordinates for soil samples with phytoremediation by either *M. pruriens* and *P. glaucum* structurally were positive in the upper right quadrant in the factorial map (**Figure 4**). Therefore, the more effective the tebuthiuron biodegradation is, the less probable the contaminant is to become toxic during the germination and primary growth of *L. sativa* seeds. The second component, attributable to physiological anomaly by phytotoxicity, had positive and negative loadings with germination and hypocotyl length, respectively. Soil samples with *P. glaucum* and hypocotyl were closer together in the lower right quadrant. Thus, this species should be of greater relevance to ensure plant growing and development without any severe physiological anomaly by tebuthiuron in site without vinasse.

CONCLUSION

Green manure species and vinasse can remediate soils with tebuthiuron. Preliminary evidence of *M. pruriens* and *P. glaucum* show their increased capabilities of phytoremediating sites where the target herbicide exists. These species, in association with vinasse as source of soluble carbon, can decontaminate the system more effectively than *C. cajan* and *C. ensiformis*, thus enabling the bioindicator *C. juncea* to grow and develop healthier in the presence of residual tebuthiuron. As long as the manuring by fertilizing agents is effective in remediating the soil, the less probable tebuthiuron persists at high concentrations in soil and, thus, becoming harmful to non-target organisms as shown in ecotoxicity bioassays. Undergerminated seeds and severe physiological anomalies due to phytotoxicity in roots and hypocotyl of *L. sativa* are likely to decrease quickly with phytoremediation by *M. pruriens* and *P. glaucum*, which is the best chance to do this. Findings of this study are timely and should be of great importance to development and implementation of cost-effective strategies to assist in mitigating contamination of soil by tebuthiuron in sugarcane crops with vinasse application as biofertilizer. The fate of this herbicide and its potential metabolites in soil and into tissues of green manure species, especially *M. pruriens* and *P. glaucum*, is prone to scaled up designs toward an effective and safe industrial usage and could be the focus of further investigations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article could be made available by the authors.

AUTHOR CONTRIBUTIONS

LF, EP, RT, EB, and PL: conceptualization. LF, RM, YF, and PL: methodology. LF, BM, RM, RV, and PL: validation. LF, BM, and PL: formal analysis, data curation, and writing—original draft. LF and YF: investigation. PL: resources, supervision, project administration, and funding acquisition. RM, EP, RV, RT, JC, EB, YF, and PL: writing—review & editing. BM, JC, and PL: visualization. All authors contributed to the article and approved the submitted version.

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Challenges and Current Status of the Biological Treatment of PFAS-Contaminated Soils

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Per- and polyfluoroalkyl substances (PFAS) are Synthetic Organic Compounds (SOCs) which are of current concern as they are linked to a myriad of adverse health effects in mammals. They can be found in drinking water, rivers, groundwater, wastewater, household dust, and soils. In this review, the current challenge and status of bioremediation of PFAs in soils was examined. While several technologies to remove PFAS from soil have been developed, including adsorption, filtration, thermal treatment, chemical oxidation/reduction and soil washing, these methods are expensive, impractical for *in situ* treatment, use high pressures and temperatures, with most resulting in toxic waste. Biodegradation has the potential to form the basis of a cost-effective, large scale *in situ* remediation strategy for PFAS removal from soils. Both fungal and bacterial strains have been isolated that are capable of degrading PFAS; however, to date, information regarding the mechanisms of degradation of PFAS is limited. Through the application of new technologies in microbial ecology, such as stable isotope probing, metagenomics, transcriptomics, and metabolomics there is the potential to examine and identify the biodegradation of PFAS, a process which will underpin the development of any robust PFAS bioremediation technology.

Keywords: PFAS-contaminated soils, bioremediation, mycoremediation, bioaccumulation, bacteria, phytoremediation

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INTRODUCTION

As a result of continued production and use, per- and polyfluoroalkyl substances (PFAS) have become widespread in the environment, including drinking water, rivers, groundwater, wastewater, household dust, and soils (Kim et al., 2007; Eriksson and Karrman, 2015; Shi et al., 2015; Eriksson et al., 2017; Von Der Trenck et al., 2018). PFAS are highly stable organic compounds that contain multiple carbon-fluorine bonds. They are used in various commercial products, including aqueous fire-fighting foams and products with non-stick coatings. These compounds are also likely to be present in foods (Schaider et al., 2017) and are known to be present in humans, including pregnant women (Lauritzen et al., 2016).

Human exposure to PFAS occurs through several pathways, including ingestion of contaminated drinking water, food and household dust, inhalation of indoor air, and contact with other contaminated media (Trudel et al., 2008). Drinking water sources include rivers, lakes and groundwater may also be contaminated with PFAS originating from industrial sources. There may

also be significant exposure risk from PFAS-contaminated sewage sludge (biosolids) and recycled water from wastewater treatment plants, which are often used in agriculture, with exposure through contaminated soils and crop foods (Sunderland et al., 2019). PFAS have been shown to have bioaccumulation potential, which tends to increase with increasing chain length. Specific PFAS compounds have been shown to impact human health through altered kidney and thyroid function, immunosuppression and deleterious effects on reproduction and development. Perfluorooctane sulfonate (PFOA)-related chronic diseases include kidney and testicular cancers, ulcerative colitis, and high cholesterol have also been observed (Darrow et al., 2013; Steenland et al., 2013; Starling et al., 2017; Sunderland et al., 2019). PFOS and PFOA are readily absorbed through the gut and are not metabolized, meaning body loads become excessive before they are excreted. PFAS are believed to act as endocrine disruptors through alterations in estrogen- and androgen-receptor functions (Mora et al., 2017). Research conducted by Tao et al. found that PFOS and PFOA accumulate in the serum of adults and blood of newborn babies, which indicates that breast milk is a major pathway for transferal (Tao et al., 2008a,b). Research indicated that the milk of mothers who have given birth to multiple children tend to have slightly higher levels of PFAS (Mora et al., 2017).

PFAS are considered to be stable and amphiphilic, exhibiting both hydrophobic and lipophobic tendencies (Giesy et al., 2010) resulting in ready accumulation within lipids (fats) and proteins (Mora et al., 2017; Seo et al., 2018). Based on the above health issues, there is an urgent need to remove these compounds from soils. Current methods to remove PFAS from contaminated soils are expensive, impractical for *in situ* treatment, use high pressure and temperatures, and/or result in toxic waste. Biodegradation has the potential to form the basis of a cost-effective, large scale *in situ* remediation strategy for PFAS. However, information about the biodegradation of PFAS by fungal and bacteria is limited. Consequently, this review aims to review chemical properties, the source of PFAS contamination in soils and summarize the remediation technologies, focussing on the potential of bioremediation for the safe and effective removal of PFAS from soils.

CHEMICAL PROPERTIES OF PER-AND POLYFLUOROALKYL SUBSTANCES (PFAS) COMPONENTS

Per-and polyfluoroalkyl substances (PFAS) are a group of synthetic man-made compounds manufactured for their ability to interact between two immiscible fluid phases acting as a surfactant (Buck et al., 2011; Rahman et al., 2014). PFAS are highly polar and contain strong carbon-fluorine bonds (C-F) which display unique amphiphilic properties (**Figure 1**). Generally, most PFAS exhibit (i) high thermal resistance, (ii) high chemical stability, and (iii) resistance to biotic degradation (Buck et al., 2011; Lindstrom et al., 2011; Rahman et al., 2014). Two broad categories of PFAS have been defined:

- ***Perfluoroalkyl substances*** typically comprise of short and long carbons chains (C2-C13+) and have a charged functional group head which is attached to one end. Generally, this functional group will be a carboxylic or sulfonic acid. Fluorine atoms attach to all bonding sites on the carbon chain except for the last carbon group head forming multiple carbon-fluorine (C-F) bonds (**Figure 1**). C-F bonds have the dissociation energy of 450 kJ mol^{-1} compared to carbon-chlorine and carbon-bromine bonds at 330 and 194 kJ mol^{-1}, respectively (Parsons et al., 2008).
- ***Polyfluoroalkyl substances*** are not fully fluorinated. These substances have at least one lapse in the chain which is not a fluorinated atom—typically hydrogen or oxygen—which attaches to one of the carbon-chain tails. Polyfluoroalkyl chains contain carbon-hydrogen (C-H) bonds which create weak chains that are susceptible to biotic or abiotic degradation (Buck et al., 2011).

Three specific PFAS compounds, perfluorooctane sulfonate (PFOS), perfluorooctane acid (PFOA) and perfluorohexane sulfonate (PFHxS) (**Figure 1**) are known to have been contained in much older Aqueous Film Forming Foams (AFFF). Within environmental pH values, both PFOA and PFOS exist as anions (Rahman et al., 2014). Generally, PFAS do not degrade in water or soil under normal conditions, although it is thought that they can undergo physiochemical changes and breakdown into smaller alkyl chains (Teaf et al., 2019). The physicochemical properties of these three major PFAS compounds are presented in **Table 1** and further discussed below:

- ***Perfluorooctane Sulfonate (PFOS),*** CAS number 1763-23-1, is a completely fluorinated compound that contains eight carbon atoms and a sulfonate group head. PFOS has been used as coatings and protectants. PFOS is produced commercially from perfluorooctanesulfonly fluoride (POSF) which was used as an intermediate to produce other fluorochemicals. PFOS is formulated by $C_8HF_{17}O_3S_1$ which has a molecular mass of 500.13 g/mol. PFOS is produced through Simons Electro-Chemical fluorination (SECF), which produces 70% linear chains and 30% branched chains isomers. PFOS can also be created through telomerization which produces linear chains. As PFOS has major impurities in the form of other POSF-derived fluorochemicals it can be formed in the environment through the degradation of POSF-based products (Buck et al., 2011).
- ***Perfluorooctanoic Acid (PFOA),*** CAS number 335-67-1, is a completely fluorinated organic acid with seven-carbon (C7) atoms and a carboxyl functional group head. PFOA has been a main constituent of AFFFs, as well as non-stick coats, and waterproofing. PFOA is formulated by $C_8HF_{15}O_2$ which has a molecular mass of 414.07 g/mol (Buck et al., 2011).
- ***Perfluorohexane Sulfonate (PFHxS),*** CAS number 355-46-4, has 6 carbons (C6) and is a completely fluorinated organic acid that is capable of repelling oil and water which have been used in the manufacturing of AFFF. PFHxS displays similar properties to both PFOS and PFOA. PFHxS is formulated by

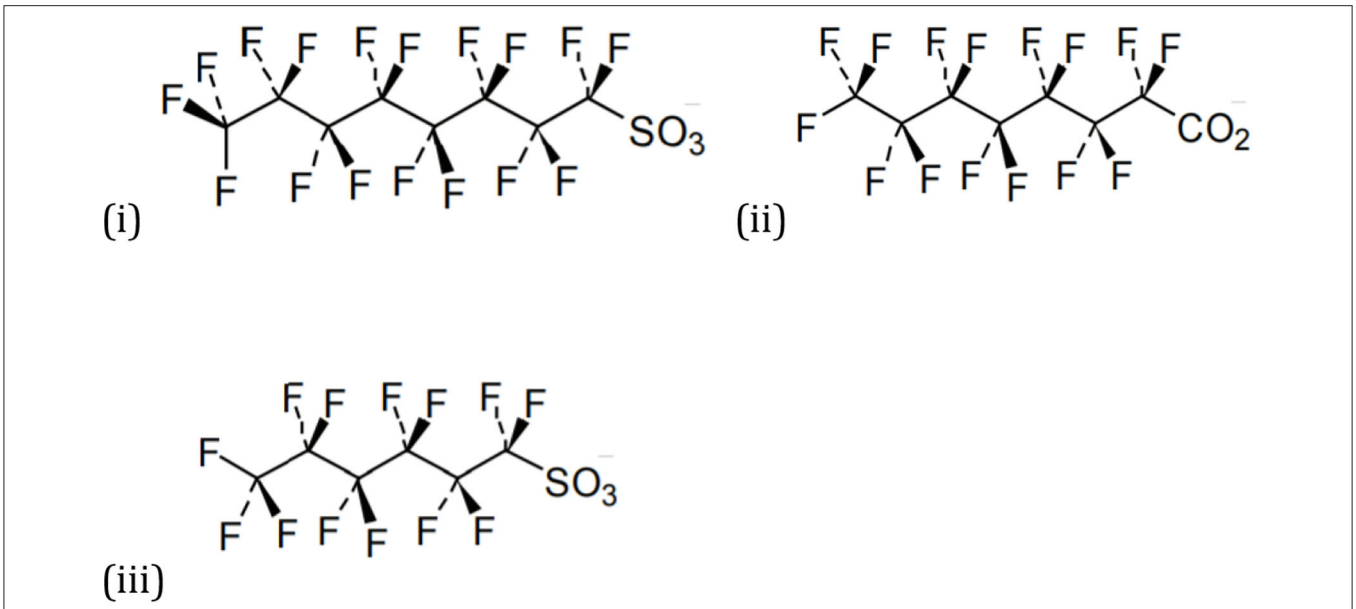

FIGURE 1 | Structure of PFAS (i) PFOS (ii) PFOA, (iii) PFHxS structure (Buck et al., 2011).

TABLE 1 | Physiochemical properties of PFAS (Buck et al., 2011).

Characteristic	PFOS	PFOA	PFHxS
Appearance	White powder (potassium salt)	White to off-white powder	White crystalline powder
Melting point	>400°C (potassium salt)	54.3°C	No data
Boiling point	258–260°C	192.4°C	114.7°C >400°C
Density	~0.6 (potassium	1.7292 g/mL at 20 °C	1.84 g/mL at 20°C
Water solubility	519 mg/L at 20°C; 680 mg/L at 24–25°C	Soluble, 9.5 g/L at 25°C	Slightly soluble
Organic solvent solubility	56 mg/L	Soluble in polar organic solvents	No data
Log Kow	Not measurable	6.30 (estimated) in octanol-water mixture	Not measured
pKa:	−3.3 (estimated)	Debated; values of 2.8 and 3.8 have been reported. 0.5 has been estimated.	0.14

$C_6HF_{13}O_3S$ and has a molecular mass of 400.12 g/mol (Buck et al., 2011).

Based on the physiochemical properties, PFAS have been identified to have bioaccumulation potential, which tends to increase with increasing chain length. Most health research has been conducted on individuals with high levels of PFAS accumulated in their organs due to work on containment sites, airbases, and in response firefighting. A significant number of PFOA-related chronic diseases which include kidney and testicular cancers, ulcerative colitis, high cholesterol have been reported among PFAS-exposed individuals (Darrow et al., 2013; Steenland et al., 2013; Starling et al., 2017; Sunderland et al., 2019). PFOS and PFOA are readily absorbed through the gut and are not metabolized, meaning body loads become excessive before they are excreted. PFAS are believed to act as endocrine disruptors through the alteration in estrogen- and androgen-receptor functions (Mora et al., 2017). Human exposure to PFAS, produced by industry, occurs through ingestion of contaminated drinking water, food and household dust, inhalation of indoor air, and contact with other contaminated media (Trudel et al., 2008). Drinking water sources include rivers, lakes, and ground water, which can all be contaminated from industrial sources. In addition, there appears a significant exposure risk from contaminated treated sewage sludge (biosolids), as fertilizer, and recycled water from wastewater treatment plants, which are often used in agriculture, providing potential human exposure through contaminating crop foods (Sunderland et al., 2019).

SOURCES OF CONTAMINATION OF PFAS AND FATE IN THE ENVIRONMENT

The following section describes major sources of PFAS in the environment.

Aqueous Film Forming Foams (AFFFs)

Aqueous Film Forming Foams (AFFFs) are intended to be used on flammable liquid fires through the process of combining hydrocarbon foaming agents with fluorinated surfactants when mixed with water (Backe et al., 2013). This creates interfacial tension that spreads across the surface of a hydrocarbon fuel, which extinguishes the flame, and forms a vapor barrier between the fuel and atmospheric oxygen, preventing re-ignition (Backe et al., 2013; Weiner et al., 2013; Harding-Marjanovic et al., 2015). The fluorotelomer AFFF, although not directly made with PFOA, and therefore less toxic to the environment has precursors that breakdown into PFOA in the natural environment (Backe et al., 2013; Weiner et al., 2013; Harding-Marjanovic et al., 2015). Typically, fluorotelomer based AFFF contains short-chain (C6) PFAS which can range from 50 to 98% short chains balanced with long-chain PFAS which can break down to PFOA.

Fire training facilities undergo extensive and prolonged use of AFFFs, which has caused large volumes of PFAS to be released into adjacent soils during short periods (Dauchy et al., 2019). From there PFAS leaches into groundwater supplies. Soils that do not contain high amounts of Total Organic Matter (TOC) through a lack of vegetation, land clearing, and anthropogenic abuse make it difficult for the chemicals to bind within the soil substrate (Allred et al., 2015; Gallen et al., 2018). Dauchy et al. (2019) sampled 44 soil cores and 17 groundwater samples from a firefighting drill sites active for more than 30 years and detected PFOS, 6:2 FTSA and 6:2 FTAB as the most predominant PFAS in surface soil; the highest total concentrations detected were 357 $\mu g\ g^{-1}$, despite the presence of clay layers. However, the highest total PFAS concentrations were detected in the wells at the perimeter of the firefighter training site as well as the spring located downgradient of the groundwater flow. These concentrations ranged from 300 to 8,300 ng L^{-1}; 6:2 FTAB was detected in water table 20 m belowground indicating these chemicals are not contained.

Landfill Leachate

Landfills are designed to undergo large amounts of decomposition from natural and man-made organic compounds. Realistically, landfill serves as temporal and spatial storage. In a landfill, soil chemistry is heavily compromised which impacts natural degradation processes due to the number and nature of pollutants present. PFAS within waste can become mobile and leach into pore water creating contaminated leachate. Fortunately, modern sanitary landfills typically have stringent mechanisms for preventing and mitigating leachate from entering groundwater. However, the controlled discharge of leachate to wastewater treatment plants is allowed. Reinforcement of smaller and older sites to stop the threat of local point source contamination into surrounding soil and groundwater is paramount. PFAS will continue to persist in the landfill and continue to increase over time (Gallen et al., 2018). Studies examining landfill leachate confirmed that PFHxS was detected at high concentrations (mean 1,700 ng L^{-1}; range 73–25,000 ng L^{-1}); PFOA contamination was on average 690 ng L^{-1} (range of 17–7,500 ng L^{-1}) and PFOS was detected at concentrations with a mean of 310 ng L^{-1} (range 13–2,700 ng L^{-1}). Samples from sites with higher levels of PFAS profiles generally had greater proportions of construction and demolition waste. Dealing with landfill chemistry will require novel treatment pathways to deal with the existing PFAS loads on-site (Hamid et al., 2018).

Hepburn et al. (2019) stated that groundwater systems are at risk from increased urban re-development on former industrial land and this would lead to increased human exposure to PFAS. Their research indicates that legacy landfills are poorly constructed in major Australian urban developmental precincts. PFOS, PFHxS, PFOA, and PFBS were all detected in samples surrounding 13 sample locations including sites directly on waste material and down-gradient of landfills indicating evidence of leachate contamination. Many urban areas contain unknown amounts of legacy landfills which may lack any former leachate control, increasing the probability of contaminating local aquifers.

Biosolids and Recycled Water

Point sources of PFAS transmission to agriculture occurs through the application of recycled water from wastewater treatment plants, landfill leachates and biosolids applied to agricultural land (Blaine et al., 2014; Ghisi et al., 2019). Venkatesan and Halden (2014) monitored soil amended with PFAS-containing biosolids over 3 years. They observed a loss of short-chain PFAS compounds within 100 days of application, due mainly to groundwater and surface water leaching. In a laboratory-based study by Allred et al. (2015) on the physical and biological release of PFAS from landfill leachate, they reported that increased leaching occurred from biological reactors under methanogenic conditions compared to abiotic reactors.

Once in agricultural lands, PFAS can be taken into the root systems of plants including cereals, fruits, and vegetables. PFAS with higher chain lengths are usually restricted to the roots, whereas shorter chains compounds can extend further (Ghisi et al., 2019). Generally, the physicochemical properties of the soil together with the plant uptake system will determine the rate and accumulation of PFAS; however, generally, PFOS accumulates at greater concentrations compared to PFOA. Pérez et al. (2013) showed that the PFAS in human tissue was 263 and 807 ng g^{-1} in the kidney and lung. In plants, the amount varied; however, most experiments used the spike method for contamination of soil. For example, Stahl et al. (2009) showed that ryegrass accumulated PFAS ranged between 408 and 7,520 $\mu g\ kg^{-1}$ dry weight when the soil was contaminated with 0–50 $mg\ kg^{-1}$ PFAS.

PFAS in Soil Systems

As a consequence of these major sources of PFAS, these compounds are almost ubiquitously detected in the environment (Xiao et al., 2015; Lu et al., 2018). Research has indicated that soil organic carbon content is the dominant solid-phase parameter which affects the adsorption of PFAS. Solid matrices influence the environmental fate of hydrophobic organic contaminants (Higgins and Luthy, 2006). However, the different behavior of PFAS in comparison to traditional ionisable organic pollutants is due to their hydrophobic and hydrophilic functionalities

(Li et al., 2018). Adsorption to soil or sediment can occur through two-mechanisms: interaction of their hydrophobic fluorinated carbon tails with the organic carbon fraction of the soil, or to a lesser extent by electrostatic interactions of the polar head group with the charged clay fraction (Kucharzyk et al., 2017). Longer-chained PFAS appear to sorb to soils more readily. PFAS with sulfonate groups sorb more than carboxylates. In comparison to PFOS which has a higher sorption capacity, PFOA is usually found in the dissolved phase. Perfluorinated acids appear to bind to soils with higher total organic carbon (TOC) and iron oxide concentrations; Li et al. (2018) achieved an adsorption equilibrium in ∼48 h. Their results indicated that both PFOS and PFOA adsorption are influenced by TOC, proteins and saccharides. Similarly, iron and aluminum oxides also appear to be key parameters for adsorption of PFAS. Some forest soil vegetation shows greater ability to accumulate PFAS; the background levels of PFOA and PFOS in 28 forest soils suggested that PFOA concentrations were greater in precipitation at higher altitudes (Cabrerizo et al., 2018). In contrast, the concentration of PFOA in temperate grasslands appears to be much lower (Wang et al., 2018).

In summary, both bioaccumulation and translocation of PFAS occur from both natural terrestrial and aquatic environments and anthropologically built-up areas (Giesy and Kannan, 2001, 2002; Giesy et al., 2010; Xiao et al., 2012; Hu et al., 2016; Hepburn et al., 2019). The distribution of PFAS is enhanced by leaching and discharge into adjacent locations from treatment plants and urbanized redevelopments; eventually reaching oceans, including the North Pacific and the Arctic Ocean (Cai et al., 2012; Hepburn et al., 2019). However in addition, PFAS are now thought to be able to travel through airborne particles and wet and dry atmospheric deposition (Nakayama et al., 2019).

There is only limited information regarding the fate of PFAS in the environment. This is in part due to the difficulty associated with the detection of PFAS in the environment. Avoiding cross-contamination in the sample is difficult due to ambient atmospheric contamination. Most materials will at some point directly come in contact with fluorocarbons (Nakayama et al., 2019).

REMEDIATION APPROACHES

Technological approaches looking at the removal of PFAS from waste streams or contaminated environments tend to be expensive or impractical for the *in situ* removal of the contamination (Kucharzyk et al., 2017) (**Table 2**). Energy-intensive methods, such as high pressures and temperatures can disrupt and harm the balance of delicate ecosystems. Non-energy-intensive technologies such as granular activated carbon adsorption, sonolysis (generating chemical reactions using an acoustic field in a solution) and reverse osmosis have all shown some potential application for PFAS removal during field studies (Kucharzyk et al., 2017; Sorengard et al., 2019). Unfortunately, however, most treatment methods appear to collect rather than dismantle the C-F bonds, resulting in a residue containing PFAS that inevitably needs to be placed in a landfill.

Several methods, including adsorption, filtration, thermal, chemical oxidation/reduction and soil washing have been developed for the removal of PFAS from environments. An outline of these approaches is shown in **Table 2**. Like all methods, there are both advantages and disadvantages related to each method. For example, soil washing is an *ex situ* technology which requires low technology input. However, it is expensive and may lead to water contamination (de Bruecker, 2015). These technologies have been thoroughly reviewed (Kucharzyk et al., 2017; Mahinroosta and Senevirathna, 2020). While some of these technologies have shown promising outcomes in laboratory-based studies, their cost-effectiveness, field applicability and feasibility are open to question (Mahinroosta and Senevirathna, 2020). Current commercial methods for remediating PFAS-contaminated environments, based on the use of one or more of the above treatments are only available for groundwater and not soils. There is therefore an urgent need to develop methods for the *in situ* bioremediation in the soil at sites contaminated by PFAS. Chemical and physical methods tend to be more expensive than bioremediation approaches, since bioremediation often treats contamination in place, allowing post-clean-up costs to be substantially reduced (Shahsavari et al., 2019).

Bioremediation, which is the use of a biological agent to breakdown contaminants, could represent a simple, environmentally safe and cost-effective technology to treat PFAS-contaminated soils. The commercial application of bioremediation has been successfully applied to remediate a variety of organic contaminants such as petroleum hydrocarbons, chlorinated substances and pesticides (Adetutu et al., 2015; Uqab et al., 2016; Khudur et al., 2019). However, the ability of biological agents to degrade PFAS is poorly studied (Kucharzyk et al., 2017).

Bioremediation Options

Biodegradation of PFAS may involve enzymes that directly remove fluorine atoms from these compounds either (i) by adding oxygen across the F-C bond, i.e., oxidation, or (ii) adding electrons across the F-C bond, i.e., reduction, allowing other normal assimilation enzymes to breakdown the rest of the compound. The F-C bond is very strong and therefore difficult to destroy, which leads to its environmental stability. Therefore, it significant energy is required to catalyze reaction; biologically this can be provided *via* oxidative or reductive processes. There are known microbes that can break a F-C bond, either under aerobic or anaerobic conditions (Goldman and Milne, 1966; Tiedt et al., 2016, 2017); these are further discussed below.

It has been shown that some bacteria are able to bioaccumulate PFAS in aerobic and to a lesser degree, anaerobic conditions (**Table 3**); most of these bacteria have been identified as *Pseudomonas* sp. While there have been no confirmed reports of the biological removal of fluorine atoms from PFAS, the defluorination of monofluorinated compounds by many bacteria has been reported (Huang and Jaffé, 2019). For example, under aerobic conditions, pseudomonads have been isolated which can utilize fluoroacetate as a sole carbon source. In this case, the

TABLE 2 | Removal technologies of PFAS from the environment.

Technologies	Process	Site	Advantages	Disadvantages	Source
Adsorption	Removal of PFAS compounds *via* adsorption to selective materials of adsorbing potential (e.g., Biochar, Resin, and modified clays)	*Ex situ/in situ*	Low operational cost and uses several materials which are commercially available	Ineffective for short-chain PFAS removal Interfere with other pollutants May require a large quantity of the adsorbent may be required, which causes a change in the land use.	Zhang et al., 2011
Filtration	Uses Reverse osmosis or Nanofiltration to remove PFAS compounds	*Ex-situ*	Effective under a wide range of pH	Expensive PFAS molecular weight dependant Creates high concentration waste	Tang et al., 2007
Thermal	Vaporizing the contaminants through increasing temperature to about 600 −1,000°C.	*Ex situ*	High destruction potential of the PFAS compounds	Time-consuming, high-cost and energy-intensive approach. Disturbs the soil and the ecosystem.	Yamada et al., 2005
Chemical oxidation/ reduction	Using chemical oxidants/reducing agents for the abiotic breakdown of contaminants	*In situ and ex situ*	Potential for PFAS mineralisation; effective in PFOA removal	Very expensive as it requires a large volume of chemicals and centralized equipment. Not applicable to treat all PFAS compounds. Short-chain PFAS could result. Interferes with other contaminants.	Yates et al., 2014; Arvaniti et al., 2015
Soil washing	Detaching PFAS from the soil by washing with water	*Ex situ*	Requires low technology Land reuse could be possible.	Expensive and time-consuming. Contaminated water results.	de Bruecker, 2015
Bioremediation	Use of biological agents (e.g., Microorganisms and Plants) to breakdown or accumulate PFAS compound	*In situ* and *ex situ*	Simple, cost-effective, and environmentally safe (Green) approach	Limited evidence that PFAS can be degraded. It could take a long time due to the slow biodegradation of PFAS.	Presentato et al., 2020

defluorination occurs through:

$$FCH_2COO^- + OH^- > HOCH_2COO^- + F^-, \text{where the oxygen of the hydroxyl group is derived from water.}$$

Indigenous bacterial species isolated from PFAS-contaminated environments have shown the ability to remediate PFAS compounds; two strains of *Pseudomonas* (PS27 and PDMF10) were able to remove 32 and 28% of PFAS compounds, respectively, within 10 days of incubation under alkanotrophic conditions (Presentato et al., 2020). Further, a decrease of around 32% in PFAS was also reported during a 96 h incubation of *Pseudomonas parafulva* (Yi et al., 2016) along with a 67% decrease in PFAS concentration over 48 h incubation of *Pseudomonas aeruginosa* (Kwon et al., 2014). In another study, *Pseudomonas plecoglossicida* utilized PFAS as an energy source, producing perfluoroheptanoic acid and releasing fluorine ions as a result (Chetverikov et al., 2017). A recent publication reported that following incubation of the ammonium oxidizing bacterium, *Acidimicrobium* sp. strain A6 with hydrogen as the sole electron donor for 100 days a 60% reduction in PFAS concentration was observed (Huang and Jaffé, 2019).

While these studies focussed on relatively small organic compounds that contained fluorine atoms, they may help us to understand how selected microbes may break F-C bonds in PFAS. In these terms, it may be also useful to consider microbial activities known to break Cl-C bonds. For

TABLE 3 | Bacteria reported to be capable of bioaccumulating PFAS.

Bacterial sp.	Process	Conditions	Concentration removed (%)	Initial PFAS concentration	Treatment time	Source
Pseudomonas sp. strain PS27	Bioaccumulation	Aerobic	32	200 ng L^{-1}	10 days	Presentato et al., 2020
Pseudomonas sp. strain PDMF10	Bioaccumulation	Aerobic	28	200 ng L^{-1}	10 days	Presentato et al., 2020
Pseudomonas parafulva	Biodegradation	Aerobic	32	500 mg L^{-1}	96 h	Yi et al., 2016
Pseudomonas aeruginosa strain HJ4	Biodegradation	Aerobic	67	1,400–1,800 μg L^{-1}	48 h	Kwon et al., 2014
Pseudomonas plecoglossicida 2.4-D	Biodegradation	Aerobic	75	1 g L^{-1}	6 days	Chetverikov et al., 2017
Acidimicrobium sp. strain A6	Defluorination/ biodegradation	Anaerobic	60	100 mg L^{-1}	100 days	Huang and Jaffé, 2019
Gordonia sp. strain NB4-1Y	Biodegradation	Sulfur-limiting	70	n.d.	7 days	Shaw et al., 2019

example, *Dehalobacter* sp. strain TeCB1 was able to carry out the reductive dechlorination of 1,2,4,5-tetrachlorobenzene to 1,3- and 1,4-dichlorobenzene with 1,2,4-trichlorobenzene being the intermediate daughter product (Alfán-Guzmán et al., 2017). A key enzyme is PceC, and the C subunit of the tetrachloroethene (PCE) reductive dehalogenase is encoded by the conserved pceABCT gene cluster identified in the microbial strain *Dehalobacter restrictus* PER-K23 (Buttet et al., 2018). Importantly, providing an electron donor can improve reductive dehalogenation catalyzed by specific bacteria (Holliger and Schumacher, 1994). To grow, these microbes utilize organohalide respiration (OHR), which is the energy metabolism of anaerobic bacteria able to use halogenated organic compounds as terminal electron acceptors (Buttet et al., 2018).

In terms of potential enzymes capable of degradading PFAS, reduction could be undertaken by a P450-type enzyme or similar. In organic chemistry F, in F-C bonds, can be replaced by transition metals (Kiplinger et al., 1994), and therefore transition metal-dependent enzymes can release F from F-C bonds (**Figure 2**). The F in F-C bonds is significantly electronegative, and therefore can promote attraction to transition metal cations. The value of a transition metal in an enzyme reaction is to allow the recycling of its charge state. For example, P450-type enzymes contain a transition iron cation, with activity modified by a heme group for its reaction. Some mixed-function oxidases and horseradish peroxidases have been reported to defluorinate monofluorinated compounds (Goldman and Milne, 1966).

Recently a mode of oxygen-independent defluorination was identified for the complete degradation of para-substituted fluoroaromatics by the denitrifying bacterium *Thauera aromatica*. This microbe utilizes a class I benzoyl-coenzyme A (BzCoA) reductase (BCR), which catalyzes the ATP-dependent defluorination of 4-F-BzCoA to BzCoA. Other enzymes involved are 1,5-dienoyl-CoA hydratase (DCH) and bifunctional 6-oxo-1-enoyl-CoA hydrolase (OAH). The outcome of the complete degradation of 2-F-benzoate is the production of HF and CO_2 (Tiedt et al., 2016, 2017) (**Figure 2**).

Both F-1,5-dienoyl-CoA isomers (compounds 2/2*) are hydrated to different F-OH-1-enoyl-CoA isomers (compounds 11/11*) by DCH and OAH, respectively. Unstable 6-F-6-OH-1-enoyl-CoA (11*) spontaneously decomposes to 6-oxo-1-enoyl-CoA (compound 7) by HF-expulsion. This, in the presence of OAH becomes immediately hydrated presumably to 2-oxo-6-OH-cyclohexanoyl-CoA (compound 12) before hydrolysis to 3-OH-pimeloyl-CoA (compound 8). Stable 2-F-6-OH-1-enoyl-CoA (compound 11) can also only be further hydrated by OAH, apparently to the unstable 2-F-2,6-di-OH-cyclohexanoyl-CoA intermediate, which spontaneously decomposes to compound 12 before ring hydrolysis by OAH. Intermediates illustrated with brackets probably only occur transiently (Tiedt et al., 2017).

Microbial Interaction With PFAS

Perfluorinated chemicals are chemically very stable and metabolically either completely stable or barely biodegradable so that they can be classified as persistent substances (Von Der Trenck et al., 2018). However, investigations have inferred that limited biotransformation of these chemicals can occur in natural and industrial environments. It is, therefore, important to understand what biotransformation occurs in practice, given the variation in toxicity across the range of potential PFAS products that may be produced due to biotransformation. A further goal is to find organisms that can significantly remove fluorine atoms from these compounds to substantially reduce their toxicity and stability.

It has been demonstrated that fluorinated precursors can be transformed to PFAS, with variable efficiency by a range of biological systems (soil/wastewater; Liu and Avendano, 2013; Lee et al., 2014). Attempts have been made to infer pathways for the biotransformation of fluorinated precursors (D'eon and Mabury,

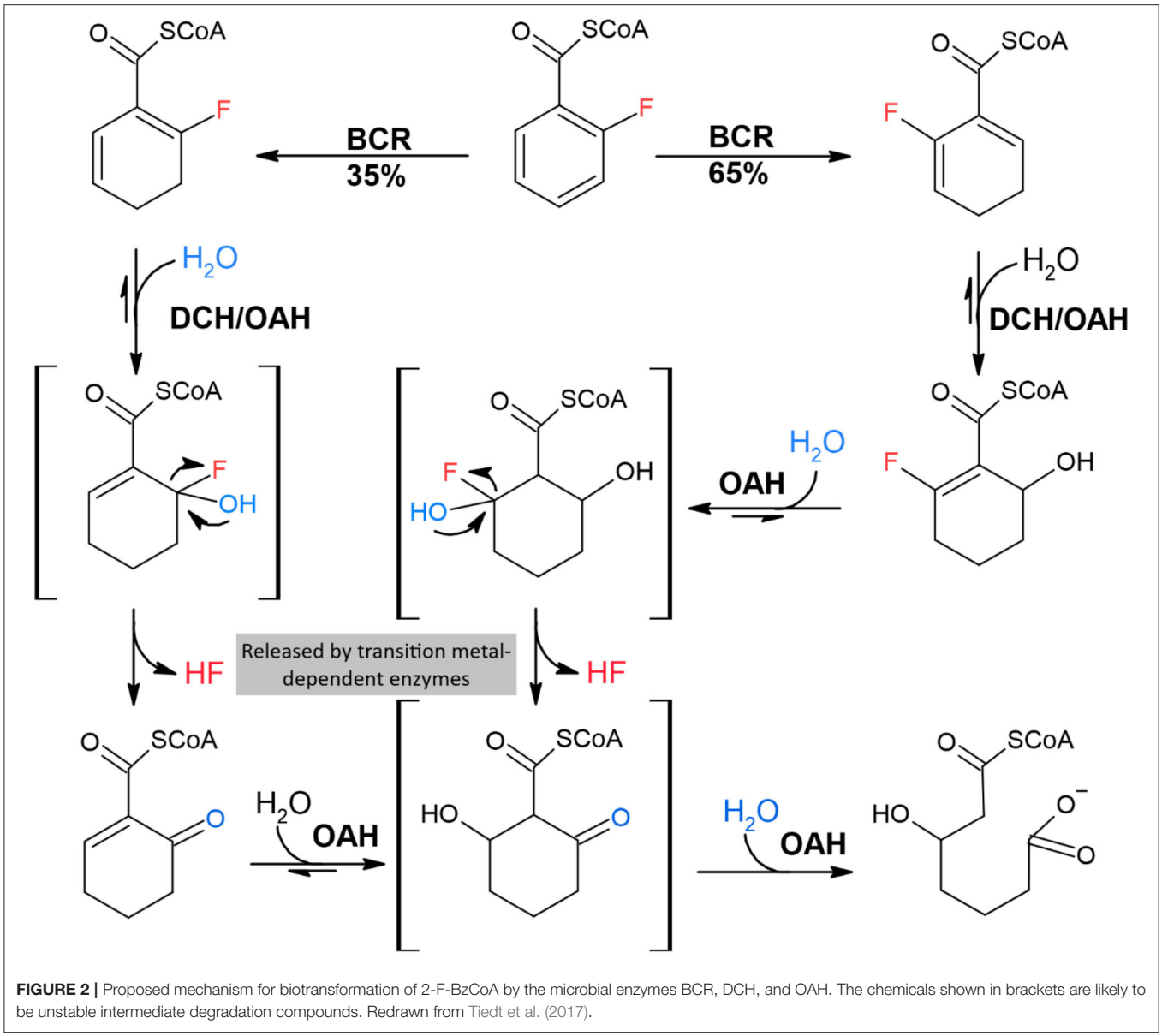

FIGURE 2 | Proposed mechanism for biotransformation of 2-F-BzCoA by the microbial enzymes BCR, DCH, and OAH. The chemicals shown in brackets are likely to be unstable intermediate degradation compounds. Redrawn from Tiedt et al. (2017).

2007; Rhoads et al., 2008; Wang et al., 2009; Liu and Avendano, 2013; Lewis et al., 2016) (**Figure 3**). However, these tend to be limited to side chains, without significant removal of fluorine atoms. This is unfortunate, in terms of potentially increased toxicity, though may provide a useful basis to further investigate biodegradation of PFAS.

In a further study, two different microbial consortia were isolated from two river sediments in Saitama and Osaka, Japan, known for long term pollution with PFOS and PFOA (Beskoski et al., 2018). Amicrobial chemoorganoheterotrophic consortia which included bacteria, yeast and molds was able to significantly decrease concentrations of PFOS and PFOA between 46–69% and 16–36%, respectively. However, defluorinated PFOS and PFOA products were not detected, though several metabolites were found only in samples from consortia with PFOS and PFOA. It was suggested these were associated with unsaturated monofluorinated fatty acids and hydrocarbons with multiple unsaturated bonds or ring structures (Beskoski et al., 2018). Nevertheless, if confirmed, this is problematic as the fluorine is still attached to an organic molecule that could be simply transferred to other organisms through a food web.

While specific biodegrading pathways for some PFAS have been investigated (Liu and Avendano, 2013), for many PFAS, the pathways are unknown. Moreover, the types of enzymes and associated genes involved have not been reported. A degradation pathway for a particular PFAS may be investigated by assessing the intermediate products in a sampling time series. Organisms able to degrade these compounds may optimally be selected from environmental areas that have been polluted with the particular PFAS. This approach has been utilized for developing

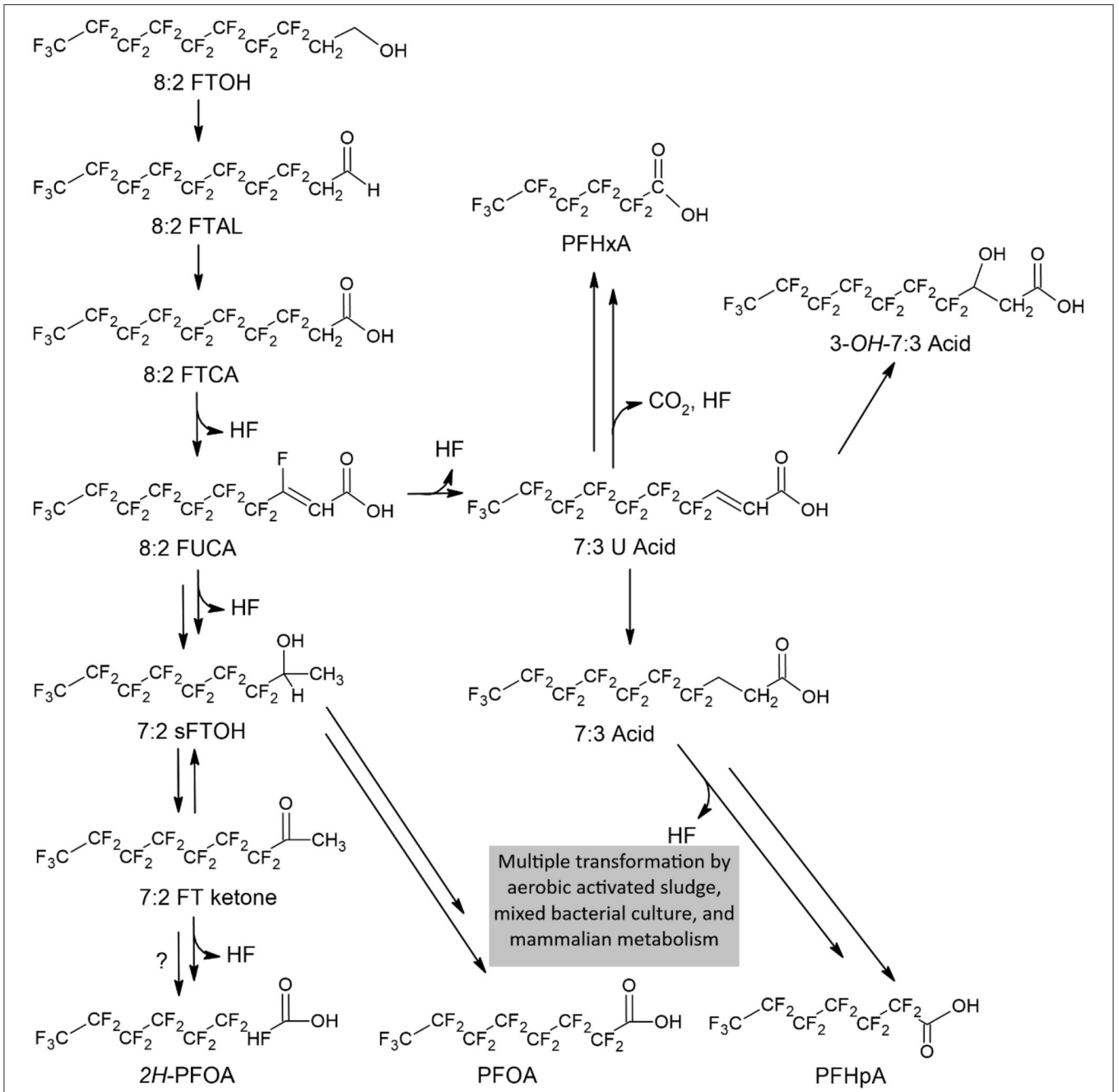

FIGURE 3 | Proposed aerobic biodegradation pathways of the precursor 8:2 FTOH in soil. The double arrows indicate multiple transformation steps. Defluorination reactions are indicated by the release of fluoride ions (HF). Stable and semi-stable compounds are shown inside dashed boxes. 2H-PFOA has been proposed, but it has not been successfully validated as a PFOA degradation product Obtained. Redrawn from Liu and Mejia Avendaño (2013).

bioremediation of soils polluted with oil products (Moliterni et al., 2012). The types of enzymes involved in biodegradation may be inferred by assessing the intermediate products, followed by a search of the KEGG database (Kanehisa et al., 2018) to confirm the type of enzymes and infer the structural genes that produce these enzymes.

It is also important to understand the biodegrading pathways of PFAS precursors in more detail, to potentially support bioremediation of PFAS and their precursors. It is also useful to recognize that transformations of PFAS may be potentially caused by chemical factors, as well as biological activities.

While biotransformation of PFAS commonly occurs, high concentrations may reduce biotransformation rates due to chemical toxicity. The impact of PFOA on the activated sludge process has been assessed using a lab-scale sequencing batch reactor, which was continuously exposed to PFOA (Yu et al., 2018). This method used a representative concentration for PFAS (20 mg L^{-1}) to mimic extreme conditions from industrial waste or groundwater from fire-fighting practice sites. The results indicate that PFOA restrained microbial growth which affected dissolved organic carbon removal. Also, continued exposure to PFOA resulted in a significant shift in community structure, leading to the presence of more PFOA-tolerant species (*Bacteroidetes*, *Proteobacteria*, and in particular *Acidobacteria*) (**Table 3**).

It is important to investigate biodegradation of PFAS under both aerobic and anaerobic conditions and in particular, to assess biotransformation intermediates in a reductive environment (Liu and Avendano, 2013), given the current limited PFAS biodegradation reported in aerobic environments. An oxygen-independent pathway may lead to enhanced degradation (Tiedt et al., 2016, 2017).

Mycoremediation

To date, research is limited on their ability of fungi to degrade PFAS. This is perhaps surprising given they are known to degrade lignin, one of the most recalcitrant natural compounds along with many toxic natural and xenobiotic compounds including organochlorines [e.g., DDT and DDE, organophosphates, pesticides, including chlorpyrifos and polychlorinated biphenyls (Beaudette et al., 2000) andpolyaromatic hydrocarbons (Moghimi et al., 2017)].

White-rot fungi in particular have displayed relative success in terms of the biotransformation of organic toxicants, including polychlorinated biphenyls, organophosphate pesticides and polycyclic aromatic hydrocarbons (Kaur et al., 2016; Stella et al., 2017; Harry-Asobara and Kamei, 2019). There are very few studies examining their ability to degrade PFAS. Tseng et al. (2014) reported some promising preliminary results looking at the effects of wood-rotting fungus on 6:2 FTOH, using the ligninolytic fungi, *Phanerochaete chrysosporium*. *P. chrysosporium* was capable of transforming 50% of 6:2 FTOH and 70% 8:2 FTOH in 28 days. Major metabolites of 6:2 FTOH included 5:3 polyfluorinated acid (40%), 5:2 FTOH (10%), PFHxA (4%). In contrast, the non-lignolytic fungus *Aspergillus niger* was unable to transform 6:2 FTOH over 35 days. while the same study reported that *P. chrysosporium* was capable of transforming 20% PFOS within 28 days. However, this research was conducted in a laboratory; it remains to be determined whether ligninolytic fungi are capable of degrading PFAS in the environment.

Phytoremediation

Phytoremediation represents another potential bioremediation approach for PFAS removal from contaminated environments due to the ability of several plants to bioaccumulate PFAS. Although PFAS are not extensively degraded during phytoremediation, bioaccumulation in plants creates a potential route for removal of PFAS from contaminated environments. Phytoremediation has been successfully used for the removal of several environmental contaminants including heavy metals and chlorinated substances (Huff et al., 2020).

Several plants have been used to accumulate PFAS. The wetland species *Juncus effuses* accumulated 11.4% of seven PFAS compounds from PFAS-spiked soil (Zhang et al., 2019). *Betula pendula* and *Picea abies* were reported to accumulate up to 97 and 94 ng g^{-1}, respectively, during a study at a firefighting training site near Stockholm, Sweden, contaminated with 26 PFAS compounds (Gobelius et al., 2017). The phytoremediation of PFAS contaminated soils using herbaceous and woody plant species has also been reported (Huff et al., 2020). The potential of several plants in a greenhouse study to bioaccumulate 6 PFAS compounds over 14 weeks has been reported (**Table 4**).

Future Prospects and Conclusion

Using a single bioremediation approach for PFAS may not be successful duet to the process is very slow therefore using a combination of bioremediation techniques to maximize the remediation of PFAS may offer a better approach (Ji et al., 2020). In one study, a combination of phytoremediation and PFAS-degrading bacteria in a constructed wetland was recommended as an effective and environmentally friendly approach that integrates optimum physio-chemical conditions and enhanced microbial degradation. The effectiveness of this "treatment train" approach has previously been reported in removing several emerging contaminants, such as pesticides, pharmaceutical and personal care products (Lv et al., 2016; Liu et al., 2019).

Constructed wetlands consist of three main components which are substrates, plants and microorganisms. The substrate, such as biochar, works as an absorbent of long-chain PFAS as well as media for plant growth and provides surface area for microbial biofilm production (Yang et al., 2018). Plants are another essential component of the constructed wetlands due to their ability to accumulate PFAS in different plant parts, including leaves and roots tissues (Zhang et al., 2019). Plant and substrate disposal, however, remains a great challenge. Thus, thermal treatment could be required for the complete mineralization of adsorbed and bioaccumulated PFAS (Gagliano et al., 2020). Microorganisms are the most important component of the wetland; however, the indigenous microbes have limited ability to biodegrade PFAS. The introduction of microorganisms that can degrade a certain contaminant has been proven to enhance the biodegradation of several emerging contaminants, such as antibiotics and personal care products (Li et al., 2019). Therefore, the introduction of defluorinating microorganisms that can use methane and hydrogen as an electron donor, to the constructed wetlands could enhance the breakdown of the C-F bond and the biodegradation of PFAS compounds (Huang and Jaffé, 2019). However, further investigation is required to assess the effectiveness of this approach (Ji et al., 2020).

Microalgae have shown the ability to remediate several emerging contaminants, including PFAS, through

TABLE 4 | Bioaccumulation of PFAS compounds by herbaceous plant species [Adapted from Huff et al. (2020)].

Plant species	Bioaccumulated PFAS compounds											
	PFPeA		PFHxA		PFOA		PFBS		PFHxS		PFOS	
	μg	%	μg	%	μg	%	μg	%	μg	%	μg	%
Amaranthus tricolor	446	30.9	153	8.1	66	7.7	4	0.4	1	4	0	0
Brassica juncea	114	11.8	72	5.7	15	2.7	9	1.7	8	1.6	4	0.7
Cynodon dactylon	434	22.6	427	16.9	55	4.9	156	14.1	51	4.8	20	2
Equisetum hyemale	759	39.5	557	22.1	36	3.2	1	0.1	7	0.6	4	0.4
Festuca rubra	717	37.4	652	25.9	122	10.8	224	20.3	141	13.2	39	3.8
Helianthus annuus	52	5.5	8	0.6	4	0.8	2	0.4	3	0.6	1	0.2
Schedonorus arundinaceus	807	42	696	27.6	60	5.3	262	23.8	92	8.6	14	1.4
Trifolium incarnatum	29	3.1	11	0.8	50	8.9	13	2.3	10	1.9	1	0.2

bioaccumulation, biodegradation and bio-adsorption. However, to date, most of the studies that have been conducted on microalgal-bioremediation are laboratory-based experiments under control conditions and the transition to field applications remains a challenge. Therefore, further research is required to employ microalgal species to bioremediate PFAS, which demonstrate increased biodegradation potential (Sutherland and Ralph, 2019).

The other role that microalgae may play in remediating emerging contaminants is enhancing bacterial biodegradation. Microalgal cells provide oxygen, an essential electron acceptor, *via* photosynthesis for the aerobic bacterial species, which in turn, produces CO_2 which is required for microalgal photosynthesis (Sutherland et al., 2015). Microalgae release dissolved organic matter (DOM) which can biostimulate bacterial degradation of the contaminants although the mechanism for the bacterial biostimulation is not fully understood. Thus, investigating the relationship between microalgae and bacteria and the optimum physico-chemical conditions are crucial steps to enhance the bioremediation process (Sutherland and Ralph, 2019).

Both fungal and bacterial strains have been isolated that are capable of degrading PFAS; however, degradation is slow and incomplete. In addition, information regarding the biodegradation and bioaccumulation of PFAS using bacteria and fungi is limited. Thus, more research needs to be undertaken. This is a crucial limitation to the development of any robust bioremediation strategy. However, with the current array of approaches and tools available to microbial ecologists, including stable isotope probing, metagenomics, transcriptomics and metabolomics, the identification of degradative pathways and the subsequent harnessing of PFAS-exposed microbial communities for remediation remains a possibility and further work needs to be performed to underpin the degradation process. Further studies could lead to an understanding of the pathways of degradation, by comparison to proposed published pathways in soil, for example (Liu and Avendano, 2013). In turn, the possible enzymes involved in degrading PFAS can be inferred by comparing the structures of PFAS in the proposed pathways. It would also be of value to list the potential genes in key bacteria that express the types of enzymes involved in degrading It would be useful to find and characterize microbes in contaminated soils that are capable of degrading PFAS and to quantify bioaccumulation and biomagnification of PFAS in trophic levels of marine ecosystems, in particular, to improve the assessment of health risks in human consumption of seafood contaminated by PFAS. Stable isotope probing (SIP) has been an extremely useful tool to link microbial identity to function; this technique has been used to elucidate the microbes responsible for the degradation of a variety of xenobiotics (Dumont and Murrell, 2005). The technique can be performed with DNA-SIP (Uhlík et al., 2009) or RNA-SIP which has been used during the degradation of benzene (Aburto, 2007; Aburto and Ball, 2009) and naphthalene (Huang et al., 2009) in groundwater, phenol in a bioreactor (Manefield et al., 2002), phenol in sludge (Sueoka et al., 2009) and tetrachloroethene in river sediments (Kittelmann and Friedrich, 2008) among other types of studies involving trophic interactions, biogeochemical processes or ecosystem functioning (Gutierrez-Zamora and Manefield, 2010). More recently it has been used to identify pesticide degraders (Jiang et al., 2018) and 1-4 dioxane degraders (Aoyagi et al., 2018). Therefore, SIP is a powerful technique that can also be combined with metagenomics (Vo et al., 2007) and transcriptomics (Lueders et al., 2016) and could also aid during the bioremediation of PFAS.

Metagenomics is a valuable tool that has been used recently to assess the stress of polyfluorinated alkyl substances on the microbial community (Cai et al., 2020) as well as their dynamics and structure (Zhang et al., 2020) in different environments such as soil and freshwater ponds. It is also one of the tools of

synthetic biology (synbio) which has been recently proposed to help in the bioremediation of xenobiotics, among them PFAS (Rylott and Bruce, 2020). Systems biology and protein design will also be critical tools for synbio, that in the future should allow the synthesis of proteins by reprogramming the genetic code and aid in the remediation of the persistent contaminants (Rylott and Bruce, 2020).

AUTHOR CONTRIBUTIONS

ES, DR, and LK wrote the manuscript. DT and AA-M contributed to the collection of literature and summarization. ES and AB guided throughout the preparation of the paper, proofreading the paper, and revised it. All authors fully agreed for publication of the paper.

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REVIEW
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Synthetic Organic Compounds From Paper Industry Wastes: Integrated Biotechnological Interventions

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Synthetic organic compounds (SOCs) are reported as xenobiotics compounds contaminating the environment from various sources including waste from the pulp and paper industries: Since the demand and production of paper is growing increasingly, the release of paper and pulp industrial waste consisting of SOCs is also increasing the SOCs' pollution in natural reservoirs to create environmental pollution. In pulp and paper industries, the SOCs *viz*. phenol compounds, furans, dioxins, benzene compounds etc. are produced during bleaching phase of pulp treatment and they are principal components of industrial discharge. This review gives an overview of various biotechnological interventions for paper mill waste effluent management and elimination strategies. Further, the review also gives the insight overview of various ways to restrict SOCs release in natural reservoirs, its limitations and integrated approaches for SOCs bioremediation using engineered microbial approaches. Furthermore, it gives a brief overview of the sustainable remediation of SOCs via genetically modified biological agents, including bioengineering system innovation at industry level before waste discharge.

Keywords: synthetic organic compounds, bioremediation, xenobiotics, pollution, pulp and paper industry

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INTRODUCTION

The paper and pulp industry consumes various raw materials i.e., wood, cellulose-based products, etc. The main aim of the paper and pulp industry is to produce on large scale to figure out the demand. This review insight into the environmental pollution caused by SOCs produced at various processing stages (**Table 1**). Deforestation for wood has caused a decline in oxygen level worldwide, directly responsible for floods and droughts. Water pollution by waste discharge from pulp industries also contaminates the water bodies with dissolved organic compounds (DOCs), synthetic organic compounds (SOCs), and suspended particles (Gupta and Gupta, 2019; Ramírez-García et al., 2019). The organic compounds reaching humans via water consumption leads to health issues, which are not immediate but show long term effects. The waste discharge also disturbs aquatic life (Karbalaei et al., 2018; Gupta et al., 2019). The emission of harmful chemicals and gases i.e., sulfur dioxide, nitrogen oxide, carbon monoxide will cause acid rain as they are water-soluble and reaches the water bodies indirectly (Gupta and Shukla, 2020). Metlymercaptans, hydrogen sulfides,

TABLE 1 | Types of SOCs from the paper industry.

S.no	Synthetic organic pollutant released	Source/Activity/Stage	References
1.	Nitrogen Oxides	Drying process	Zifang et al., 2017; Deviatkin et al., 2019
2.	TRS (i.e., Toxic sulfides)	Fiber paper mill	Sailwal et al., 2020
3.	Carbon Monoxide	Drying process	Mukhametzyanov et al., 2018; Man et al., 2020
4.	Nitrous Oxide	Drying process	Deviatkin et al., 2019
5.	Carbon dioxide	Drying process	Man et al., 2020
6.	VOCs (Volatile Organic Compounds)	Fiber paper mill	Sailwal et al., 2020
7.	Sulfur Oxides	Drying process	Zifang et al., 2017
8.	Dioxins	Pulp effluent	Xiao et al., 2017; Xia et al., 2020
9.	AOX (Adsorbable Organic Halogens)	Pulping stage	Kumar et al., 2020
10.	Furans	Pulp effluent	Hubbe et al., 2016; Romo et al., 2019
11.	Organo siloxane	Washing stage	Zhong et al., 2017; Li and Rabnawaz, 2018
12.	Hydrogen peroxide	Washing stage	Biswas et al., 2019; Elakkiya and Niju, 2020
13.	Mercaptans	Pulping stage	Singh and Chandra, 2019
14.	Sodium hydroxide	Bleaching and washing stage	Yehia et al., 2018; Perzon et al., 2020

and dimethyl sulfides along with volatile organic compounds (VOCs) lead to air and water pollution (Singh and Chandra, 2019; Pino-Cortés et al., 2020). The trials for pollution prevention are in continuous use by industries (by use of alternative bleaching agents), environmentalist (by releasing norms) as well as by consumers (by recycling waste and use of sedimentation tanks). Still, these measures are not fulfilling the demand to degrade the SOCs waste from the paper and pulp industry (Zumstein et al., 2018; Liu, 2020). In recent reports, the researchers have shown their interest in the biotechnological advancements for degrading the pollutants (Ellouze and Sayadi, 2016; Tripathi et al., 2017; Sharma et al., 2020). This review covers the advancement in methodologies via engineered biological agents (mainly bacteria) that are reviewed and suggested for sustainable bioremediation of SOCs.

SOCs FROM PAPER INDUSTRY WASTES

An ecosystem polluted and damaged by human activities with increasing intensity becomes a primarily global problem. SOCs are of xenobiotic origin in nature and thus there are difficulties involved in biotransformation (Antizar-Ladislao and Galil, 2004; Kumar et al., 2019). Due to recalcitrant, it has ecotoxic effects on the biosphere. SOCs can be primarily produced by following compounds such as methane, ethylene, aliphatics, and aromatics. Among the above, most of the industrial important SOCs derived from the aromatics viz., ethylbenzene, xylene, benzene, and toluene (Fang et al., 2018). Based on their primary uses SOCs are mainly classified such as cyclic, acyclic, aromatics, or aliphatics. SOCs contain huge categories like volatile organics carbons (VOCs), and relatively emerging organic contaminants (EOCs). VOCs primarily contain industrial re-solvents, gasolene agents, trihalomethanes, etc., while EOCs have pharmaceuticals, endocrine disrupting substances, hormones, food additives, microplastics, etc. (Lapworth et al., 2012; Postigo and Barceló, 2015). SOCs are primarily present in wastewater treatment plants. Most of the SOCs pass througfh various photo-transformations or chemical reactions and many of them remain inert in an open environment system.

In the paper mill, SOCs are released during the pulping and papermaking process. Chlorine and its derivatives have been released and restrained as adsorbable organic halides (AOX) (Savant et al., 2006), while other xenobiotic agents (resin acids, chlorinated lignins, dioxins, phenolic (tannins), and furans) are produced via pulping and paper manufacturing (Chandra et al., 2011). Out of the above, polychlorinated dibenzofurans and dibenzodioxins compounds of furans and dioxins are notably resistant to degradation and are persistent in nature (Gupta and Shukla, 2020). The polar phenolic polymeric compounds (tannins) are released in wastewater during the debarking process of raw wood material, which creates 50% COD of this wastewater (Chandra et al., 2018). Another study revealed that the naturally occurring tricyclic diterpenes (resin acids) are released during the pulping operations, which have pathetic aqua-phobic acids and toxicity levels to aquatic animals at conc. of 200–800 μg/l in wastewater (Duan et al., 2020). Mainly resin acids are produced from the pulping process containing dehydroabietic acid, abietic acid, pimaric acid, isopimaric acid, levopimaric acid, and neoabietic acid (Yadav and Chandra, 2018). Out of all the resin acids, isopimaric acid is notable as highly toxic. Many SOCs are discharged into the water body during the chemical process like calendaring (coating for paper smoothness) in the paper manufacturing industry. The schematic diagram of pulp processes releasing SOCs is given in **Figure 1**. The dioxins and furans are also released when chlorine reacts with some defoamers and wood preservatives like pentachlorophenol (PCP) during the pulping, washing and pulp bleaching process (Badar and Farooqi, 2012). Additionally, most SOCs that are discharged from the bleaching process areditolyethane, bis (methylphenoxy) ethane, di-iso-propyl naphthalene, terphenyl, chloromethyl-phenoxy-methyl-phenyl-ethane, etc. (Singh and Chandra, 2019). There are a lot of dyes used for paper printing in paper mills. At the end result, approximately 200 billion liters of dye effluents are released based on fabric type and dye

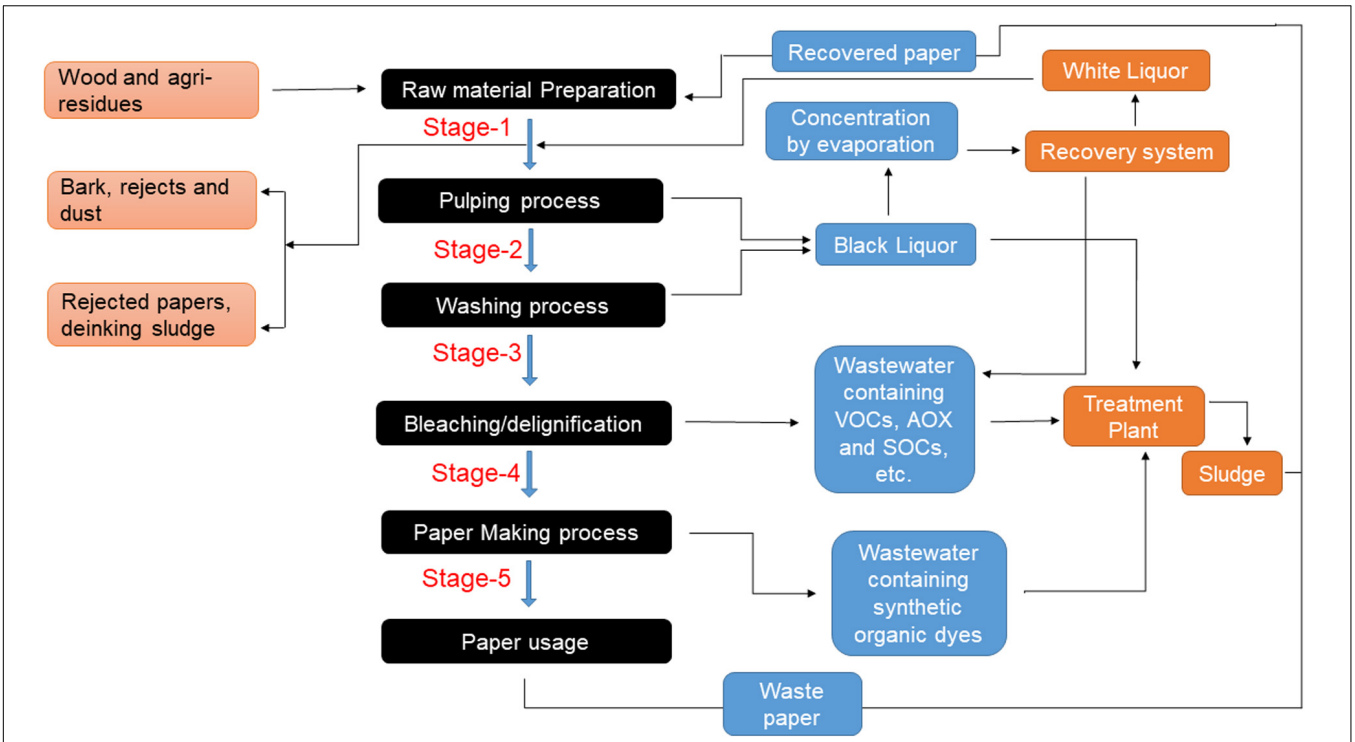

FIGURE 1 | Schematic diagram of pulp processes releasing various SOCs.

used. Many researchers reported that synthetic organic dyes such as azo, phthalocyanine and anthraquinone dyes discharged as effluents in the water body and have the most toxic effect on the environment as well as human health (Tkaczyk et al., 2020).

Ways to Restrain SOCs Production as Waste

To evaluate and mitigate the hazardous effect and load of SOCs released from the pulp and paper industries into the environment, various processes such as the use of chlorine-free bleaching process, use of ecofriendly chemicals for pulping, use of enzymatic pulping, and bleaching instead of the chemical pulping and bleaching process have been used. Among these, several other advanced and more significant methods have been adopted to reduce the SOCs load into wastewater, which is discussed below.

Many researchers have adopted many significant and ecological important methods help to remove organic pollutants from the environment, viz adsorption, biodegradation, striping, hydrolysis, photolysis, etc. (Ali et al., 2012). But significant results have not yet been obtained. Additionally, conventional adsorption techniques integrated with post-treatment using granular activated carbon (GAC) have been globally adopted for the removal of AOX for pulp mill wastewater. According to Osman et al. (2013), the treatment of paper mill wastewater GAC used with a sequenced batch biofilm reactor (GACSBBR) has significant capability to remove AOX at the longest hydraulic retention time (HRT) (Farooqi and Basheer, 2017). Currently, researchers have revealed that the use of biochar adsorption to mitigate organic pollutants has become an interesting field of research and hotspot. Biochar has a porous structure and contains functional groups of oxygen and minerals (Weber and Quicker, 2018). To eliminate dyes, these dyes go to different types of the treatment process (Puzyn and Mostrag, 2012). The biological, chemical, and physical processes can be done based on wastewater treatment stages (Primary, secondary and tertiary treatment) (Samer, 2015). The removal of organic and inorganic solids takes place in the primary treatment via sedimentation, grinding, and flocculation. While in the biological treatments (secondary treatment), organic materials are used by the aerobic or anaerobic microorganisms by the means of biological oxidation and biosynthesis processes. In the tertiary treatment, the wastewater undergoes different treatment processes like advanced oxidation processes, ion exchange, adsorption and reverse osmosis processes. For example, many researchers used ferric oxide-biochar nanocomposite absorbent extracted from paper mill sludge (Chaukura et al., 2017).

Another study reported/investigated that the biochar can be prepared from cardboard (BCPD), pig manure (BC-PM), and pinewood (BC-PW) for the use in adsorption of various synthetic organic dyes within several pyrolysis terms. Due to high ash content, BC-PM showed significant adsorption properties (Lonappan et al., 2016). Adsorption methods are amongst those used to remove dyes in comparison with other methods (Srivastava et al., 2018). During the degradation process of synthetic organic dyes, it undergoes various transformations kinetics. Some of the changes are into the more toxic agents and some of them non-toxic agents. Advanced techniques such

as oxygen cooking techniques, hydrogen peroxide, and ozone treatment for the pulp bleaching process could be options for pretreatment of primary sludge wastes, which helps with the reduction of an environmental load of SOCs production. There are mostly two types of chemical pretreatment used, alkaline and acidic. Acidic pretreatment is promoted for the hemicellulose while alkaline pretreatment for the lignocellulose, which makes it more accessible to use their products (Hendriks and Zeeman, 2009). However, lots of modified methods have been used for the pulping and bleaching process of the pulp mill. Bio-pulping is most suitable for the pulping process using eco-friendly enzymes and it can reduce the production of SOCs in waste materials. Some other techniques like innovation in the bleaching process can be adopted by many researchers. These techniques are elemental chlorine-free (ECF) bleaching techniques and a totally chlorine-free (TCF) bleaching technique (Gupta et al., 2019).

Detection and Analysis

Gas chromatography (GC) and Gas chromatography-mass spectrometry (GC/MS) have been used to detect and analyze the SOCs effluent released from the pulp and paper industries. Some metabolites formed by degradation of AOX can be identified by using GC/MS (Pronk et al., 2015). Many researchers used a multi X2500 analyzer to characterize bleaching AOX effluent. A study has stated that organic chlorides were recognized by using GC-MS incorporation with or without hot water abstraction. By these methods, AOXs were categorized into at least four main components such as macromolecular, small molecular organic chloride, chloro-phenol and chlorobenzene. Although, these methods are conventional methods and are time-consuming and expensive. Nowadays, advanced technologies like biosensors have been used, which offer an advantage over classical analytical methods due to their selectivity, sensitivity, eco-friendly, inexpensiveness and short assay time (Yao et al., 2017). However, an immobilized laccase based biosensor has been used for the detection and analysis of organic compounds. Several other electrochemical biosensors such as voltammetric sensor, amperometric laccase biosensor and optical biosensors are used for the analytical analysis of various organic effluents released from industrial operations. Among these, amperometric transducer methods have been reported as widely studied and used in laccase biosensors, while presently optical biosensors have the most significant results in terms of sensitivity (Rodríguez-Delgado et al., 2015). Additionally, a nanomaterial-based (Pena-Pereira et al., 2020) colorimetric detector has been used for the quantitative analysis of low molecular weight gaseous VOCs (Azzouz et al., 2019). Some researchers have employed high-temperature combustion to the transformation of Total organic halides (TOX) into halides and detected and quantified these halides using micro-coulometry methods. In 1977, micro-coulometry titration methods have been replaced by the more reliable ion-selective electrode (ISE) to detect the halides present in the wastes released from the paper mill (Chen et al., 2020).

Limitations and Challenges

SOCs such as aromatic compounds (phenols and biphenyls), polycyclic aromatic hydrocarbons (pyrene), are generally discharged into the water bodies. Most of the SOCs found in the environment/wastewater are recalcitrant due to their complexity compared to other effluents. However, these effluents have drawn more attention to treatment systems. These compounds are highly persistent, more toxic compounds that remain over a long period and bio-accumulated into the water body. Separation and treatment of these effluents became mandatory before releasing effluents in the marine ecosystem. For this purpose, the development of efficient techniques has been an interesting area of research for a long time (Awad et al., 2019). The use of conventional technologies has many disadvantages that limit the application area. The main environmental impact is the production of a huge amount of hazardous sludge that creates dumping problems and increases toxicity concentration in treated water (Ashrafi et al., 2013). Traditional methods are more expensive than advanced methods. However, environmental and health costs are also affected by using this classical method. Gaseous emissions, wastewater and sludge production from effluent treatments are relatively unmonitored. In developing countries, these effluents are primarily disposed of into unsecured landfills. The hazardous agents leach out over a long period from the landfills and go directly or indirectly into the environment. Constraints are in place with the purpose of limiting these effects, which have been mandatory across industries (Nimkar, 2017). However, the challenges of the reduction of SOCs production are still under investigation. Researchers have used some innovative and modified technologies for the treatment process of wastewater to help in the mitigation of hazardous compounds in the environment. Mostly SOCs are derived from the aromatic source, viz., toluene, ethylbenzene, anthracene, etc., which are persistent over the period and recalcitrant in the ecosystem because of the rigidness of their molecular structure and present thermodynamically stable aromatic ring (Postigo and Barceló, 2015). The ecotoxic impacts of SOCs on the environment have been accepted and implicit. However, water scarcity, water pollution and water recycling are serious challenges globally (Jain et al., 2020).

Economical Importance and Hindrance by SOCs for the Paper Industry

Pulp and paper are produced from cellulosic fibers, other plant material and synthetic materials may be used. Papers are mainly derived from wood fibers but cotton liners, bagasse, rags, etc are also used in some papers (Bajpai, 2018). Pulp and paper mills waste material and used papers can be further recycled and used to create economical values. The pulp and paper mills librated a substantial amount of wastewater composed of organic material such as high cellulose, hemicellulose, lignin contents (Kaur et al., 2020). Lignins are cross-linked phenolic polymers. These organic materials are suitable for the derivation of glucose and other fermentable sugars for example galactose, mannose, arabinose, and xylose. By using physical and chemical treatment methods, transformation of paper industry sludge into a glucose-rich liquid can be achieved. Enzymatic hydrolysis is a promising approach for the derivation of sugars from

paper industry sludge. Other valuable products can be obtained by causing the fermentation of sugars (Naicker et al., 2020). Production of biofuels such as bioethanol could be successfully achieved by the conversion of pulp and paper industry waste mainly composed of cellulose, hemicellulose, and lignin contents. These components require a series of reaction steps such as hydrolysis, hydrogeoxygenation alkylation, etc to be converted into biofuel. Lignin based biofuels can be produced by using one-pot depolymerization or by the upgrading of bio-oil from biomass decomposition. Pulp and paper industry waste conversion into biofuel is an interesting approach to manage paper industry waste and to create commercial value out of it (Zhu et al., 2020). The paper industry also generated sludge composed of biomass fly ash and calcareous sludge that is commonly disposed of in landfills. Calcareous sludge can be used in the manufacturing of green geopolymeric mortars for the application in construction. These components are released during the Kraft process of lignin. Biomass fly ash was reused as an alternative source of silica and aluminum, and calcareous sludge mainly constituting of calcite, was recycled and used in GP mortars construction. The implemented Mix design was outlined to maximize the incorporation of the calcareous sludge and improve the mortar's mechanical performance (Saeli et al., 2020). To accomplish a productive re-utilization of waste generated from the paper industry, waste effluent was recycled and used to produce green-composites with high strength which depends on ultra-molecular weight polyethylene, high-density polyethylene, and low-density polyethylene. The three green-composites were developed by an extrusion and injection molding named PLC, PUC, and PHC composites. The maleic anhydride grafted polyethylene, an organic compound, was used as a compatibilizer for preparing composites. The utilization of paper mill waste avoids the environmental waste and also produces the green-composites (Zhang et al., 2020). Anaerobic digestion under mesophilic condition is widely applied for the production of biogas by utilizing waste rich in suspended organic materials liberated from the paper industry. Industry waste contains a very high level of COD and BOD due to the presence of lignin, fatty acids, tannins, resin acids, and chlorinated compounds, etc. This biofilm technology is highly effective in biogas production (Bakraoui et al., 2020). Biogas production can be successfully achieved by using UASB digester technology and it can be applied on a large and small scale. Anaerobic digestion of Recycled pulp and paper industry waste can be carried out at different organic loading rates and in mesophilic conditions (Bakraoui et al., 2020). The amount of lignin is very important in paper manufacturing because lignin will affect the properties of the resultant paper. Lignin amount influences the tensile strength and elongation of cellulose fiber.

Effect on Ecological and Biological Health

The production of SOCs comes from mainly the pulping and bleaching stage of the pulp mill. These compounds have toxic properties, which may cause carcinogenic disease, allergic and dermatic disease (Puzyn and Mostrag, 2012). The production of trichlorotrihydroxybenzenes and bromomethylpropanylbenzene in the spent bleach liquor from pulp and paper industries have mutagenicity effects on the aquatic body as well as human beings. Additionally, some other SOCs such as chlorophenols and chloroguaiacols from bleach effluents notably carcinogen, reproductive toxicity in fish, and estrogenic in humans. Further, it has acute toxicity, which prevents the ATP synthesis process and oxidative phosphorylation mechanism (Singh and Chandra, 2019). Some endocrine-disrupting chemicals as residual organic compounds showed chromosomal aberration in marine animals (Chandra et al., 2018). The discharge of black liquor containing SOCs into the environment causes a direct effect on flora and fauna. In a developing country, untreated wastewater released from pulp and paper industries is discharged into the water body (Duan et al., 2020). They have to use this water for irrigation purposes so a lot of hazardous chemicals come into the fields and affected the crops due to changes to the soil properties, like alteration in pH values and beneficial microbes (Nguyen et al., 2020). The organic compounds pass through different trophic levels in the marine ecosystem and are bio-accumulated at a different level, which can be harmful to marine animals. However, the use of biochar for the adsorption of SOCs helps to retain fertilizers in the soil, promoting soil fertility, removal of heavy metals and acids, etc. (Shiralian, 2016). Based on dissipation time, SOCs can be classified into three main categories: highly persistent, moderately persistent, and low persistent. Humans are more exposed to SOCs through polluted air, water, or soil (Bilal and Iqbal, 2019). SOCs combined with their precursors employ eco-toxic effects on the environment (**Figure 2**; Jaishankar et al., 2014). An experiment was conducted which reported that the effect of SOCs on rainbow trout (*Oncorhynchus mykiss*) in the rivers of Chile, Canada, and Argentina was observed as stimuli for the development of secondary sexual properties and enhanced the intersex features in the young rainbow trout (*Oncorhynchus mykiss*) (Chiang et al., 2015). Similarly, a study conducted in China (2018) reported that long term exposure of andostenrdione has masculinization and reproductive effects in both male and female wastern mosquitofish (*Gambusiaaffinis*) (Hou et al., 2018). Another experiment demonstrated by terasaki and co-workers in 2012 stated that the effects of Dimethyldiphenylmethane and di-iso-propylnaphthalene have reproductive and tissue toxicity on marine fish (Terasaki et al., 2012). The exposure of hexachlorobutadine (HCBD) in human beings has hostile effects on human health either directly or metabolically. The nephrotoxicity effects of HCBD have been observed in animal host experiments and reported as having a necrosis effect on the renal proximal tubule, up-regulation of kidney injuring molecule-1 and lipid peroxidation in renal cells (Sadeghnia et al., 2013). In china, the approximately 8.0×10^{-6} μg/kg/day of HCBD exposure dose for human and animal risk was observed which has caused skin diseases, carcinogenicity, sexual abbreviation and mutagenicity in humans as well as aquatic communities (Zhang et al., 2014).

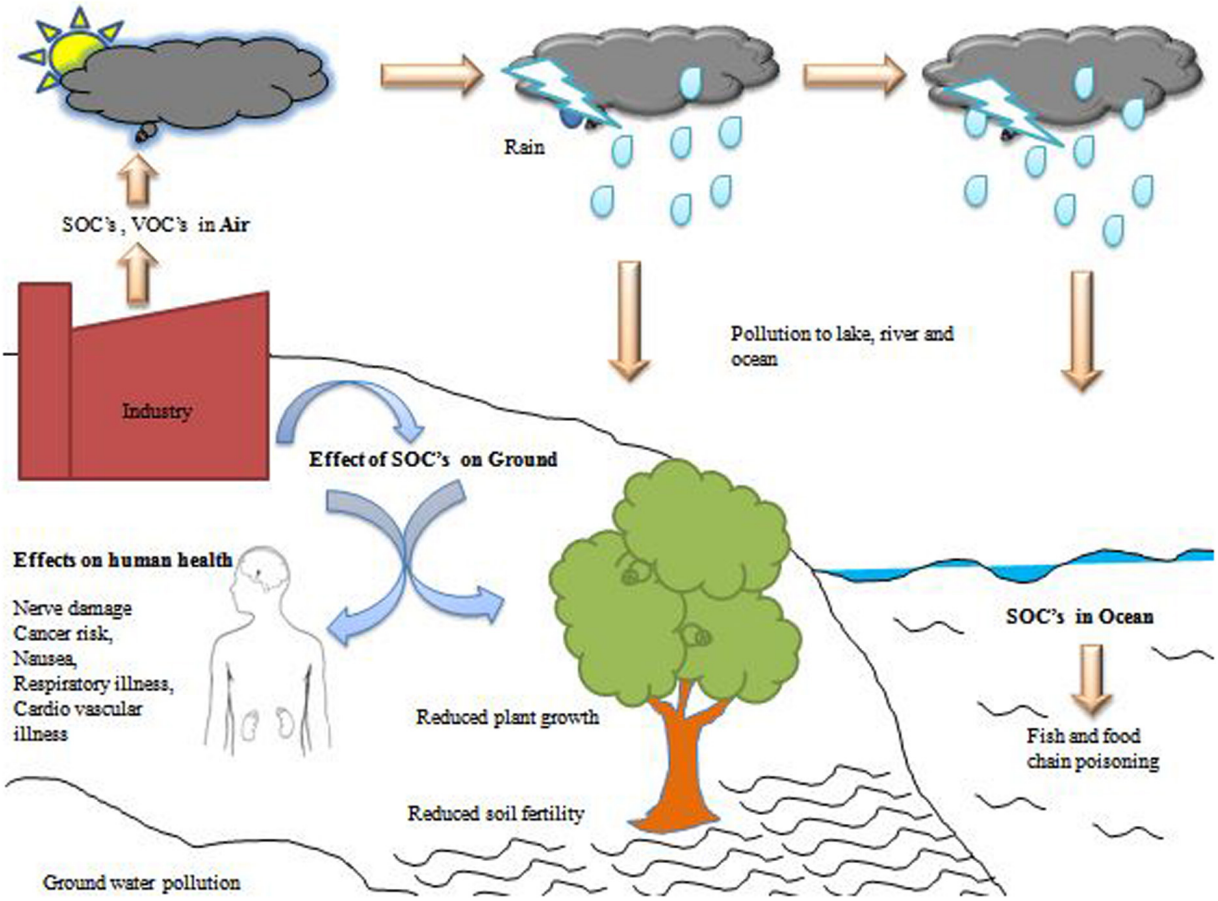

FIGURE 2 | The overall view of SOCs affecting the ecological and biological health.

BIOTECHNOLOGICAL INTERVENTIONS IN PRESERVING ENVIRONMENT THROUGH BIOREMEDIATION

The recalcitrant nature and toxicological assessment of synthetic organic compounds were not carried out at the early industrial stage. But as the industrialization sector boomed and ill-effects of various pollutants were studied then SOCs also came under scrutiny because of their presence in polluted industrial water. Since then it has become a matter of great concern to remediate these pollutants. Various biological and technological approaches have been utilized to remove SOCs from wastewater before their discharge into water bodies (Jain et al., 2020).

Bioelectrochemical systems, containing electro genesis systems, electro hydro-genesis systems, microbial electrosynthesis (MES) systems (Liu et al., 2018), and microbial desalination systems, are an emerging technology to remediate pollutants (Wang et al., 2015; Fernando et al., 2019). This technology uses electricity and microorganisms to degrade pollutants into less toxic elements. Certain value added products such as biofuels (including hydrogen, butanol, and ethanol, etc.) (Kondaveeti et al., 2019; Liu and Yu, 2020), acetates, and metals are also produced by using these techniques (Moscoviz et al., 2016; Maktabifard et al., 2018). The relatively low energy value (0.2–0.8 V) is needed for the MEC system as compared to conventional water electrolysis (Kadier et al., 2016). Rozendal and co-workers reported that approximately 7 kg COD/m^3 bioreactor volume/day could be removed by the BES which is the same as a conventional treatment system (Rozendal et al., 2008). Lab scale results reported that MEC showed COD removal efficiency was observed to be about 90–97% of synthetic wastewater at different temperature profile (ranging 5–23°C) and 0.6 kWh/kg electricity. Hence, the BSE is more suitable for small and lab scale systems due to the low energy utilization with improved byproduct production which minimizes the capital cost (Tartakovsky et al., 2018). But the implementation of BES with ordinary systems at industrial levels is more challenging due to the high capital cost which is required (Santoro et al., 2017). Microbial fuel cells (MFCs) are efficient for the biochemical conversion of energy for a useful purpose. Dual-chamber MFC has been utilized for the management of polyaromatic hydrocarbons (PAHs) contagion from diesel. The proposed system detached 82% of PAHs and generated about 31 mW/m^2 power. MFCs with tubular single- and dual- chambers were applied to reveal

ex situ and *in situ* management of refinery wastewater or groundwater having a blend of PAHs, containing benzene and phenanthrene (Adelaja et al., 2017). Fenton reaction and the microbial consortium was evaluated for the removal of tannery dye effluent. This exceptional combination was able to remove 89.5% pollutants and led to a reduction in the COD level of 93.7% (Shanmugam et al., 2019). Another advanced oxidation process of ultrafiltration and photoelectrolysis alone was found to remove total phosphorus between 90 and 97% from municipal wastewater and 44% from industrial wastewater (Gray et al., 2020).

Activated carbon has been used as a suitable adsorbent for many pollutants. Superfine powdered activated carbon is found to be more suitable as an adsorbent due to its smaller size, lesser surface oxygen amount, bigger aperture diameters, and neutral pH. An increase in adsorption of planar (phenanthrene) compounds was affected more than non-planar (2-phenyl phenol) compounds (Partlan et al., 2020). Activated carbon can also be used in supporting biofilms for pollutant removal. Due to the larger surface area provided by activated carbon, biomass was able to degrade xylene and other BTEX compounds efficiently and reduce the toxicity of up to 99% (Mello et al., 2019). In this era of machine learning, modeling strategy to check the efficient substrates of adsorption of SOCs can help in the development of efficient adsorbents. In a study, Ghosh et al. (2019) developed a regression support model quantitative structure-property relationship (QSPR). According to this model, they have calculated the adsorption coefficient of 40 SOCs on single-walled carbon nanotubes. They found that various hydrophobic and electrostatic interactions as well as hydrogen bonding help in the adsorption of SOCs on nanotubes. The interaction studies help in the development of suitable adsorbent for SOCs removal from wastewater (Ghosh et al., 2019).

Modified zeolites are also emerging as suitable adsorbents for wastewater treatment. Hashemi et al. (2019) modified a zeolite Y made from bentonite by using CTAB. Various adsorption isotherms indicated removal of 89% total organic carbon and involvement of electrostatic and hydrophobic interactions (Hashemi et al., 2019). Another Fe-nano zeolite was able to absorb phenol (Ph), 2-chlorophenol (2-CP) and 2-nitrophenol (2-NP) in the amount of 138.7, 158.9, and 171.2 mg/g, respectively. This zeolite-based adsorbent was even more cost effective than activated carbon (Tri et al., 2020).

SUSTAINABLE REMEDIATION OF SOCs VIA GENETICALLY MODIFIED BIOLOGICAL AGENTS

In the pulp and paper industry paper is derived from wood and produces a huge amount of waste effluents as sludge and polluted water. Toxic chemicals and recalcitrant organic compounds are found in this wastewater (Dixit et al., 2020). Pulp and paper industry waste released into freshwater alters aquatic habitats and adversely impacts human health. The remediation of these organic compounds is necessary to accomplish environmental sustainability. Bioremediation of pollutants is a novel technique to make the effluents less toxic and safe for discarding the waste (Gupta et al., 2019). To protect human lives, the advancement of remediation technologies for the recovery of polluted sites is of utmost importance. Sustainable remediation, which seeks to reduce concentrations to risk-based levels as well as mitigate ancillary environmental consequences such as waste generation, has recently gained significance (Cecchin et al., 2017). Bioremediation requires the use of particular microorganisms to degrade organic pollutants, a reasonable and efficient approach based on microbes' unique catabolic capacity (Dvořák et al., 2017). This has led to increased efforts using innovative biotechnological methods (**Table 2**) to develop more effective, ecologically sustainable, environmentally acceptable, and cost-effective remediation technologies (Kumar et al., 2017). Various microorganisms, mainly bacteria and

TABLE 2 | Advance biotechnological techniques for SOCs level reduction.

S.no	Approach	Methodology	Model organism used	Application	References
1.	Biotechnological approach	GMOs	Bacteria	*Pseudomonas putida*	Tahseen et al., 2019; Lindemann et al., 2020; Yang et al., 2020
				Klebsiella spp.	Shang et al., 2019; Rhie et al., 2019
				Achromobacter	Yang et al., 2020
2.	Synthetic biology	Gene editing tools	Crispr-Cas	Gene-specific nucleases	Stein et al., 2018
			TALEN		Jaiswal et al., 2019
			Zn Finger		Kumar N.M. et al., 2018; Kumar V. et al., 2018
3.	Systems biology	Biodegradation network	BioCyc	Bioremediation	Jaiswal et al., 2019
				Biotransformation	
		Metabolic network	MetaCyc	Xenobiotics metabolism;	Jassal et al., 2020
				Dynamics study;	
				Genome-scale metabolic database	
		Enzymatic reactions	KEGG	Enzymes encoding genes;	Kanehisa, 2017; Li et al., 2018
				Metabolic enzymes	
		Omics	Genomic; metagenomics; High throughput analysis; Proteomics		Malla et al., 2018; Gupta et al., 2020

fungi, play an important role to degrade synthetic organic compounds. Degradation of these compounds depends upon the secretion of enzymes by microorganisms that participate in the metabolic pathways. The traditional physicochemical bioremediation methods (*in situ* and *ex situ*) (Jaiswal et al., 2020) are inefficient for degradation and removal of new emerged compounds (Jaiswal and Shukla, 2020). With the development of genetic engineering and Recombinant DNA technology many genetically modified microorganisms were constructed by using various techniques for the remediation of synthetic organic compounds (Liu et al., 2019). Biodegradation of recalcitrant azo dye was successfully done by enzyme azoreductase encoded by gene *azoA* from *Enterococcus* sp. L2 into *E. coli* and *Pseudomonas fluorescens* using the expression vector PBBRMCS2. To further increase the degradation of azo dye NADH regenerate system depended on the formate dehydrogenase enzyme introduced into the host strain by the overexpression of *fdh* gene from *Mycobacterium vacccae* N10. For efficient dye decolorization processes the transcription fusion of *azoA* – *fdh* provided a simple genetic cassette for genetic engineering of an appropriate host (Rathod et al., 2017). Moreover, Biodegradation of phenol and p-nitrophenol was successfully done by genetically modified *Bacillus cereus* strains by introducing the *vgb* gene from *Vitrocilla stercoraria*. The gene was cloned into a pUB110 multicopy plasmid. A higher degradation rate was obtained at 37°C under aerobic conditions by genetically modified bacteria compared with wild type. p-Nitrophenol degradation was obtained high by using the strain with uni-copy of *vgb* gene (Vélez-Lee et al., 2016). *Bacillus cereus* and its recombinant strains are effectively used for biodegradation of phenols and p-nitrophenol under anaerobic and aerobic conditions. Different Phenolic compounds are effectively degraded by the action of Manganese peroxidase, an extracellular heme enzyme of white-rot basidiomycete *Ganoderma*. 1092 bp full-length cDNA of the *MnP* gene, designated *as G. lucidum MnP (GluMnP1)*, was cloned from *G. lucidum* and a eukaryotic expression vector, pAO815: GlMnP was constructed and transferred it into the methylotrophic yeast *Pichia pastoris* SMD116 by the electroporation-mediated transformation. Recombinant GluMnP1 is capable of the degradation of phenol and the degradation of four types of dyes. Great potential for the enzymatic remediation of phenolic compounds and industrial dyes was shown by the Recombinant

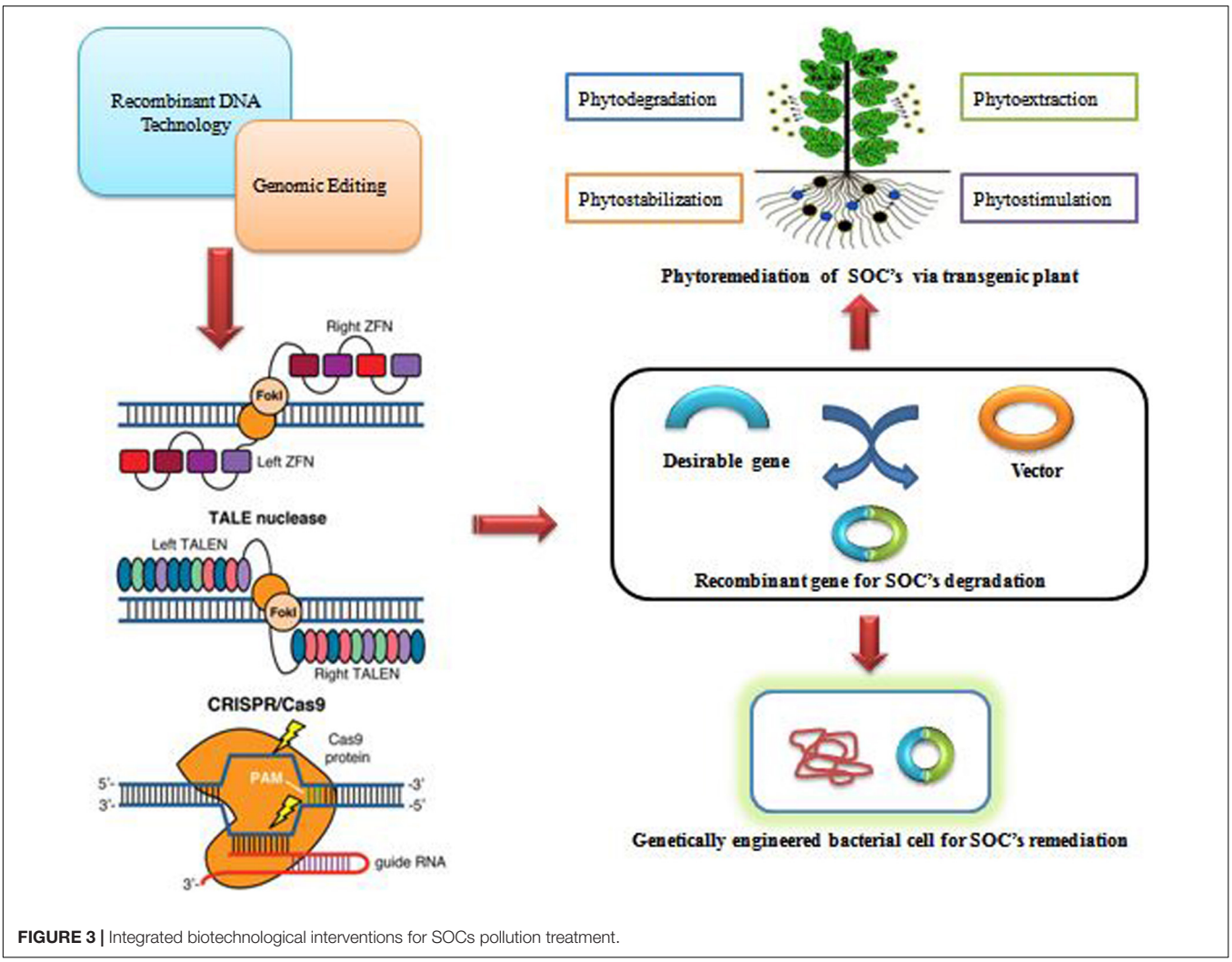

FIGURE 3 | Integrated biotechnological interventions for SOCs pollution treatment.

GluMnP1. Phenol and the main oxidation degradation products including hydroquinone, pyrocatechol, and resorcinol were analyzed by using HPLC (Xu et al., 2017). In another study for the remediation of the phenolic compound engineered *Escherichia coli* effectively used. Nine genes namely, pheA1, pheA2, catA, catB, catC, catD, pcaI, pcaJ, and pcaF were selected from different microorganisms and an oligonucleotide was synthesized. By using the modified overlap-extension PCR method, all synthesized genes were seamlessly connected with the T7 promoter and terminator to construct a gene expression cassette. All the cassettes were transformed to the host *Escherichia coli* strain BL221-AI and the transformant was named BL-phe/cat. The engineered *Escherichia coli* was effectively used for phenol degradation (Wang et al., 2019). Degradation of organophosphates, carbamates, and pyrethroids was achieved by engineering *Pseudomonas putida*. In a study, a scarless genome-editing tool was applied for the engineering of *Pseudomonas putida* KT2440. The vgb and gfp genes were transferred into the chromosome. It was observed that the genetically modified strain *Pseudomonas putida* KTUe having genes (ΔphaC1, Δvdh, ΔalgA/algF, Δfcs, Δupp, ΔphaZ/phaC2, gfp+, mcd+, cehA+, mpd+, pytH+, vgb+) was able to decompose all the pesticides screened. Also, it was found that to sequester oxygen in the soil study with the VHb gene was responsible. Thus, this engineered *Pseudomonas putida* strain is a powerful approach for the degradation of pesticides (Gong et al., 2018). Recent genetic editing technology is a promising approach for engineering the various microorganisms to perform remediation of pollutants (Dangi et al., 2019). With the help of gene-editing techniques, modified microorganisms with maximum quality can be produced by making targeted modifications in the genome using molecular scissors involving engineered nucleases. Clustered regularly interspaced short palindromic repeat (CRISPR-Cas), zinc finger nucleases (ZFNs) and Transcription-activators like effector nucleases (TALENs) are the main gene-editing tools that have the dynamic capacity to boost bioremediation of synthetic pesticides (Jaiswal and Shukla, 2020; Kumari and Chaudhary, 2020). The gene editing process involves self-designed guide sequences that are inserted complementary to the sequence of the gene of interest assisting break at a site, repaired by homologous recombination, insertion, or deletion of desired sequence fragments. A double-stranded (DSB) break can be created by Transcription-activators like effector nucleases in the target sequence on DNA and makes sticky ends. Likewise, zinc finger nucleases also introduce a DSB in the target sequence of the host genome. On another hand, CRISPR-Cas comprise of crRNA and trcRNA joined by gRNA. gRNA controls the Cas9 enzyme to create DSB in the desired sequences of DNA (Jaiswal et al., 2019). In another study plants also play a main role in the removal of various pollutants by phytoremediation. Phytoremediation is a bioremediation form that requires plants as tools for the removal of hazardous contaminants from the environment. Phytostimulation, phytoextraction, phytoextraction, phytostabilization, and phytovolatilization are different approaches of phytoremediation for the remediation of metals/metalloids and other hazardous contaminants. A plant's genome can be modified by utilizing CRISPR-Cas, ZFNs, and TALENs gene-editing tools (**Figure 3**; Aminedi et al., 2020). Indeed, clustered regularly interspaced short palindromic repeat (CRISPR-Cas) is a revolutionary genetic engineering tool in plants that provides a pragmatic approach to synthesize advanced phenotypes (Saxena et al., 2020). On another hand, progress in the development of recombinant microorganisms has created potential risks associated with the release into the open environment of such genetically engineered microorganisms (GEMs). But many attempts are being made to monitor and track genetically engineered microorganisms to address these risks. Designing genetically engineered microorganisms by employing sufficient genetic methods to contain the bacterial system will help to reduce the anticipated hazards. For example, transposition vectors are designed which are deemed to be safe in the environment. Another containment technique primarily includes the production of suicidal genetically engineered microorganisms, but the technology has yet to be applied. These advanced technologies are one of the most promising ways to mitigate the adverse effects of genetically engineered microorganisms release in the open environment (Hussain et al., 2018). But certain risks could also exist and further study will then be needed to produce acceptable technical regulatory guidelines.

CONCLUSION AND FUTURE PERSPECTIVE

The review shows the extent that the recent research in the field of environmental pollution by the paper and pulp industry has reached. The researchers and environmentalists concluded that SOCs pollutant levels must be declined, and have worked in the same direction. They found that the composition of various chemicals varies with the stage and methodologies applied for paper production. The detection and degradation of organic chemicals produced during paper production are enhanced by researchers using advanced techniques. Biotechnological intervention using synthetic and systems biology for producing genetically modified organisms specifically for potential degradation of SOCs came into consideration. Thus, this review covers the recent reports and methodologies used by the researcher for environmental sustainability.

AUTHOR CONTRIBUTIONS

SJ wrote the first draft of the manuscript with contributions from GK, M, and KP. PS read and edited the final draft. All authors approved the final draft for its submission.

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Isolation and Identification of Organics-Degrading Bacteria From Gas-to-Liquid Process Water

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The gas-to-liquid (GTL) process generates considerable amounts of wastewater that are highly acidic and characterized by its high chemical oxygen demand (COD) content, due to the presence of several organic pollutants, such as alcohols, ketones, aldehydes, and fatty acids. The presence of these organics in the process water may lead to adverse effect on the environment and aquatic life. Thus, it is necessary to reduce the COD content of GTL process water to an acceptable limit before discharging or reusing the treated water. Due to several advantages, biological treatment is often utilized as the main step in GTL process water treatment plants. In order to have a successful biotreatment process, it is required to choose effective and suitable bacterial strains that have the ability to degrade the organic pollutants in GTL process water. In this work, bacterial strains were isolated from the GTL process water, identified by 16S rRNA gene sequencing and then used in the biodegradation process. The detailed identification of the strains confirmed the presence of three organics-degrading bacteria identified as *Alcaligenes faecalis*, *Stenotrophomonas* sp., and *Ochrobactrum* sp. Furthermore, biodegradation experiments were carried out and confirmed that the pure culture as well as the mixed culture consortium of the bacterial strains has the ability to reduce the organic pollutants in GTL process water. However, the growth rate and biodegradation efficiency depend on the type of strains and the initial COD content. Indeed, the removal percentage and growth rate were enhanced after 7 days for all cultures and resulted in COD reduction up to 60%. Moreover, the mixed culture of bacterial strains can tolerate and treat GTL process water with a variety of ranges of COD contents.

Keywords: GTL process water, isolation, identification, biodegradation, COD reduction

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INTRODUCTION

Qatar is the capital of natural gas production and hosts the largest gas-to-liquid (GTL) plant in the world. During the GTL process, considerable amounts of water are often generated, due to the chemistry nature of the Fischer–Tropsch (F-T) process that is the main process in natural gas plants. The generated GTL process water contains several dissolved hydrocarbons that cannot be

directly used or discharged to the aquatic environment. GTL process water is characterized by high acidity and high chemical oxygen demand (COD) content that can reach 32,000 mg/L. The water is mainly contaminated with non-acid oxygenated (NAO) hydrocarbons, including ketones, aldehydes, alcohols, ethers, and esters (Zacharia et al., 2018). Thus, an appropriate treatment process should be applied to reduce the concentration of organics in GTL process water to the acceptable discharge limit. Since F-T water contains volatile organics and light oxygenates such as carbonyl compounds and C_1-C_3 alcohols which have boiling points less than that of water, they are typically treated using distillation or stripping columns. Therefore, the GTL wastewater treatment plant usually requires a pretreatment step such as stripping or distillation column to reduce the content of COD before the biological treatment process (Surkatti et al., 2020). The wastewater generated from the stripping/distillation column still contains large quantities of carboxylic acids and other oxygenates, which need to be treated biologically (Enyi et al., 2013).

The conventional activated sludge process, constructed wetlands, and trickling filters are nowadays among the most common biological technologies for the treatment of wastewaters, in addition to different types of membrane bioreactors (Shokrollahzadeh et al., 2008). For GTL process water, several biological treatments were combined with other treatment processes to achieve high COD reduction efficiency (Wang et al., 2016). However, the use of traditional anaerobic suspended sludge process has a major limitation in wastewater treatment. Therefore, new biological reactors were developed for the treatment of industrial wastewater, in which bacterial strains were used in the form of free or immobilized systems (El-Naas et al., 2013, 2016; Bouabidi et al., 2019).

In biological treatments, chemo-organotrophic species are considered as the main microbial degraders of organic pollutants in contaminated wastewaters (Fritsche and Hofrichter, 2008). A large range of organic compounds can be used by these chemoheterophic microorganisms, especially bacteria, as carbon and energy sources (Elbeshbishy, 2014). However, searching to extend the range of the organics that can be used, their admissible concentrations, and the efficiency of their removal is leading to continuous efforts deployed to isolate new strains or species having the ability to degrade all organics present in GTL process water. This is more needed for special situations of water generated in arid zones, like the Gulf area, characterized by harsh conditions (Disi et al., 2017). Indeed, the selection of any bacterial strain for a bioremediation process is based on its ability to tolerate harsh conditions, exhibiting a suitable activity for the existing organics in the treated water (Azubuike et al., 2016). Arid and semiarid areas are characterized by harsh weather with high temperatures that can affect bacterial populations and create a dynamic diversity of these bacteria, based on their ability to adapt by acquiring new metabolic activities and suitable surfactant production (Elazzazy et al., 2015). Local bacteria are then needed for the treatment of local GTL process water. Moreover, under harsh conditions, indigenous microorganisms have adapted to develop a specific metabolism, effective for these weathered organics (Kumar and Gopal, 2015). Indeed, many failures of bioremediation applications in areas characterized by harsh weather and soils can be attributed to the use of unacclimated bacteria and their associated activities (Disi et al., 2017).

The novelty of this work resides in the collection, isolation, and identification of indigenous bacteria from GTL process water available in Qatar. The same bacterial strains are then utilized for the biodegradation of major organic contaminants in local GTL process water. The main approach employed in this work is multidisciplinary, combining environmental microbiology, biochemistry, and bioremediation. This is important from fundamental and applied points of view for specific GTL process water generated and treated at harsh conditions.

MATERIALS AND METHODS

Industrial Water Samples

Process water samples were collected from a local GTL plant in Qatar, in which two types of water samples were analyzed and referred to as pretreated GTL process water and original GTL process water. The physical and chemical characteristics of the GTL process water samples are shown in **Table 1**. GTL process water is characterized by its high acidity and organic contents (high COD).

In order to investigate the organics present in GTL process water, a qualitative analysis using GC-MS was carried out for the GTL process water (6,000 mg/L COD) and for pretreated GTL process water. In general, GTL process water contains alcohols, ketones, volatile fatty acids, ester, and other aliphatics. Based on the analysis, the main organic contaminants are short-chain alcohols and long-chain alcohols; it also contains some fatty acids, such as propenoic acid, butanoic acid, and acetoacetic acid, in addition to the esters, aliphatics, and ketones such as methyl ketone and pentanone. However, the type of organic pollutants is varied for the raw GTL process water and the pretreated GTL process water since the pretreated wastewater contains alcohols, ketones, and some aliphatics.

Isolation and Purification of Bacterial Strains From Enrichment Cultures

Luria–Bertani (LB) medium was used for the enrichment culture, isolation, and purification of the bacterial strains (**Figure 1**).

One milliliter of the GTL process water was suspended in 20 ml liquid LB medium as the first enrichment step. The liquid cultures were incubated at 30°C in a rotating shaker set at 200 rpm for 3 days. At the end of the incubation period, 2 ml from each liquid culture was transferred to 20 ml mineral salts medium (MSM)-GTL as the adaptation step. The adaptation steps were repeated three times. Then, aliquots (100 μl) of the MSM-GTL enrichment cultures were spread on solid LB agar medium. The LB plates were incubated at 30°C for 24 h. Isolates exhibiting distinct colonial morphologies were isolated and transferred to separate LB agar plates for further purification. Consequent purification of the bacterial isolates was repeated six times using the streak plate method until pure isolates were obtained (Survery

TABLE 1 | Physical and chemical characteristics of GTL.

Characteristic	GTL process water	Pretreated GTL process water
COD (mg/L)	5,000–7,000	1,800–2,000
TOC (mg/L)	1,500–1,700	700–800
pH	2.9	2.9
Conductivity	0.435	0.430

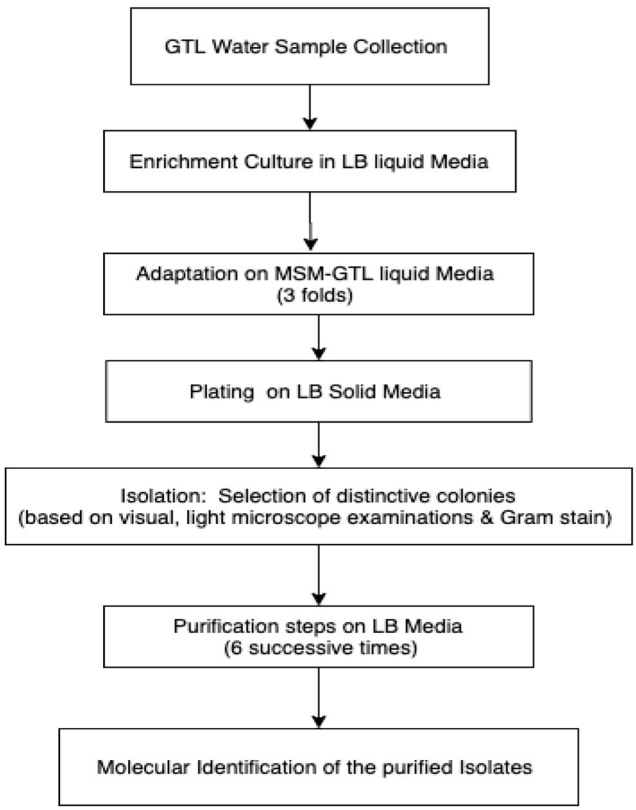

FIGURE 1 | Flowchart illustrating the isolation strategy.

et al., 2005). Stock bacterial cultures were preserved at −80°C in 30% glycerol until use.

Evaluation of the Potential of Bacterial Strains to Remove COD in GTL Process Water

Biodegradation experiments were performed using MSM liquid medium containing per liter (pH 7.2): NH_4NO_3, 4.0 g; Na_2HPO_4, 2.0 g; KH_2PO_4, 0.53 g; K_2SO_4, 0.17 g; $MgSO_4 \cdot 7H_2O$, 0.10 g; and 1 ml/L of trace element solution (per 100 ml): EDTA, 0.1 g; $ZnSO_4$, 0.042; $MnSO_4$, 0.178 g; H_3BO_3, 0.05; and $NiCl_2$, 0.1 g. The media were prepared using different concentrations of GTL process water. All media were sterilized by autoclaving at 121°C for 20 min. Analysis was performed after the incubation periods to investigate the reduction in organic pollutants present in GTL wastewater. The bacterial growth was evaluated using the Lambda 25 UV/VIS spectrophotometer at 600 nm. Samples were collected and analyzed at several time intervals to confirm the growth and the biodegradation efficiency of the isolated bacteria. All biodegradation experiments were carried out at optimal conditions, temperature of 30°C and pH 7, since there was no growth or organic removal observed at room temperature (T = 25°C) and pH values (pH 3.0 and 5.0).

COD Determination

The COD analysis was carried out using a HAC-UV spectrophotometer with COD reagents. The analysis was obtained by adding 2 ml of the water sample into the HAC LCK514 cuvettes and heating for 2 h to complete the reaction between the reagent and water sample. The sample was then transferred to the HAC 3900 to read the COD content in milligrams per liter. Each sample was analyzed in duplicate.

Molecular Identification of Isolates

The DNA was obtained from cells grown on LB solid media overnight at 30°C. Pure colonies were suspended into Eppendorf tubes filled with 0.5 ml of sterile distilled water. The Eppendorf tubes were incubated in a water bath set at 100°C for 10 min and then placed in an ice bath for 1 min. After centrifugation for 60 s at 12,300 rpm, the supernatant containing total DNA was carefully transferred to a new sterile Eppendorf tube. The amplification of the 16S rRNA gene fragments of ∼1.5 kb was carreid out using two universal primers: RibS73sp 5′-AGAGTTTGATCCTGGCTCA-3′ and RibS74sp 5′-AAGGAGGTGATCCAGCCGCA-3′ (Lane, 1991). The PCR reactions were performed in a total volume of 25 µl including $MgCl_2$ 1.5 µM, dNTP 0.8 µM, forward primer 1.35 µM, reverse primer 1.35 µM, and 0.5 IU Taq DNA polymerase; 2 µl of genomic DNA from the isolates served as template for the PCR reactions.

The thermocycler program for each PCR reaction was intiated with a 3-min denaturation step set at 94°C, followed by 35 cycles of denaturation steps at 94°C with 45 s each, annealing step at 50°C for 45 s, elongation step at 72°C for 45 s, and then one final 2-min extension step at 72°C. Consequently, the purification of PCR products was acomplished using an Invitrogen PureLink PCR Purification Kit. The DNA sequencing was performed using a Genetic Analyzer-Applied Biosystems 3,500 Series. The obtained sequences of 16S rRNA gene fragments were then compared with the most closely related species available in the GenBank database using the NCBI Blast server. Additionally, the phylogenetic tree of bacterial isolates was constructed by MEGA X software using the method of maximum likelihood (ML) based on the 16S rDNA gene sequences.

RESULTS AND DISCUSSION

Isolation and Molecular Identification of Bacteria From Qatari GTL Process Water

Only three isolates (RZ3, RZ4, and RZ5) were obtained after enrichment cultures and isolation in LB medium. In fact, they

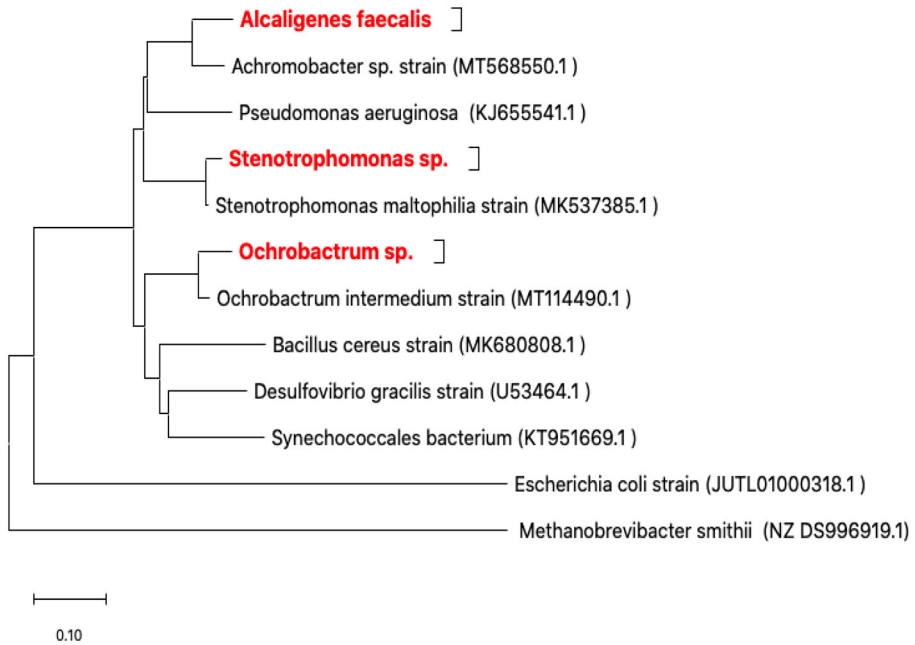

FIGURE 2 | Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of the studied strains. *Methanobrevibacter smithii* from Archaea was the domain used for outgroup rooting. *Achromobacter* sp., *Bacillus cereus*, *Desulfovibrio gracilis*, *Escherichia coli*, *Ochrobactrum intermedium*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Synechococcales* bacterium were used as reference bacterial strains. GenBank accession numbers are given in parentheses. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.51758258 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1,790 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018; Stecher et al., 2020).

TABLE 2 | List of previous works on the removal of organic compounds by the studied strains.

Bacterial type	Bacterial source	Wastewater	Contaminant	Initial concentration (mg/L)	Removal %	Reference
Alcaligenes faecalis	Durgapur steel industry wastewater	Synthetic	Phenol	1,600	100	Jiang et al., 2007
Alcaligenes faecalis	Activated sludge	Synthetic	Phenol	1,600	100	Jia et al., 2005
Alcaligenes faecalis	Activated sludge from coke factory	Synthetic	Phenol	1,000	100	Essam et al., 2010
Stenotrophomonas sp.	Polluted river	Petroleum wastewater	Phenols	998	89	Patel and Patel, 2020
Stenotrophomonas sp.	Polluted river	Petroleum wastewater	COD	15	93	Patel and Patel, 2020
Stenotrophomonas sp.	Aquifer	Synthetic	*p*-Nitrophenol	1.078	100	Subashchandrabose et al., 2013
Ochrobactrum sp.	Textile sludge	Agricultural	COD	11,707	71	Neoh et al., 2016
Ochrobactrum sp.	Textile sludge	Agricultural	Ammonium nitrogen	256	60	Neoh et al., 2016
Ochrobactrum sp.	Textile sludge	Agricultural	Total polyphenolic compounds	916	55	Neoh et al., 2016
Ochrobactrum sp.	Marine environment	Petroleum	PAHs, COD	1,000	66	Arulazhagan and Vasudevan, 2011
Ochrobactrum sp.	Phenol-activated sludge	–	Phenol	100	100	El-Sayed et al., 2003
Ochrobactrum sp.	Polluted soil	–	*p*-Nitrophenol (PNP)	100	100	Qiu et al., 2007

represent three types of colonies characterized by their form and color and the microscopic observation of the corresponding bacteria. The isolates were identified by ribotyping, based on sequencing of their 16S rDNA amplicons, after purification. The obtained 16S rDNA sequence of each isolate was used to determine the most closely related sequence of available

sequences in the GenBank database using the Blast server at NCBI. The three isolates identified were as follows: *Alcaligenes faecalis* (*RZ3*), *Stenotrophomonas* sp. (*RZ4*), and *Ochrobactrum* sp. (*RZ5*) (**Figure 2**).

It can be concluded that a few strains were isolated. There are two possible reasons for this. The water samples contained highly toxic organic compounds, so that only the three isolated strains were able to tolerate this high level of water toxicity. The enrichment medium contained low nutrient contents, which may not be enough to sustain the required nutrients for cell growth and maintenance. Therefore, a few cells would have been able to adapt and subsist. This result shows that the isolation strategy was efficient to bacteria by enriching cultures in LB containing GTL process water. This culture medium was very toxic to bacteria because of the high organic concentration (COD ranging from 6,000 to 7,000 mg/L). The aim of the isolation procedure was to isolate and purify bacteria with strong potentials to degrade and tolerate GTL process water.

The isolated bacteria have been widely applied in the treatment of different GTL process water containing a variety of organic contaminants. **Table 2** summarizes the application of these strains in wastewater treatment in previous studies. *A. faecalis* shows high performance in the removal of ammonia from wastewaters (Neerackal et al., 2016). However, it has high tolerance in phenol removal from wastewater, even at very high initial concentrations (Bai et al., 2007). It was confirmed by Essam et al. (2010) that an isolated strain of *A. faecalis* has the ability to grow in phenol; however, it has difficulty to grow and remove other phenol derivatives, such as nitrophenols and chlorophenols. Additionally, the bacterium was shown with low tolerance to grow in alcoholic compounds such as methanol and ethanol (Essam et al., 2010). *Stenotrophomonas* sp. is reported with its tolerance in heavy metal removal (Gunasundari and Muthukumar, 2013); however, it showed good performance in the removal of phenols and organic carbon present in refinery wastewaters (Patel and Patel, 2020). *Ochrobactrum* sp. is a Gram-negative, rod-shaped, aerobic and oxidase-positive bacterium (Arulazhagan and Vasudevan, 2011). This bacterium showed high treatment performance of several contaminants present in wastewaters (Qiu et al., 2007). Neoh et al. (2016) showed that *Ochrobactrum* sp. has high performance in the biotreatment of agricultural wastewaters that contain high COD and nitrogen contents. In addition, *Ochrobactrum* sp. has high performance in the removal of phenol and its derivatives (El-Sayed et al., 2003; Qiu et al., 2007). The removal of the organic compounds present in the GTL process water was not documented using the isolated strains.

Biodegradation of Organic Pollutants From GTL Process Water

Organic Removal Using Several Strains

The potential of the isolated bacterial strains in the removal of organic compounds present in the Qatari GTL process water using pure and mixed cultures was evaluated. The GTL process water used in this section was obtained after the pretreatment process and has COD content of 1,800 mg/L. A set of 14 experiments were performed using the MSM-GTL medium to screen the isolated strains for the biodegradation of organic pollutants in GTL wastewater. All samples were kept in the incubator in which the COD content and biomass growth (biomass production) were evaluated at 3, 7, 10, and 14 days. The growth curves of single and mixed cultures are shown in **Figure 2**. It is clear that the three strains reach their maximum growth after 3 to 4 incubation days. Interestingly, the growth started immediately after inoculation, without a clear lag phase. This means that all the strains are highly adapted to the substrates, which are the organic compounds in GTL process water including alcohol, ketones, esters, and aliphatics. However, a clear decline in growth was observed after this period. The period of high growth was marked with a fast decrease of the COD content, which continued up to the 7th day (**Figure 3**). Nevertheless, there was no further COD reduction observed after

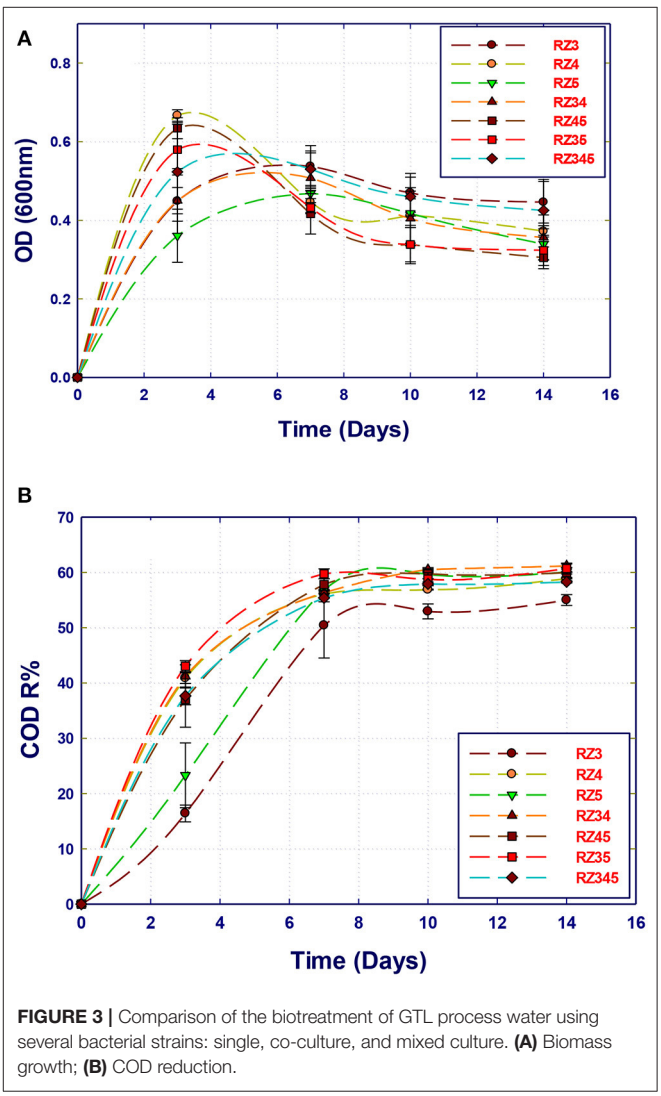

FIGURE 3 | Comparison of the biotreatment of GTL process water using several bacterial strains: single, co-culture, and mixed culture. **(A)** Biomass growth; **(B)** COD reduction.

a 1-week incubation. When comparing the growth curves and corresponding COD reduction curves for each single or mixed culture, it can be noticed that the strain *Stenotrophomonas* sp. (RZ4) showed the highest growth rate as a single culture, followed by the mixture *Stenotrophomonas* sp. with *Ochrobactrum* sp. (RZ5). Although *Ochrobactrum* sp. growth was the least as a single culture, a clear symbiotic and cooperation can improve the growth of *Ochrobactrum* sp. by the concomitant growth of *Stenotrophomonas* sp. A pure culture of *Ochrobactrum* sp. can also achieve around 56% COD reduction similar to that obtained by *Stenotrophomonas* sp. alone or by *Stenotrophomonas* sp. and *Ochrobactrum* sp. after 14 incubation days. However, the yield of *Ochrobactrum* sp. in biomass production was lower than that of *Stenotrophomonas* sp.

The strain *A. faecalis* (RZ3) also showed less growth rate than that of *Stenotrophomonas* sp. alone, which is reflected in a slightly lower COD reduction (52%). Mixing *A. faecalis* with *Stenotrophomonas* sp. did not improve its growth, although the COD reduction was improved to the best level. In contrast, the combination of the three strains resulted in biomass growth and COD reduction similar to that of *Stenotrophomonas* sp. alone. It seems that the strain *Stenotrophomonas* sp. can be used alone to remediate the Qatari GTL process water at these conditions (COD 1,800 mg/L).

Effect of COD Concentration on Biodegradation Performance

The performance of the isolated strains to grow and remove organics in GTL process water at three initial COD contents (1,800, 3,800, and 5,500 mg/L) was investigated. The initial COD content of the raw GTL process water was 5,500 mg/L, which was pretreated to reduce its concentration to 1,800 mg/L. The third COD content (3,800 mg/L) was obtained by diluting the initial raw GTL process water. The biodegradation performance of pure and mixed culture of the three strains was evaluated at

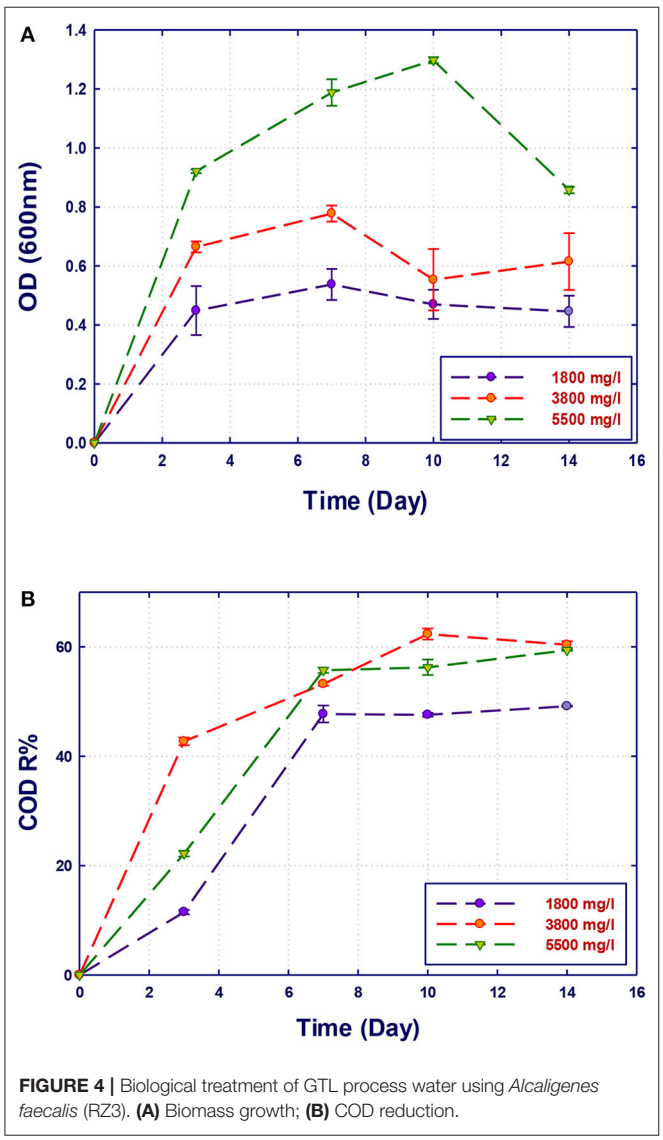

FIGURE 4 | Biological treatment of GTL process water using *Alcaligenes faecalis* (RZ3). **(A)** Biomass growth; **(B)** COD reduction.

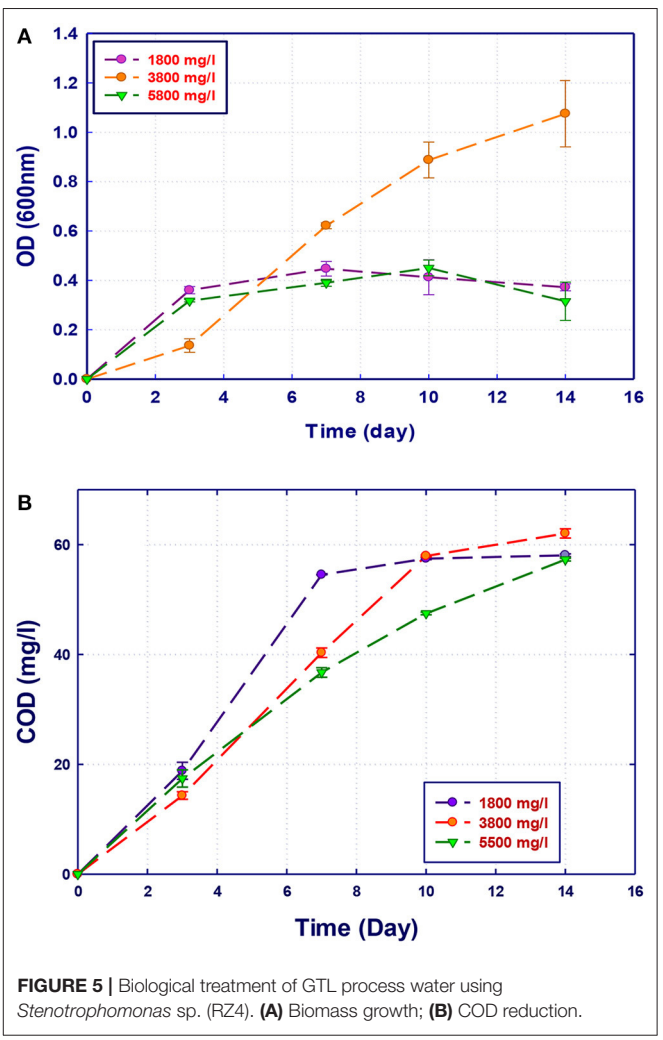

FIGURE 5 | Biological treatment of GTL process water using *Stenotrophomonas* sp. (RZ4). **(A)** Biomass growth; **(B)** COD reduction.

each COD content. The biomass growth and COD reduction for each water sample (with different COD concentrations) are shown in **Figures 4**–**7**. Interestingly, the results of **Figure 4** show that the strain *A. faecalis* (RZ3) is not inhibited by the excess of organic pollutants (high COD process water) since an increase of the growth rate is related to COD increase. Moreover, the overall removal of COD after 14 days of incubation was almost similar (60%). This may be attributed to the ability of *A. faecalis* to remove several organic pollutants in GTL wastewater including fatty acids that are available in the raw GTL process water.

The growth of both strains *Stenotrophomonas* sp. (RZ4) and *Ochrobactrum* sp. (RZ5) was negatively affected by the increase of the COD content in the growth medium. This means that these strains may be inhibited by the excess of substrates or inhibited by several organics present in GTL process water. The combination of the three strains showed high ability in the degradation of organic pollutants present in GTL process water, which indicates the synergy effect for several strains in the GTL wastewater treatment.

Although the three isolates were capable of degrading the organic pollutants in GTL process water at various COD contents, the removal of these organic contaminants by mixed cultures was improved compared with individual strains as shown in **Figure 7**. Each individual microorganism may have the ability to metabolize limited types of substrates; thus, the combination of different bacterial strains with wider enzymatic capabilities will result in the degradation of more organic pollutants giving higher COD reduction. This was confirmed by Senthilvelan et al. (2014) when they tested phenol degradation using single strains and mixed culture. In their study, a significant increase in the biodegradation rate and phenol removal was obtained using mixed microbial culture.

Since short-chain alcohols (SCA) represent about 82% of the COD content of GTL process water (Majone et al., 2010), GC analysis was carried out to determine their concentrations in

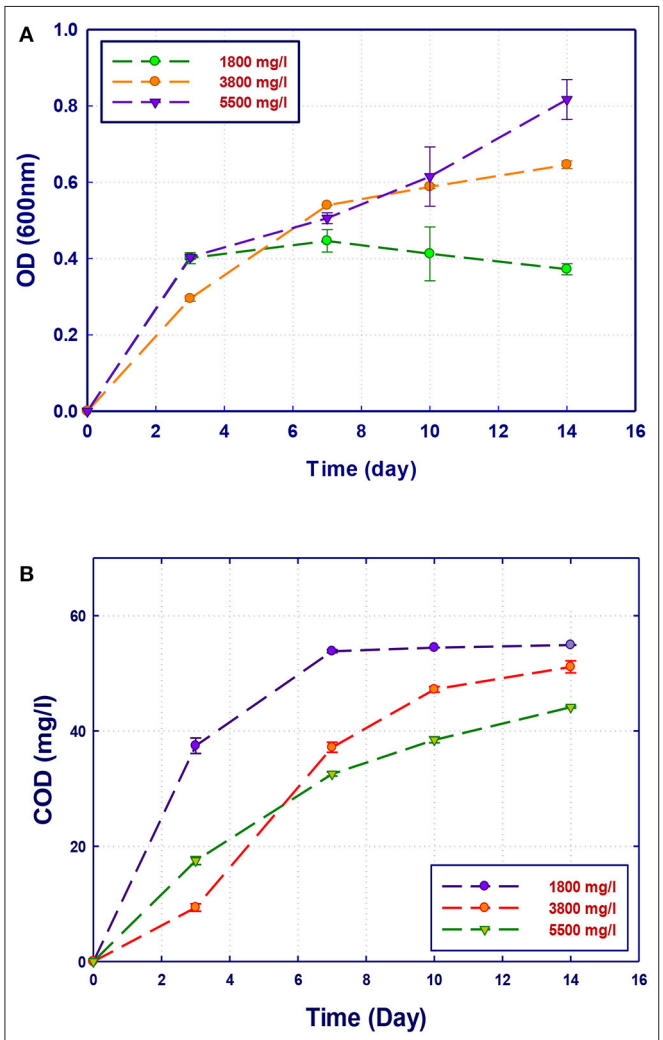

FIGURE 6 | Biological treatment of GTL process water using *Ochrobactrum* sp. (RZ5). **(A)** Biomass growth; **(B)** COD reduction.

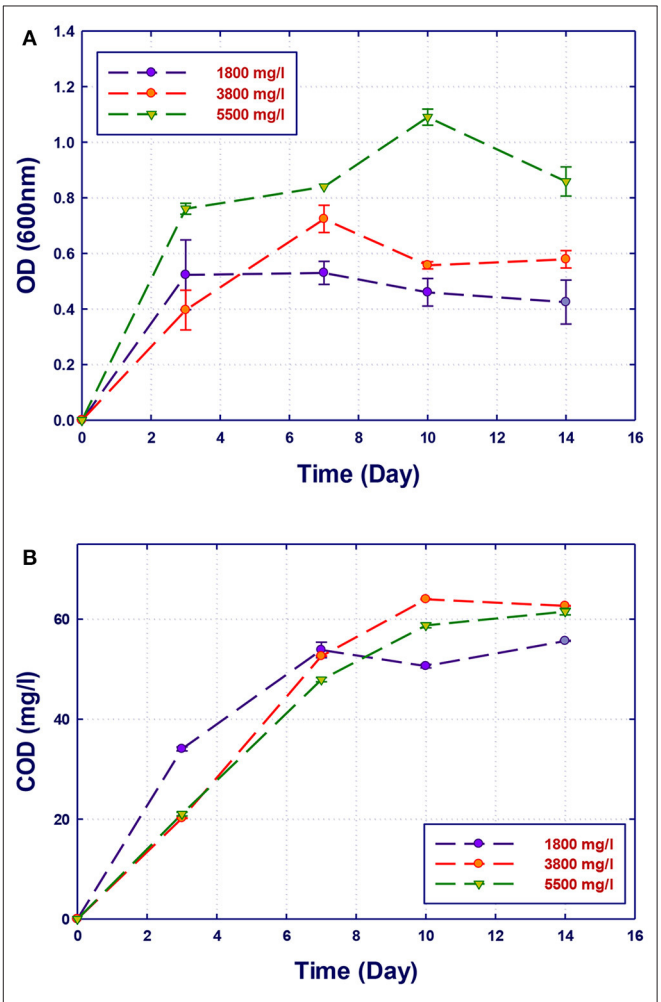

FIGURE 7 | Biological treatment using mixed culture of *Alcaligenes faecalis*, *Stenotrophomonas* sp., and *Ochrobactrum* sp. (RZ 345). **(A)** Biomass growth; **(B)** COD reduction.

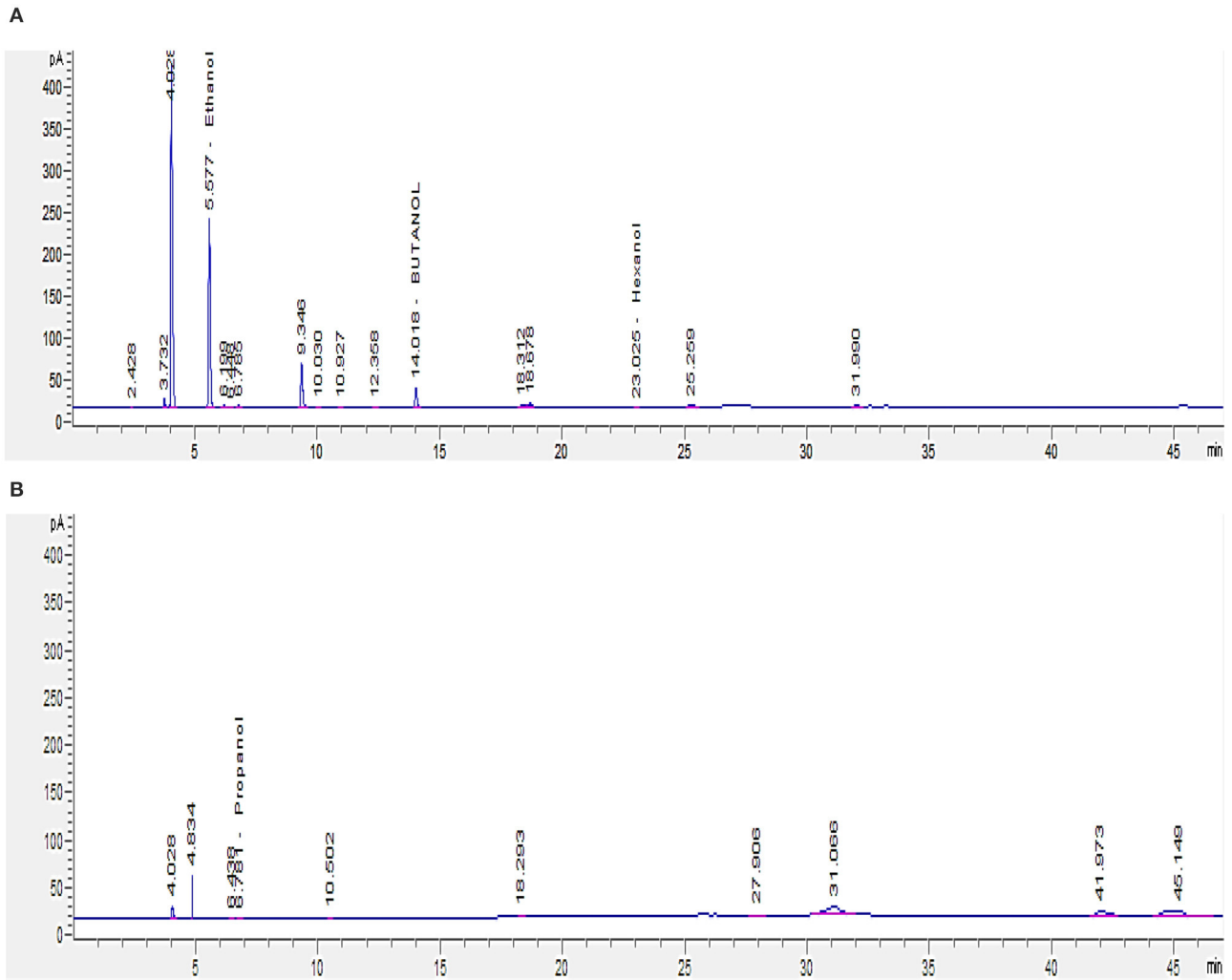

FIGURE 8 | GC analysis for short-chain alcohols (C1–C6) in GTL process water: **(A)** before and **(B)** after biotreatment.

GTL process water before and after biotreatment. GTL process water and biotreated water contain SCA (C1–C6) concentration of 2,465 and 813 mg/L, respectively (**Figures 8A,B**). The analysis showed that the isolated bacteria were able to degrade around 60% of the short-chain alcohols.

Since the SCA are the major contributor to the COD content in GTL process water, it is important to reduce their concentration through biotreatment, in order for the treated water to be reused or discharged safely.

It is obvious that GTL process water has a broader range of organic pollutants. This may require the application of several types of microorganisms in order to achieve high degradation performance. Thus, it is recommended to use mixed bacterial strains for the biological treatment of GTL process water, at different ranges of COD contents. The main novelty of this study is the application of the isolated strains to remove the organic pollutants in the same GTL process water and achieve high COD reduction under stress conditions, which has not been reported before.

CONCLUSIONS

It is evident from this study that GTL process water is a rich source of organic components that are rather difficult to remove or degrade. However, the isolation, identification, and testing of organics-degrading bacterial strains, such as *A. faecalis*, *Stenotrophomonas* sp., and *Ochrobactrum* sp., from GTL water showed that these strains have the capability to degrade the organic contaminants in GTL water and can be a good alternative for the conventional activated sludge systems, which are currently applied in the biological treatment of GTL process water. Each bacterial strain has the ability to work as a single strain or in a mixed culture to remove the organic pollutants

present in GTL process water. Regardless of the complex composition of GTL process water, the isolated strains resulted in high COD reduction (up to 60%) under stressed conditions and, consequently, had high growth performance in such industrial wastewater.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

RS, ME-N, and ZA conceived the original idea and designed the experimental program. RS and ZA carried out the experiments and prepared the initial draft. ME-N and NZ revised the manuscript with support from ZA and RS. MV and UO helped with discussion of the results and manuscript revisions.

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SYSTEMATIC REVIEW
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Recent Advanced Technologies for the Characterization of Xenobiotic-Degrading Microorganisms and Microbial Communities

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Global environmental contamination with a complex mixture of xenobiotics has become a major environmental issue worldwide. Many xenobiotic compounds severely impact the environment due to their high toxicity, prolonged persistence, and limited biodegradability. Microbial-assisted degradation of xenobiotic compounds is considered to be the most effective and beneficial approach. Microorganisms have remarkable catabolic potential, with genes, enzymes, and degradation pathways implicated in the process of biodegradation. A number of microbes, including *Alcaligenes*, *Cellulosimicrobium*, *Microbacterium*, *Micrococcus*, *Methanospirillum*, *Aeromonas*, *Sphingobium*, *Flavobacterium*, *Rhodococcus*, *Aspergillus*, *Penecillium*, *Trichoderma*, *Streptomyces*, *Rhodotorula*, *Candida*, and *Aureobasidium*, have been isolated and characterized, and have shown exceptional biodegradation potential for a variety of xenobiotic contaminants from soil/water environments. Microorganisms potentially utilize xenobiotic contaminants as carbon or nitrogen sources to sustain their growth and metabolic activities. Diverse microbial populations survive in harsh contaminated environments, exhibiting a significant biodegradation potential to degrade and transform pollutants. However, the study of such microbial populations requires a more advanced and multifaceted approach. Currently, multiple advanced approaches, including metagenomics, proteomics, transcriptomics, and metabolomics, are successfully employed for the characterization of pollutant-degrading microorganisms, their metabolic machinery, novel proteins, and catabolic genes involved in the degradation process. These technologies are highly sophisticated, and efficient for obtaining information about the genetic diversity and community structures of microorganisms. Advanced molecular technologies used for the characterization of complex microbial communities give an in-depth understanding of their structural and functional aspects, and help to resolve

issues related to the biodegradation potential of microorganisms. This review article discusses the biodegradation potential of microorganisms and provides insights into recent advances and omics approaches employed for the specific characterization of xenobiotic-degrading microorganisms from contaminated environments.

Keywords: bioremediation, microorganisms, xenobiotics, omics, bioinformatics

INTRODUCTION

The environment is everything that naturally surrounds us and affects our daily lives on Earth. A safe and healthy environment is essential for the existence of life on this planet. However, in the era of advanced industrialization and urbanization, various anthropogenic activities are largely responsible for the introduction of toxic and hazardous pollutants such as environmental xenobiotics (Embrandiri et al., 2016; Malla et al., 2018; Bhatt et al., 2020a; Rodriguez et al., 2020). Xenobiotics are chemical substances not naturally produced or expected to be present within organisms. The term "xenobiotic" is usually used in the context of environmental pollutants to refer to synthetic compounds produced in large volumes for industrial, agricultural, and domestic use (Embrandiri et al., 2016; Atashgahi et al., 2018; Dinka, 2018). There is growing public concern over the wide range of xenobiotic compounds being introduced, deliberately or accidentally, into the environment, which involves high potential risk to humans and animals (Jacob and Cherian, 2013; Hashmi et al., 2017; Zhu et al., 2017; Dinka, 2018). Environmental xenobiotics include pesticides, polycyclic aromatic hydrocarbons (PAHs), pharmaceutical active compounds (PhACs), personal-care products (PCPs), phenolics, chlorinated compounds, and other industrial chemicals. Their increasing frequency in different environmental compartments has raised concerns about their potential adverse effects (Crinnion, 2010; Kim et al., 2013; Embrandiri et al., 2016; Tsaboula et al., 2016; Dhakal et al., 2017). Their toxicity results in unprecedented health hazards and risks to environmental safety and security (Godheja et al., 2016; Dovrak et al., 2017; Burgos-Aceves et al., 2018; Ravindra and Haq, 2019). Once xenobiotics are released into the environment, they can bioaccumulate within the food chain due to their high affinity toward organic substances, and produce toxic adverse effects toward natural ecosystems, humans, and animals (Pedersen et al., 2003; Iovdijova and Bencko, 2010; Maurya, 2016). Consequently, they can cause severe chronic effects such as respiratory tract infections, damage to the immune system, pulmonary bronchitis, dysfunction of the nervous system, disruption of the endocrine system, behavioral and developmental disorders, and carcinogenic and mutagenic effects (Sajid et al., 2015; Zhu et al., 2017; Dinka, 2018; Catron et al., 2019; Mishra et al., 2019; Bertotto et al., 2020). Thus, xenobiotic contamination represents a persistent anthropogenic threat and raises serious environmental concerns. Various physical and chemical treatment methods such as coagulation, filtration, adsorption, chemical precipitation, electrolysis ozonation, etc. have been used for the degradation and detoxification of such xenobiotic compounds, but not all these methods are very useful due to their high cost, waste-disposal problem and generation of toxic by-products that are sometimes more hazardous than the parent compound. In contrast, the biological remediation method, "bioremediation," is a widely accepted clean-up strategy for the degradation of xenobiotics from contaminated environments without producing harmful products (Paul et al., 2005; Perelo, 2010). Bioremediation involves the metabolic capabilities of microorganisms in the removal of pollutants and thus, is the most suitable and promising technology these days (Gilliespie and Philp, 2013; Azubuike et al., 2016).

Microbial remediation of xenobiotic compounds is regarded as a superficial, proficient, economically feasible approach that uses a wide range of microorganisms to consume organic pollutants as carbon or nitrogen supplements to sustain their developmental activities (Chen et al., 2013; Mahmoud, 2016; Arora et al., 2018; Ortiz-Hernandez et al., 2018; Siles and Margesin, 2018; Zhan et al., 2018; Bhatt et al., 2020b). Microorganisms are ubiquitous in nature, and diverse microbial communities thrive in natural and extreme stress environments, including soil, water, the human gut, hydrothermal vents, acid mine runoff, and oil reservoirs (Cycoń and Piotrowska-Seget, 2016; Jalowiecki et al., 2016; Ding et al., 2017; Aguinga et al., 2018; Wang Y. F. et al., 2018; Zierer et al., 2018; Delegan et al., 2019; Arora, 2020; Shekhar et al., 2020). Microbial populations exhibit potential for the remediation of any contaminated environment because of their genetic diversity and functionality (Chen et al., 2015; Bastida et al., 2016; Bhatt and Barh, 2018; Dangi et al., 2018). Therefore, the study of microbial population existing in contaminated environments provides a significant knowledge of specific microbial characteristics that improve degradation rates. However, effective implementation of microbial remediation strategies needs advanced technical approaches, which provide an in-depth understanding about the dynamics aspects of microbial activity and survival under stressed environment (Rastogi and Sani, 2011; Lima-Morales et al., 2016; Mao et al., 2019). The development in molecular, biotechnological, bioinformatics and system biology tools pertaining to bio-remedial problems have provided gene level mechanisms of bioremediation (Ahmad and Ahmad, 2014; Aora and Bar, 2014; Singh D. P. et al., 2018; Jaiswal and Shukla, 2020; Nkongolo and Kotha, 2020; Wolf et al., 2020). Moreover, the direct study of microorganisms in a contaminated environment including the whole microbial population granted a new frontier of the scientific community to share the knowledge of the uncultured microbial world (Zepeda et al., 2015; Zhao Q. et al., 2017; Panigrahi et al., 2019; Yan et al., 2020).

The development of advance molecular tools and a better understanding of microbial metabolic and genetic structures and functions have accelerated encroachment in recombinant engineering techniques to enhanced bioremediation for removal of environmental pollutants (Ram et al., 2005; Temperton and Giovannoni, 2012; Singh V. et al., 2018; Stein et al., 2018; Delegan et al., 2019; Marco and Abram, 2019; Puckett et al., 2020). Soil is the most dynamic environment for the enormous microbial population of immense diversity. It has been estimated that one gram of soil approximately contains 10^9 bacterial cells, but only <1% of these may be culturable in the laboratory (Rossello-Mora and Amann, 2001). Culture based identification of diverse microbial population in a contaminated environment, is a challenging task, which is limited to fast-growing microbial diversity (Gillbride et al., 2006). Thus, modern culture-independent molecular techniques represent a feasible approach to unrevealing the diversity and functional dynamics of microbial population in contaminated environments. Moreover, the advanced innovation in molecular tools and techniques provides new insights and changes the traditional research trend in the field of bioremediation (Malik et al., 2008; Shah et al., 2011; Devarapalli and Kumavnath, 2015; Mahmoud, 2016; Biswas and Sarkar, 2018; Shakya et al., 2019). Omics technologies are the result of advanced molecular techniques, which involved direct characterization of genome structure of microorganisms, devoid of their culture sample (Segata et al., 2013; Biswas and Sarkar, 2018; Jaiswal et al., 2019; Yu K. et al., 2019). Therefore, the applications of modern molecular techniques like metagenomic, transcriptomic, proteomic generates relevant information on genes and proteins expression levels in whole microbial communities under contaminated environments attempted to unravel the mechanism of microbial degradation and successful execution of bioremediation (Keller and Hettich, 2009; Yang, 2013; Malla et al., 2018; Bharagava et al., 2019; Marco and Abram, 2019; Rodriguez et al., 2020). These methods are comparatively efficient, quicker, and accurate, which overcome the limitations of conventional molecular techniques. It explores the advanced microbial degradation mechanism of xenobiotics, their metabolic activities, genetic regulation and molecular-biology aspects (Cycoń et al., 2017; Gutierrez et al., 2018; Gutleben et al., 2018; Mishra et al., 2020). Hence, this review article highlights the biodegradation potential of microorganisms and provides insights into recent advance methods of "omics" technologies employed in microbial degradation and remediation purpose of xenobiotics and their perspectives in modern biological research.

BIOREMEDIATION POTENTIAL OF MICROORGANISMS FOR XENOBIOTIC COMPOUNDS

The application of microorganisms in removing xenobiotics from soil, water or sediments through complete transformation or mineralization into harmless end products like CO_2 and H_2O is a basic concept of bioremediation strategy (Ortiz et al., 2013; Singh et al., 2016). Different microorganisms including bacteria (*Pseudomonas*, *Alcaligenes*, *Cellulosimicrobium*, *Microbacterium*, *Micrococcus*, *Methanospirillum*, *Aeromonas*, *Bacillus*, *Sphingobium*, *Flavobacterium,* and *Rhodococcus*), fungi (*Aspergillus*, *Penecillium*, *Trichoderma*, and *Fusarium*), and yeasts (*Pichia*, *Rhodotorula*, *Candida*, *Aureobasidium*, and *Exophiala*) have been reported to be involved in the efficient biodegradation of xenobiotic compounds from contaminated soil/water environments, due to their exceptional bioremediation potential (Sathishkumar et al., 2008; Nzila, 2013; Sunita et al., 2013; Zhao Q. et al., 2017; Bharadwaj, 2018; Yang J. et al., 2018; Yang T. et al., 2018; Yu Y. et al., 2019; Bhatt et al., 2020c). The biodegradation ability of microbes is greatly influenced by interactive ecological factors including soil, salinity, temperature, carbon source, moisture content, pH, nitrogen sources, inoculums concentration, etc. (Megharaj and Naidu, 2010; Wu et al., 2014; Bhatt et al., 2019). Microorganisms harbor remarkable catabolic potential, genes, enzymes, and degradation pathways implicated in the process of bioremediation, which might be responsible in the evolution of novel traits and characters (Widada et al., 2002; Scholer et al., 2017; Yan et al., 2018; Zhu et al., 2020). Moreover, microbial plasmids believed to be responsible for the continuous progression, evolution, and distribution of novel biodegradable genes/enzymes (Zhang et al., 2016; Jeffries et al., 2019). These novel genes/enzymes have endowed microorganism's biodegradation capability to remove or detoxify a wide variety of environmental pollutants due to their inheritance horizontal gene transfer property (Singh V. et al., 2018; Jaiswal et al., 2019; Li et al., 2019; Phale et al., 2019; French et al., 2020). The microbial remediation process can be further improved via successful application of genome editing and biochemical techniques that modify existing strains and result in the development of a genetically modified organism capable of simultaneously degrading several xenobiotics (Shanker et al., 2011; Zhang et al., 2016; Hussain et al., 2018; Janssen and Stucki, 2020). The advancement of genetic manipulation technology gives more clear information and explores future prospects of bioremediation of xenobiotics through highly proficient microorganisms (Sayler and Ripp, 2000; Shapiro et al., 2018; Wong, 2018; Liu et al., 2019). The chemical structures of several xenobiotic compounds are presented in **Figure 1**.

Synthetic pesticides are the major example of xenobiotics especially the organochlorine pesticides (OCPs) that used extensively worldwide for a long period of time in agriculture as well as in insect control program. Several OCPs such as aldrin, dieldrin, dichloro diphenyl trichloro ethane (DDT), benzene hexachloride (BHC), and hexachlorocyclohexane are highly toxic in nature due to their stability and bioaccumulative property (Aktar et al., 2009; Jayaraj et al., 2016; Awasthi and Awasthi, 2019; Pang et al., 2020). Lindane (γ-hexachlorocyclohexane) is one of the highly toxic organochlorine xenobiotic compound, which is well-studied for its microbial biodegradation through physical (soil structure, carbon and oxygen gradients, pH, and temperature) and chemical (dechlorination, dehydroxylation, and dehydrogenation) interactions (Kaur and Kaur, 2016; Bashir et al., 2018; Zhang et al., 2021). The increasing concentration of lindane residues into the environment imposes severe health hazards such as carcinogenicity, mutagenicity, endocrine disruption, and immune-suppression diseases into the humans and other organisms (Cuozzo et al., 2017; Zhang et al.,

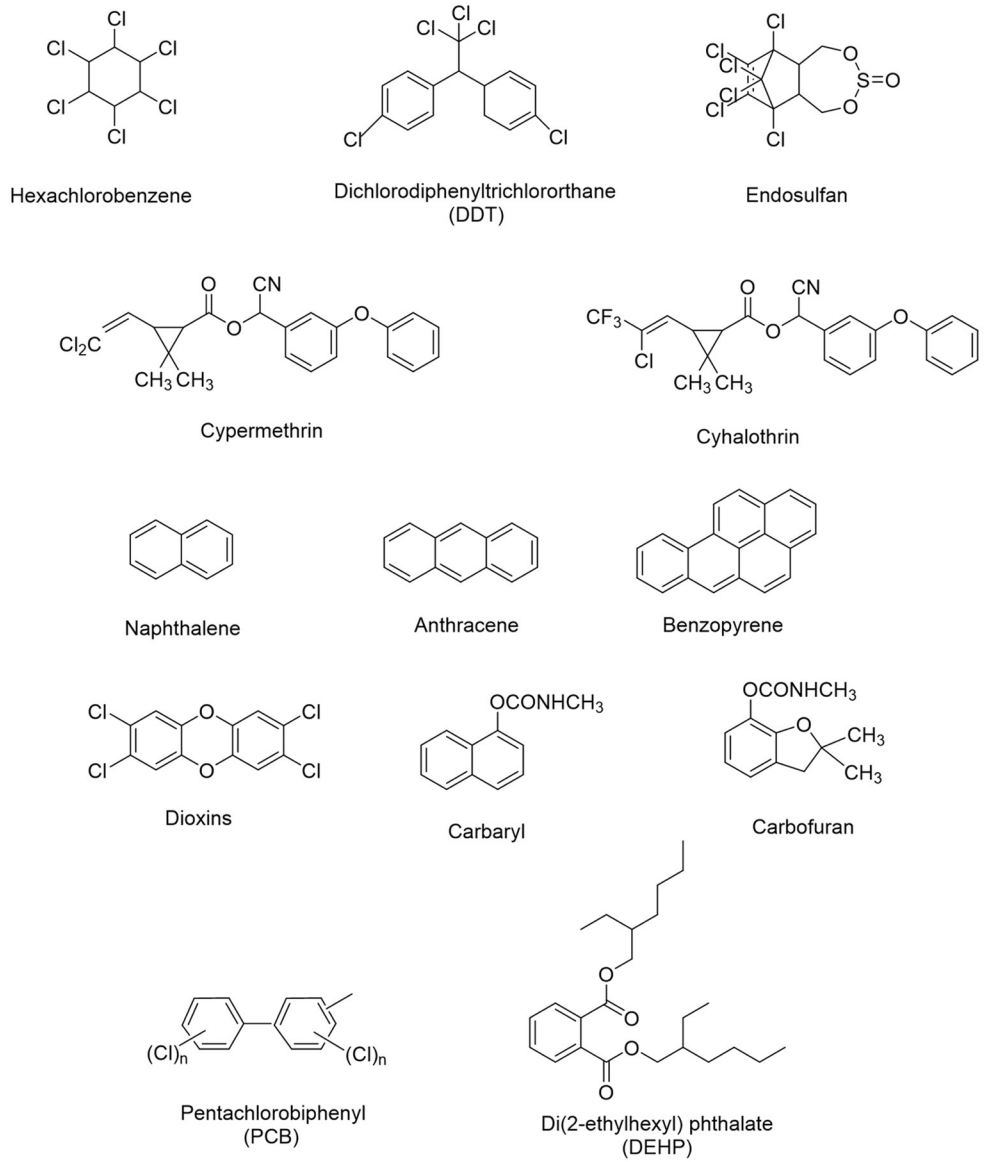

FIGURE 1 | Chemical structures of several xenobiotic compounds.

2020). Bacterial strains genera such as *Bacillus, Burkholderia*, *Pseudomonas*, *Kocuria*, *Archromobacter*, *Sphingomonas*, *Chromohalobacter*, demonstrated lindane biodegradation under axenic as well as anoxic conditions via dehydrogenation, dehydrochlorination, and hydroxylation, results in complete degradation or mineralization (Giri et al., 2014; Cuozzo et al., 2017; Wang W. et al., 2018; Nagata et al., 2019; Zhang et al., 2020). A simplified catabolic pathway of lindane is presented in **Figure 2**.

Pyrethroids are broad spectrum pesticide mainly used against agricultural and household pests. Cypermethrin, cyhalothrin, deltamethrin, cyfluthrin, bifenthrin are the common example of synthetic pyrethroids (Chen et al., 2012, 2014; Bhatt et al., 2019; Zhan et al., 2020). These pesticides are highly toxic and persistent and can cause molecular toxicity, neurotoxicity, and reproductive toxicity (Sharma et al., 2018; Bhatt et al., 2019; Gammon et al., 2019). One of the pyrethroid i.e., cypermethrin can cross the blood-brain barrier and induces neurotoxicity and motor deficits (Singh et al., 2012). The persistence of these pesticides in the environment poses a severe threat to humans and other non-target terrestrial and aquatic organisms (Burns and Pastoor, 2018; Ullah et al., 2018; Lu et al., 2019). Microbial strains such as *Acinetobacter*, *Trichoderma*, *Roultella*, *Pseudomonas*, *Cunninghamella*, and *Bacillus* have been reported for their efficient degradation of broad spectrum pesticides like cypermethrin and other pyrethroid pesticides through pyrethroid hydrolases (Cycoń and Piotrowska-Seget, 2016; Zhan et al., 2018; Bhatt et al., 2019; Chen and Zhan, 2019).

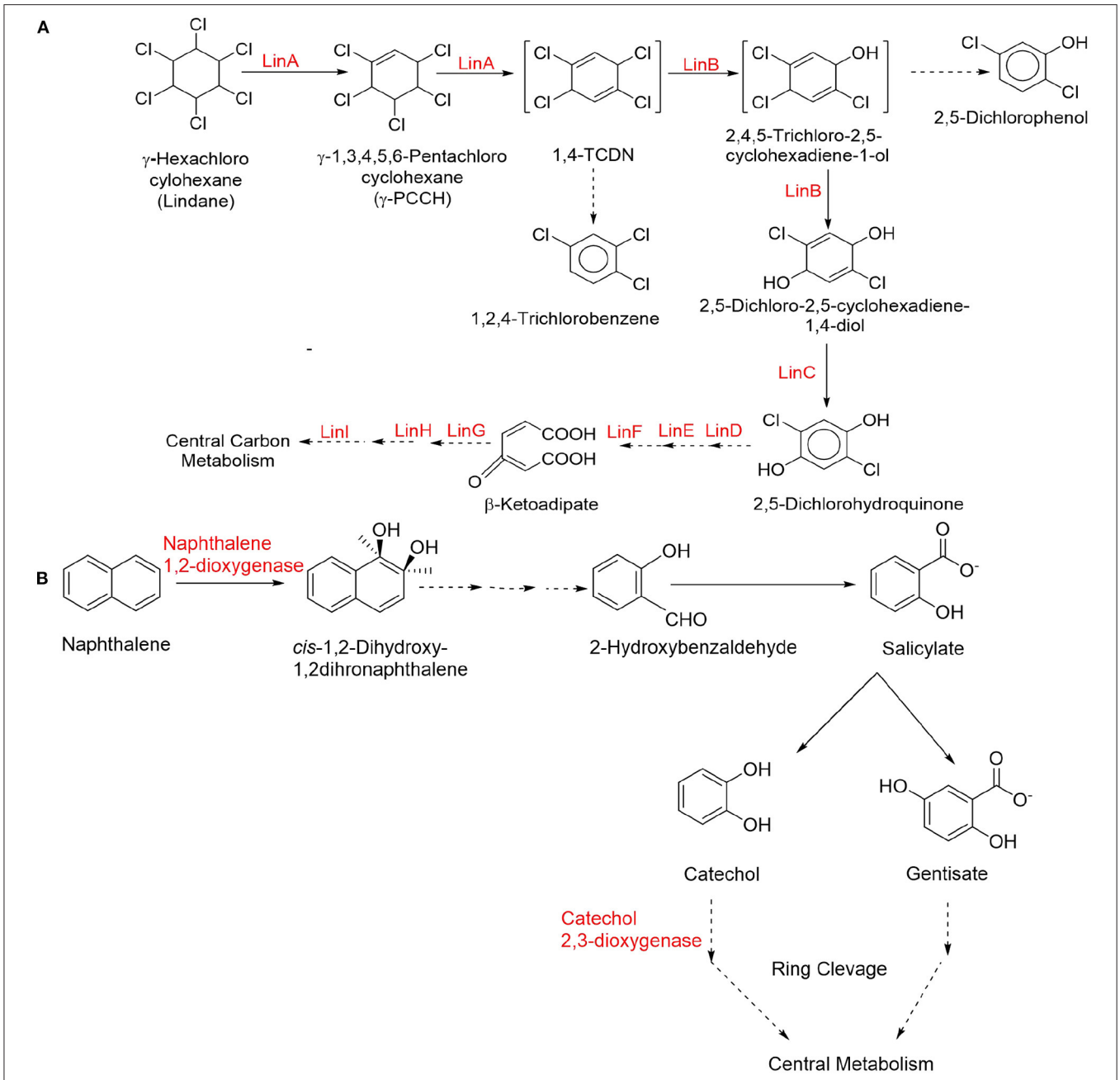

FIGURE 2 | Simplified catabolic degradation pathways of organochlorine and polycyclic aromatic hydrocarbon (PAH). Compounds **(A)** lindane (organochlorine) (Endo et al., 2005; Geueke et al., 2013) and **(B)** naphthalene (PAH) (Kiyohara et al., 1994; Dutta et al., 1998; Bamforth and Singleton, 2005; Baboshin et al., 2008).

Co-metabolism exhibiting strains include *Flavobacterium*, *Sphingomonas*, *Arthrobacter*, *Azotobacter*, *Achromobacter*, *Microbacterium*, *Brevibacterium*, *Rhodococcus*, *Trichoderma*, and *Aspergillus* demonstrated their pollutant degradation capability, exclusive of pollutant consumption as an energy resource (Nzila, 2013).

Polychlorinated bis-phenyles (PCBs) are classified as persistent organic pollutants with high toxicity (Lallas, 2001; Pathiraja et al., 2019). They have been linked to chronic effects in humans including immune system damage, decreased pulmonary function, bronchitis, and interference with hormones leading to cancer (Schecter et al., 2006). Microbial degradation of polychlorinated bis-phenyles (PCBs) is a promising remediation technology involving two major pathways: aerobic degradation and anaerobic dehalogination (Abraham et al., 2002; Pathiraja et al., 2019). Bacterial strains *Pseudomonas*, *Rhosocossus*, *Comamonas*, *Burkholderia*, and *Bacillus* have been characterized for the oxidative degradation of PCBs through ring cleavage resulting into a common by product of chlorbenzoic acid (Anyasi and Atagana, 2011; Jing et al., 2018).

Polycyclic aromatic hydrocarbons (PAHs) are potent environmental contaminants and xenobiotics that are widely distributed in the environment due to the incomplete combustion of organic matter. PAHs have moderate to high acute toxicity to aquatic life and birds (Abdel-Shafy and Mansour, 2016; Pandey et al., 2017). Some PAHs such as Anthracene, benzo(a)pyrene, phenanthrene, and naphthalene are well-known to produce harmful biological effects such as genotoxicity, mutagenicity, and carcinogenicity and therefore pose a serious threat to the human health (Kim et al., 2013; Lin et al., 2020). Microorganisms belonging to *Sphingomonas*, *Sphingobium*, and *Novosphingobium* have been found as efficient degrader of PAHs (Lee et al., 2016b; Fida et al., 2017; Auti et al., 2019). *Rhodococcus*, *Cunninghamella*, *Pleurotus ostreatus*, *Oscillatoria*, *Agmenellum quadriplicatum*, *Brevibacterium*, and *Nocardiodes* have been widely shown to metabolize particularly naphthalene and phenanthrene (Ghosal et al., 2016; Siles and Margesin, 2018). Ortega-Gonzalez et al. (2015) demonstrated that *Amycolaptosis* sp. Poz14 degraded 100% of naphthalene and 37.87% of anthracene within 45 days. A PAH-degrading marine bacterium *Cycloclasticus* sp. has been isolated from sea sediments capable to breakdown xenobiotic naphthalene, pyrene, phenanthrene, and other aromatic hydrocarbons into their supplementary products through enzymatic pathways (Wang W. et al., 2018). A simplified catabolic pathway of naphthalene is presented in **Figure 2**.

Phthalates or esters of phthalic acids are synthetic xenobiotic chemicals, which are extensively used as plasticizers added to polyvinyl chloride to improve its flexibility and hardness (Crinnion, 2010; Singh and Li, 2011; Przybylinska and Wyszkowski, 2016). Phthalates are readily released into the environment and create a risk of exposure for humans and other living organisms due their endocrine disrupting behavior (Przybylinska and Wyszkowski, 2016). They cause infertility, reproductive and developmental toxicity in humans and animals (Singh and Li, 2011; Przybylinska and Wyszkowski, 2016). Aerobic and anaerobic microbial degradation of xenobiotic phthalic acid, and isophthalaic acid considered as the most effective means of their removal from the environment (Junghare et al., 2019; Boll et al., 2020). Di-2(ethyehxyl) phthalate (DEHP) is the most common member of phthalates, which are extensively used as plasticizer in plastics and disposable medical materials (Singh and Li, 2011). DEHP is best known as an endocrine disrupter and can produce neural, heptotoxic, cardiotoxic, and carcinogenic effects on humans and animals (Rowdhwal and Chen, 2018). *Arthrobacter*, *Pseudomonas, Gordonia, Providencia, Acinetobacter, Microbacterium,* and *Rhodococuus* have been identified as efficient DEHP degrading bacteria (Nahurira et al., 2017; Yang T. et al., 2018).

In addition, the microbial consortium gained greater attention in bioremediation rather than pure microbial monocultures. The consortia cultures are better equipped in terms of metabolic and pollutant removal capability owing to their constant revelation of contaminant and promising mutual relationship with other available strains (Patowary et al., 2016). Interestingly, microbial consortia can alleviate the metabolic limitations of single microbial culture and enhance biodegradation process by their miscellaneous assemblage of bacterial populations employed with extensive degradation potential (Zafra et al., 2016; Li et al., 2020).

RECENT ADVANCED TECHNOLOGIES EMPLOYED IN BIOREMEDIATION FOR IDENTIFICATION AND CHARACTERIZATION OF MICROORGANISMS AND MICROBIAL COMMUNITIES

There are several revolutionary advanced molecular practices, including genomics, metagenomics, proteomics, transcriptomics, and metabolomics, which deliver deeper insights into microbial activities with respect to their genes, proteins, mRNA expression levels, enzymes and metabolic pathways with changing environments (**Figure 1**). The integrated approach of these multiple technologies in the field of bioremediation is termed as the "omics approach," used for the undeviating characterization of biological macromolecules, and their specific genetic and molecular structures and function mechanisms in a set of microorganisms/microbial communities (Desai et al., 2009; Yang, 2013; Godheja et al., 2014; Franzosa et al., 2015; Chandran et al., 2020). The application of omics technologies provides comprehensive insights into microbial populations, their mechanisms of interaction with pollutants, metabolic activities, and genetic-regulation and molecular-biology aspects (Akinsanya et al., 2015; Kaul et al., 2016; Misra et al., 2018; Marco and Abram, 2019; Huang et al., 2021). Moreover, these approaches can broaden our knowledge of the so-called "viable but non-culturable (VBNC)" bacteria and their potentially novel pathways for degrading environmental pollutants (Oliver, 2010; Bodor et al., 2020). It is believed that these uncultured bacteria may play an important role in the biodegradation of environmental pollutant. However, little is known about the VBNC bacteria as these bacteria cannot be cultivated on conventional media and are very different from cells (Su et al., 2013). VBNC cells exhibit metabolic and respiratory activities and may perform transcription and gene expression, which allows them to recover culturability (Oliver et al., 2005; Zhao X. et al., 2017). The VBNC bacteria could be resuscitated in favorable conditions by an autoinducer (AI-2) (Bari et al., 2013) or resuscitated promoting factor (Rpf) (Li et al., 2015). The VBNC bacterial cells are detected on the basis of their viability. Several culture-independent molecular-based methods such as denaturing and temperature gradient gel electrophoresis (DGGE/TGGE), fluorescent *in situ* hybridization (FISH), terminal restriction fragment length polymorphism (T-RFLP), fatty acid methyl ester (FAME), and next-generation sequencing (NGS) technology are used for obtaining important information about the structural composition and genetic diversity of unculturable microorganisms (Zhao X. et al., 2017; Bodor et al., 2020). Selective gene amplification is an emerging approach to detect viable cells. Zhong et al. (2016) developed a real-time fluorescence LAMP technique combined with PMA (propidium monoazide), a high-affinity photolysis DNA nucleic acid dye

applied for the detection of VBNC *V. parahaemolyticus*. Thus, the combined use of these advanced molecular technologies with bioinformatic approaches increases understanding and brings in a new era of unrevealed soil microbial communities, as well as their associated mechanisms of biodegradation for their future applications in bioremediation (**Figure 3**; Mocalli and Benedetti, 2010; Kumar et al., 2016; Dangi et al., 2018; Pandey et al., 2019; Pinu et al., 2019).

Genomics and Metagenomics

Genomics and metagenomics are powerful tools for analyzing microbial communities at the genomic level from various contaminated environments. This technology gives a new array to environmental microbiologists for understanding unculturable microbiota with a genetic variability of microbial communities (Devarapalli and Kumavnath, 2015; Zhu et al., 2018; Awasthi et al., 2020). It gives more details about the particular degradation potential of microbial communities, as it directly entails the whole-genome sequence from environmental samples (**Table 1**). Metagenomic studies unblock traditional ways of uncultured microorganisms and explore their genetic advantage in the process of bioremediation (Rahimi et al., 2018; Nascimento et al., 2020). Complete genome-sequence data of some important microbial strains, including *Pseudomonas aeruginosa* KT2440, *Shewanella oneidensis* MR-1, *Deinococcus indicus* R1, and *Dehalococcoides mccartyi* WBC-2 have already been given, which is pertinent to successful bioremediation (http://www.tigr.org). The new genes can tell too much about the degradation capability and substrate specificity.

Current metagenomic practices allowed for identifying the whole-genome structure of microorganisms and specifying particular genes that are attributed to encode degradative enzymes for the mineralization of xenobiotics (Zafra et al., 2016; Zhu et al., 2020). Thus, metagenomics clearly highlights the crucial role of novel genes in connecting the entire microbial population with functional diversity and structural identity. Metagenomics involves the manufacturing of metagenomic libraries that include (I) production of the proper size of DNA fragments, and ligation of these fragments into a suitable cloning vector; (II) further recombinant vectors introduced into an appropriate bacterium cloning host; (III) clones that harbor specific characters, functions, or sequences were screened for libraries (**Figure 3**). Moreover, the screening of metagenomic libraries can be performed by two processes, i.e., sequence-driven analysis using high-throughput sequencing, and functional analysis using phenotypic expressions (Handelsman, 2004). However, recent sequence-based metagenome analyses (such as SOLiD system of Applied Biosystems, Roche 454 sequencing) are performed without the construction of cloned libraries (Kumar et al., 2020).

Function-driven metagenomics is a potent method for studying the functional aspect of genes. It is widely used for discovering novel genes with desired functions or exploring the sequence diversity of protein families (Taupp et al., 2011; Lam et al., 2015). A function-driven analysis involves the construction and screening of metagenomic libraries to identify novel enzymes (Chakraborthy and Das, 2016; Kumar et al., 2020). Using functional metagenomics, many novel antibiotic-resistant genes were identified from environmental sources (Ngara and Zhang, 2018). The majority of metagenome-derived hydrolytic enzymes, mainly esterases and glycoside hydrolases, have been characterized biochemically and mainly originated from functional metagenomics (Steele et al., 2009; Taupp et al., 2011). A novel functional screening method, a metagenome extract thin layer chromatography (META) system, was developed by Rabausch et al. (2013) for the rapid detection of glycosyltransferase (GT) and other flavonoid-modifying enzymes from metagenomic clone libraries. It involves the screening of 38,000 clones from two different metagenomic libraries and allowed for the identification of two novel UDP glycosyltransferase (UGT) genes. Bouhajja et al. (2017) utilized function-based screening of metagenomic libraries to explore the diversity of genes and microorganisms involved in the monooxygenase-mediated toluene degradation in a hydrocarbon-polluted sediment sample.

Metagenomic approaches offer broad and reliable microbial identification on the species and strain levels, but they are much costlier methods, and the most challenging part is data investigation, which requires all short DNA sequences to pair together to assemble the final genome structure (Bragg and Tyson, 2014). The involvement of indigenous soil microorganisms in the degradation of PAHs was undertaken by Zafra et al. (2016) through a metagenomic approach. This study demonstrated the biodegradation efficiency of microbial consortia with their degradative enzymes and metabolites generated during the remediation process. The microbial-community dynamics of refined- and crude-petroleum-contaminated soil by next-generation sequencing (NGS), and their capability to degrade hydrocarbons and plant-growth-promotion potential through *in-silico* analysis was investigated by Auti et al. (2019). In this study, 16S rRNA amplicon sequencing on the Illumina MiSeq platform and PICRUSt revealed that both types of soil contained microbial communities with excellent metabolic potential for petroleum hydrocarbon (PHC) degradation. Using KEGG orthology, the abundance of functional genes involved in hydrocarbon degradation showed the presence of 61 enzyme-encoding genes, such as alkane monooxygenase, alcohol dehydrogenase, and aldehyde dehydrogenase (Auti et al., 2019). 16S rDNA or 16S rRNA gene sequencing has led to evolutionary insights into the phylogenetic and taxonomic identification of microorganisms. The 16S rRNA gene consists of several highly conserved regions interleaved with variable regions in all microorganisms and thus is highly suited as a target gene for sequencing DNA (Fuks et al., 2018; Gursoy and Can, 2019). The bacterial 16S rRNA gene generally contains nine "hypervaiable regions" that demonstrate the considerable sequence diversity of bacterial species and can be used for species identification. The 16 S rRNA gene sequence similarity between two strains provides a simple yet robust criterion for the identification of newly isolated strains, whereas phylogenetic analyses can be used to elucidate the overall evolutionary relationship between related taxa (Johnson et al., 2019). Thus, 16S rRNA gene sequencing analysis is a highly recommended cost-effective technique for the phylogenetic

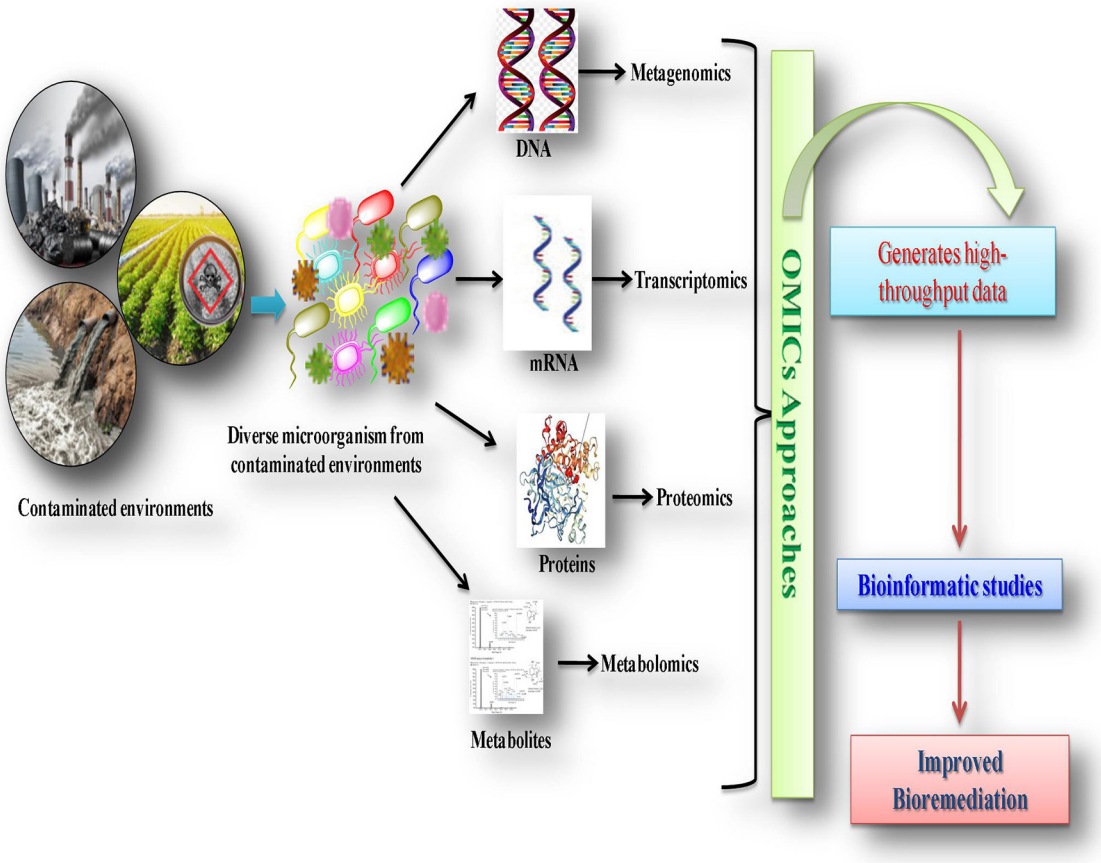

FIGURE 3 | Graphical presentation of integrated approach of advanced technologies in biodegradation of xenobiotic compounds.

resolution and taxonomic profiling of microbial communities (Auti et al., 2019).

The NGS approach has completely changed microbial-community analysis, as it provides comparative details in terms of temporal and spatial data (Hidalgo et al., 2020). There are several NGS technologies, including Illumina, Ion Torrent, SOLiD, and 454 (Caporaso et al., 2012; Liu et al., 2012; Knief, 2014; Salipante et al., 2014; Machado et al., 2019). These are high-throughput sequencing techniques of ribosomal genes that quantify community structures and functions at a higher resolution, e.g., 16S rRNA in prokaryotes, and 5S or 18S rRNA genes, or the internal-transcribe-spacer (ITS) region in eukaryotes (Luo et al., 2012). The effectiveness of such NGS technologies in analyzing microbial communities from diverse environments was elucidated, validated, and documented in many studies (Brown et al., 2013; Shokralla et al., 2014; Zhou et al., 2015; Niu et al., 2016; Scholer et al., 2017). In addition, PacBio (Pacific Biosciences) and Oxford Nanopore are highly advanced, reliable, and accurate third-generation sequencing platforms applied to microbial community analysis (Lu et al., 2016; Chandran et al., 2020). Oxford Nanopore Technologies has launched a portable MinION USB nanopore that does not rely on DNA replication and has the advantage of reading full-length molecules in real time. PacBio RS II, the first commercialized genomic sequencer, developed by Pacific Biosciences, uses single-molecule, real-time (SMRT) technology and is able to sequence single DNA molecules in real time without PCR amplification (Wagner et al., 2016; Nakano et al., 2017). The complete genome sequence of atrazine-degrading *Arthrobacter* sp. ZXY-2 (Zhao X. et al., 2017) and organophosphate-degrading *Sphingobium fuliginis* ATCC 27552 (Azam et al., 2019) was analyzed using the PacBio RSII sequencing platform to gain more insight into the genetic basis and unravel its degradation potential.

Jeffries et al. (2019) performed functional metagenomic studies in pesticide-contaminated soil to explore the degradation rates of organophosphorus xenobiotic compounds. Their study demonstrated that two distinct soil groups had different functional and taxonomic profiles, and predicted biodegradation potential in rapidly and slowly degrading soil clusters. *Burkholderia*, *Acidomicrobium*, *Koribacter*, and *Bradyrhizobium* were most abundantly present in rapidly degrading clusters, whereas *Singulisphaera*, *Solibacter*, and *Desulfomonile* were in slowly degrading clusters. The degradation assays of organophosphorus also suggested that slow-degradation clusters had significantly higher abundances of virulence genes and metabolic pathways for fatty acids and carbohydrates. In contrast,

TABLE 1 | Microorganisms and microbial communities using genomic and metagenomic approaches in biodegradation.

S. no.	Microorganisms/ microbial communities	Isolation source	Xenobiotics/ pollutants	Comments/result	References
1.	*Sphingomonas and Sphingobium*	Soil	Aromatic hydrocarbons	Whole-genome sequence gives insights into the presence of *bph* and *xyl* gene clusters in six bacterial strains for degradation of aromatic hydrocarbon and other xenobiotics.	Zhao Q. et al., 2017
2.	*Gordonia* sp. 1D	Oil-refinery soil	Naphthalene	Genomic analysis of *Gordonia* sp. 1D showed that it contains gene clusters of alkaline hydroxylase, genes of dibenzothiophene and naphthalene metabolism intermediates, which are involved in the degradation of naphthalene and other environmental pollutants.	Delegan et al., 2019
3.	*Bacillus megaterium* STB1	Contaminated soil	Xenobiotics	Genomic analysis of *Bacillus megaterium* STB1 demonstrated that it contains genes responsible for degradation of xenobiotics and developing stress-resistance mechanisms.	Nascimento et al., 2020
4.	*Cycloclasticus sp.* P1	Deep sea sediments	Polycyclic aromatic hydrocarbons (PAHs)	Genomic analysis of strain P1 revealed that six ring hydroxylating dioxygenase (RHD) enzyme were responsible for the degradation of PAH compounds like pyrene and phenanthrene.	Wang W. et al., 2018
5.	Microbial community	Hydrocarbon contaminated soil	Petroleum hydrocarbon	Gammaproteobacteria and Bacteroid classes within bacterial communities were found to be actively involved in total petroleum hydrocarbon (TPH) degradation.	Siles and Margesin, 2018
6.	Microbial community	Sugarcane farms, Australia	Organophosphates	Functional metagenomics explores the capability of different microbial profiles to predict the degradation of organophosphates in different soil types.	Jeffries et al., 2019
7.	Microbial communities	River sediments		Metagenomic study explored the correlation between diverse microbial communities and environmental factors, and their resistance mechanism under stress environments.	Yan et al., 2020
8.	Microbial community	Contaminated soil	Di (2-ethylhexyl) phthalate (DEHP)	Members of *Actinomycetales* seemed to be the dominant degraders of di (2-ethylhexyl) phthalate (DEHP) under aerobic conditions.	Zhu et al., 2020
9.	Mixed bacterial consortium	Polycyclic aromatic hydrocarbon (PAH)-contaminated soil	PAHs	Functional metagenomics demonstrated alteration on gene clusters, and predicted intermediary degradation pathway for the mineralization of PAH compounds.	Zafra et al., 2016
10.	Microbial community	Lindane-contaminated pond sediments	Lindane	Metagenomic analysis revealed that microbial populations such as Actinobacteria, Acidobacteria, Planctomycetes, and Proteobacteria, abundantly present in contaminated pond sediments, showed lindane-degradation capabilities.	Negi and Lal, 2017
11.	Microbial community	Jet-fuel-contaminated site	Toluene and benzene	Metagenomic analysis characterized degradation potential and metabolic pathway for toluene and benzene by members of *Geobacteraceae* and *Peptococcaceae* microbiota present in jet-fuel-contaminated site.	Hidalgo et al., 2020
12.	Microbial communities	Insecticide plant soil	Organochlorines	Metagenomic analysis revealed the response of microbial community composition and diversity to organochlorine pesticide (OCP)-contaminated sites and offered potential bioremediation strategies for the mitigation of OCP-contaminated sites.	Sun et al., 2019
13.	Microbial consortium	Contaminated soil	Biphenyl	Metagenomic analysis of a bacterial consortium revealed the metabolic role of strains belonging to the genera *Pseudomonas*, *Rhodococcus*, *Bordetella*, *Achromobacter,* and *Varivorex* in the bioremediation of biphenyl to benzoate and benzoate to the tricarboxylic acid cycle (TCA).	Garrido-Sanz et al., 2018

rapid-degradation clusters contain more abundant genes related to the transposable elements, membrane transport, and nutrient cycling of nitrogen and phosphorus enzymes potentially involved in phosphorus metabolism. Moreover, rapid-degradation soils also showed a higher abundance of genes encoding phosphodiesterase enzymes, which cleave phosphodiester bonds present in organophosphorus and play a major role in pesticide degradation. Overall, this study gives an overall framework of metagenomic approaches to predict the microbial degradation of xenobiotic organophosphorus compounds.

Metagenomic analysis of a complex community of lindane-contaminated pond sediment was conducted by Negi and Lal (2017) through comparative genomics. The results of this study revealed genomic variation present in pond sediment with degradative enzymes (hydrolases, isomerases, lyases, and oxidoreductases) involved in the biodegradation of

hexachlorocyclohexane and chlorobenzene (ko00361), and other xenobiotic compounds. *Cellulomonas*, *Micrococcus*, *Nocardioides*, *Kribbella*, *Isoptericola*, *Clavibacter*, *Gutenbergia*, *Streptomyces*, *Sanguibacter*, and *Kineococcus* were found to be the most dominating genera present in the aromatic-hydrocarbon-contaminated pond sediment. Genes involved in lindane metabolization, enriched with sequences for *linA* and *linB,* were also found in the pond-sediment metagenome.

Whole-metagenome sequencing of e-waste-contaminated microbial populations was conducted by Salam and Verma (2019), who demonstrated that the functional diversity and structural composition of microorganisms significantly changes due to the detrimental impact of e-waste. Denaturing gel gradient electrophoresis (DGGE) community-profiling results revealed that bacterial groups such as *Proteobacteria, Firmicutes*, *Bacteroidetes*, and *Chloroflexi* were decreased. Zhu et al. (2020) explored microbial assemblage and functional genes potentially involved in upstream and downstream phthalate degradation in soil via a metagenomic approach. Results indicated that bacterial taxon Actinobacteria (*Pimelobacter*, *Nocardioides*, *Gordonia*, *Nocardia*, *Rhodococcus*, and *Mycobacterium)* was a major degrader under aerobic conditions, and bacterial taxa Proteobacteria (*Ramlibacter* and *Burkholderia*), Acidobacteria, and Bacteroidetes were involved under anaerobic conditions. The members of *Geobacteraceae* and *Peptococcaceae* microbiota present in the jet-fuel-contaminated site could be exploited for their remarkable metabolic potential for the mitigation of toluene and benzene, as exposed by metagenomic analysis (Hidalgo et al., 2020).

Transcriptomics

Transcriptomics is a remarkable tool that addresses the division of genes transcribed in any organism known as the transcriptome. It provides functional insight links involving the genome, proteome, and cellular phenotype by studying their mRNA transcriptional profiles, directly extracted from individual microbes or microbial communities (Singh and Nagaraj, 2006; McGrath et al., 2008; Bashiardes et al., 2016). Significant changes were seen in gene-expression level and their regulation in microbial communities under stressful environments for their survival. Thus, transcriptomics and metatranscriptomics provide deep analysis of a genome wide range of differently expressed genes, either of the individual cell or the entire microbial community at a specific time (**Table 2**; Li et al., 2014; He et al., 2015; Shakya et al., 2019). RNA seq and DNA microarrays are significantly powerful technologies to determine the mRNA expression level of every gene (Diaz, 2004). GeoChip uses key enzymes or genes to spot various microbe-mediated mechanisms for biogeochemical cycles, resistance mechanism for heavy metals, and degradation pathways of xenobiotics (He et al., 2010; Xiong et al., 2010; Xie et al., 2011). Similarly, DNA- and RNA-SIP (Stable Isotope Probing) technologies are also valuable to uncover the microbial taxa and catabolic genes that are important for the bioremediation of polluted environments (Lueders, 2015).

Dual RNA-seq transcriptional profile is a better approach to understand the basic nature and mechanism of differently expressed genes in the host and symbiotic microbes at a time (Kaul et al., 2016). RNA seq allows for the detection of more differently expressed genes than a microarray alone does. Thus, recent advancements and developments in microarrays, RNA seq technology, transcriptomics, and metatranscriptomics revealed unexpected microbial diversity in aquatic and terrestrial environments with their synergistic relationships with humans, animals, plants, and other microorganisms (Perez-Losada et al., 2015; White et al., 2016; Moniruzzaman et al., 2017; Berg et al., 2018; Crump et al., 2018).

RNA seq technology is considered more efficient than traditional microarray platforms in gene expression profiling as it provides a wider quantitative range of expression level changes compared to microarrays (Roh et al., 2010; Shakya et al., 2019). The microarray technique requires a lot of effort and money to prepare custom-made microarrays. Furthermore, the target genes to be analyzed are limited in number and cannot cover the whole set of genes in the community. In contrast, a number of kits are now commercially available to carry out RNA-seq analysis, whereby a whole set of genes in the community can be quantitatively analyzed. Therefore, many studies are now performed by RNA-seq technology.

Lima-Morales et al. (2016) investigated the microbial organization and catabolic gene diversity of three types of contaminated soil under continuous long-term pollutant stress with benzene and benzene/toluene/ethylene/xylene (BTEX) to identify shifts in community structure and the prevalence of key genes for catabolic pathways. Moreover, *de novo* transcriptome synthesis gives new insights into and reveals basic information about non-model species without a genome reference. Hydrocarbon-degrading bacterium *Achromobacter* sp. was isolated from seawater, and indicated that the upregulation of enzymes such as dehydrogenases and monooxygenases, and novel genes associated with fatty acid metabolism is responsible for its enormous capability for hydrocarbon degradation and survival (Hong et al., 2016).

Metatranscriptomic analysis of the wheat rhizosphere identified dominant bacterial communities of diverse taxonomic phyla, including Acidobacteria, Cyanobacteria, Bacteroidetes, Steptophyta, Ascomycota, and Firmicutes, having functional roles in the degradation of various xenobiotic pollutants (Singh D. P. et al., 2018). Multiple enzymes such as isomerases, oxygenases, decarboxylases, reductases, kinases, and inner membrane translocators were identified that were associated with 21 different pathways for benzoates, aromatic amines, phenols, bisphenols, and other xenobiotics (Singh et al., 2016). An et al. (2020) elucidated the study of the transcriptome for the characterization of hexaconazole degrading strain *Sphingobacterium multivorum,* obtained from activated sludge. This strain was capable of degrading 85.6% hexaconazole in just 6 days and of generating three different metabolites, M1, M2, and M3, recognized as (2-(2,4-dichlorophenyl)-1-(1H-1,2,4-triazol-1-yl)hexane-2,5diol), (2-(2,4-dichlorophenyl) hexane-1,2-diol), and (1H-1,2,4-triazol), respectively. The results of transcriptome sequencing revealed the presence of 864 differential genes in which dehydrogenases, aldehydes, monooxygenases, and RND and AC transporters were upregulated. The M1

TABLE 2 | Microorganisms and microbial communities using transcriptomic and metatranscriptomic approaches in biodegradation.

S. no.	Microorganisms/ microbial communities	Isolation/ culture source	Xenobiotics/ pollutants	Comments/result	References
1.	*Dehalococcoides mccartyi* within trichloroethene-dechlorinating community	Dechlorinated enrichment culture	Trichloro-ethane (TCE)	Transcriptomic approach identified genes encoding for rRNA, and reductive dehalogenases *tceA* and *vcrA* as the most expressed genes for TCE-dechlorinating community, while in *D. mccartyi*, hydrogenases *hup* and *vhu* were identified.	Mao et al., 2019
2.	*Rhodococcus* sp. CS-1	Drinking-water treatment plant	Phenol	Transcriptomic analysis showed that *Rhodococcus* sp. CS-1 was capable of phenol degradation via ketoadipate pathway.	Gu et al., 2018
3.	*Rhodococcus erythropolis* D310-1	Activated-sludge sample	Chlorimuron-ethyl	RNA-seq results suggested that cyt P450 carboxylesterase and glycosyltransferase genes are key genes expressing degradation of chlorimuron-ethyl.	Cheng et al., 2018
4.	*Burkholderia zhejiangensis* CEIB S4-3	Pesticide-contaminated soil	Methyl parathione	Transcriptomic analysis of CEIB S4-3 strain showed transcriptional changes occurred in response to methyl parathion, and identified expressed genes related to its biodegradation.	Castrejon-Godinez et al., 2019
5.	*Sphingomonas haloaromaticamans* P3.	Wastewater disposal site soil	Polyphenol	Transcriptomics analysis of strain P3 revealed expression patterns of catabolic genes of *ortho*-phenyl phenol degradation pathway.	Perruchon et al., 2017
6.	*Novosphingobium* sp. LH128	Contaminated soil	Phenanthrene	Transcriptomics analysis showed remarkably higher expression of phenanthrene degradation.	Fida et al., 2017
7.	*Novosphingobium resinovorum* strain SA1	Contaminated soil	Sulphanilic acid	Transcriptomic analysis showed that the strain SA1 was capable to degrade suphanilic acid into sulfonated aromatic compounds.	Hegedus et al., 2018
8.	*Pseudomonas putida* KT2440	–	2,4,6-trinitrotoluene (TNT)	Transcriptomic analysis revealed that strain KT2440 showed a high level resistance to TNT. Significant expression level changes were observes in 65 genes. Of these 39 genes were upregulated and 26 were downregulated. Detoxification related enzymes and genes encoding nitroreductases (*pnrA, xenD,* and *acpD)* were induced in response to TNT detoxification.	Farnandez et al., 2009
9.	*Cycloclasticus* sp. P1	Deep sea sediments	Naphthalene/ phenanthrene/ pyrene and other aromatic hydrocarbons	Transcriptomic analysis confirms that five gene clusters were involved in biodegradation of PAH compounds in strain P1.	Wang W. et al., 2018
10.	Microbial communities	Agricultural and organic soil	Aromatic hydrocarbons	Metatranscriptomic analysis showed that agricultural soil has high expression of resistant proteins, dioxygenases, metapyrocatechases, 4-hydroxyphenylpyruvates, and ring hydroxylating dioxygenases for aromatic-hydrocarbon-degrading pathways in comparison to organic soil.	Sharma et al., 2019
11.	Microbial communities.	Wheat rhizosphere	Aromatic and xenobiotic compounds	Metatranscriptomic analysis of wheat rhizosphere deciphered taxonomic microbial communities and their multi-functionalities linked with degradation of aromatic and xenobiotic compounds.	Singh D. P. et al., 2018
12.	Activated-sludge microbiome.	Activated sludge	Heavy oil	*De novo* RNA seq strategy deciphered the high performance of nitrifers in degradation of heavy oil performance of reactor revealing unexpected linkage between carbon and nitrogen metabolisms in complex microbiomes.	Sato et al., 2019

metabolite was perhaps generated due to the participation of monooxygenases.

Genomic and transcriptomic approaches were used by Sengupta et al. (2019) for gaining mechanistic insight into 4-nitrophenol (4-NP) degrading bacterium *Rhodococcus* sp. strain BUPNP1. This study identified a catabolic 43 gene cluster named *nph* that harbors not only mandatory genes for the breakdown of 4-NP into acetyl co-A and succinate by nitrocatechol, but also for other diverse aromatic compounds. An integrated approach of metagenomics and metatranscriptomics revealed the metabolic capabilities and synergistic relationship between *Sphingomonas* spp., *Pusillimonas* sp., and *Pseudomonas* sp. in the degradation of bisphenol A (BPA) (Yu K. et al., 2019).

Metatranscriptomic analysis of this interaction model demonstrated genes encoding the transcription of 1,2-bis(4-hydroxyphenyl)-2-propanol (1-BP) into 4-hydroxybenzaldehyde (4-HBD) and 4-hydroxy-acetophenone (4-HAP) via 3,4-dihydroxybenzoate (3,4-DHB) and 3-oxoadipate (3-ODP), respectively, to the tricarboxylic acid cycle (TCA)cycle. Marcelino

et al. (2019) identified fungal species and subspecies in a mixed community by using metatranscriptomics. This study suggested a strain-level discrepancy between the *Cryptococcus* fungal species and their *in-situ* mock communities. Thus, transcriptomic analysis provides a large amount of gene information about the potential function of microbial communities in adaptation and survival in extreme environments (Singh D. P. et al., 2018; Mao et al., 2019; Marcelino et al., 2019).

Proteomics

Proteins are crucial effectors of biological responses, stabler than RNAs in living organisms, and possibly confer an integral view of biological function expressed *in situ*; the term proteomics is put forward to study the entire set of proteins expressed in an organism (Ram et al., 2005; Singh, 2006; Hettich et al., 2013). Thus, proteomics has emerged as an interesting and fruitful technology to study protein expression (post-translational modifications, protein turnover, proteolysis, and changes in the corresponding gene expression) of the microbial world (Keller and Hettich, 2009; Aslam et al., 2017). Proteomics is a promising aspect of omic technologies in the field of microbiology, allowing for investigating the complete protein profile obtained in a straight line from a composite microbial population in a contaminated environment (Williams et al., 2013; Arsene-Ploetze et al., 2015; Wang et al., 2016). However, metaproteomics is used to decipher crucial information regarding the protein profiling of two diverse ecological units (Arsene-Ploetze et al., 2015). Proteomics has been used to identify microbial communities/microorganisms in various ecosystems including soil and sediment, activated sludge, marine and groundwater sediment, acid mine biofilms, and wastewater plants, as illustrated in **Table 3** (Williams et al., 2013; Colatriano et al., 2015; Grob et al., 2015; Bastida et al., 2016; Jagadeesh et al., 2017). These studies revealed secret information related to protein synthesis, gene-expression stability, mRNA turnover, and protein–protein interaction networks in microbial communities in stress environments (Aslam et al., 2017).

The inclusion of a proteomic approach helps to identify related enzymes and their metabolic pathways in the bioremediation of xenobiotics from various contaminated sites (Kim et al., 2004; Liu et al., 2017; Wei et al., 2017). Basically, there are four primary steps that involve proteomic analysis: (1) preparation of a biological sample; (2) extraction and separation of proteins by using two-dimensional gel electrophoresis (2D-GE); (3) protein gel images are examined by means of image-analysis software such as ImageMaster 2D or PDQuest; and (4) proteins are identified by using mass spectroscopy (MS)/MALDI-TOF/MS or LC-MS (Yates et al., 2009; Chakka et al., 2015; Velmurgan et al., 2017).

A combined protein profile of 20 PAH-induced proteins was studied by proteomics in *Mycobacterium vanbaalenii* PYR-1 grown in a PAH-supplemented culture medium (Kim et al., 2004). PAH exposure of five different compounds, i.e., pyrene, pyerene-4,5-quinone, phenanthrene, anthracene, and fluoranthere causes variation in protein composition, showing upregulation of multiple proteins for PAH treatment compared to an uninduced control sample. Several PAH-induced proteins were identified by LC-MS, including a catalase-peroxidase, a putative monooxygenase, a dioxygenase, a small subunit of naphthalene-inducible dioxygenase, and aldehyde dehydrogenase. The metaproteomic approach was employed by Bastida et al. (2016) to illustrate changes in metabolic activities during compost-treated bioremediation with the help of differential protein expressions in hydrocarbon-polluted soil. Metaproteomic analysis indicated that *Sphingomonadales* and uncultured bacteria are responsible for the degradation of hydrocarbons in compost-treated soil due to the higher expression of catabolic enzymes such as 2-hydroxymuconic semialdehyde, *cis*-dihydrodiol dehydrogenase, and catechol 2,3-dioxygenase, dioxygenases involved in the first oxygenation step of aromatic rings. Moreover, biphenyl-2,3-diol 1,2-dioxygenase, estradiol dioxygenase, and naphthalene 1,2-dioxygenase were identified in compost-treated samples. By using metaproteomics, this study explored the functional and phylogenetic relationship of contaminated soil, and the microbial key players involved in compost-assisted bioremediation.

Another study, undertaken by Vandera et al. (2015) demonstrated the comparative proteomic analysis of *Arthrobacter phenanivorans* Sphe3 on aromatic compounds phenanthrene and phthalates. The proteomic approach confirmed the involvement of several proteins in aromatic-substrate degradation by identifying those mediating the initial ring hydroxylation and ring cleavage of phenanthrene to phthalate. This study also revealed the presence of both the *ortho-* and the *meta-* cleavage pathway for the degradation of these aromatic compounds, and it also identified all proteins that take part in these pathways and are highly upregulated upon phthalate growth in comparison to phenanthrene growth.

Proteomic analysis of pyrene-degrading bacterium *Achromobacter xylosoxidans* PY4 was performed by Nzila et al. (2018), who identified a total of 1,094 proteins. Among these, 95 proteins were detected in glucose supplementation, and 612 proteins were detected in the presence of pyrene. Furthermore, 25 upregulated proteins were found to be involved in stress response and the progression of genetic information. Two upregulated proteins, 4-hydroxyphenylpyruvate dioxygenase and homogentisate 1,2-dioxygenase, are implicated in the lower degradation pathway of pyrene. Enzyme 4-hydroxyphenylpyruvate dioxygenase may catalyze the conversion of 2-hydroxybenzalpyruvic acid (metabolite of pyrene) to homogentisate. Homogentisate 1,2-dioxygenase is involved in the incorporation of 2 oxygen atoms to produce 4-maleyacetoacetate, which is an intermediate in several metabolic pathways.

Lee et al. (2016b)performed proteomic analysis of PAH-degrading bacterial isolate *Sphingobium chungbukense* DJ77. This strain exhibited outstanding degradation capability for various aromatic compounds. This study demonstrated the degradation of three xenobiotics compounds, i.e., phenanthrene, naphthalene, and biphenyls (PNB), and their associated proteins was analyzed by 2-DE and MALDI-TOF/MS analysis. During PNB biodegradation by bacterial cells, an alteration was observed in protein expression to cope with the stress condition.

TABLE 3 | Microorganisms or microbial communities using proteomic and metaproteomic approaches in biodegradation.

S. no.	Microorganisms	Xenobiotics/ pollutants	Technique used	Comments/result	References
1.	*Microbacterium* Y2	Decarbodimethyl ether (BDE-209)	iTRAQ labeling and HRMS	Proteomic analysis showed that the over expression of haloacid dehalogenases, glutathione-S-transferases, and ATP-binding cassette (ABC) involve in the degradation of BDE-209 in *Microbacterium* Y2.	Yu Y. et al., 2019
2.	*Phanerochaete chrysosporium*	Terabromobisphenol A	High-performance liquid chromatography–mass spectrometry (HPLC–MS)	Proteome analysis showed upregulation of cyt P450 monooxygenase, glutathione-s-transferase, *O*-methyltransferase, and other oxidoreductases for the biotransformation of terabromobisphenol A via oxidative hydroxylation and reductive debromination.	Chen et al., 2019
3.	*Sphingobium chungbukense* DJ77	Polycyclic aromatic hydrocarbons (PAHs)	LC-MS	Proteomic analysis of PAH-degrading bacterial isolate predicted putative dehydrogenases, dioxygenases, and hydrolases involved in catabolic pathway of xenobiotic degradation.	Lee et al., 2016b
4.	*Burkholderia* sp. K24	Benzene, toluene, xylene, and aniline	LC-MS	Proteogenomic characterization of strain K24 revealed four independent degradation pathways for monocyclic aromatic hydrocarbons: benzene, toluene, xylene, and aniline.	Lee et al., 2016a
5.	*Acinetobacter* sp. KS-1	Benzoate	MALDI-TOF/MS	Proteome analysis of bacterium *Acinetobacter* sp. KS-1 showed two benzoate-degrading enzymes (catechol 1,2-dioxygenase, β-ketoadipate succinyl CoA transferase), and suggested that it degrades benzoate through β-ketoadipate pathway.	Kim et al., 2003
6.	*Acinetobacter radioresistens* S13	Aromatic compounds	Two-dimensional gel electrophoresis (2-DE) with *N*-terminal sequencing	Proteomic analysis revealed that six proteins were actively synthesized during aromatic-compound degradation, and enzymes of the β-ketoadipate pathway were observed and identified. DHB dehydrogenase, DBHDH, phenol hydroxylase oxygenase, PHO, catechol 1,2 dioxygenase, ketoadipyl-CoA thiolase, and muconolactone isomerase.	Mazzoli et al., 2007
7.	*Sphingomonas wittichii* RW1	Dioxins	MALDI-MS	Proteomic profiling of dioxin-degrading bacterium *Sphingomonas wittichii* RW1 identified a dioxin dioxygenase, *meta*-cleavage product hydrolase, and 2,3-dihydroxybiphenyl 1,2-dioxygenase proteins related to dioxin/dibenzofuran degradation.	Colquhoun et al., 2012
8.	*Sphingomonas haloaromaticamans* strain P3	*ortho*-phenylphenol	MALDI-TOF	Proteomic analysis of strain P3 explored the role of catabolic operons on the microbial bioremediation of *ortho*-phenylphenol (OPP). A total of 229 protein spots were identified that were differentially expressed in the presence of OPP. Among these, only 13 upregulated protein spots were associated with proteins having a putative role in OPP transformation.	Perruchon et al., 2017
9.	*Paecilomyces* sp. strain SF-8	PAHs	MALDI-TOF	Proteomic analysis revealed the expression of salicylaldehyde dehydrogenase, which is a key control protein of PAH degradation, especially overexpressed in strain SF-8.	Velmurgan et al., 2017
10.	*Mycobacterium* sp. strain 6PY1	Pyrene	2-DE using *N*-terminal sequencing	Proteomic analysis identified 40 induced pyrene-specific protein spots; nine proteins were detected. Two pyrene induced dioxygenases were differentially regulated.	Krivobok et al., 2003
10.	*Bacillus thuringiensis*	Cypermethrin	MALDI-TOF MS	Proteome analysis evaluated differential expressions of proteins during cypermethrin degradation.	Negi et al., 2016
11.	*Escherichia coli*	*p*-nitrophenol	MALDI-TOF/TOF-MS	Proteomic analysis showed differential expression of proteins during degradation of aromatic compounds such as *p*-nitrophenol.	Chakka et al., 2015
12.	Activated-sludge microbial community	PAHs	MALDI-TOF-MS	Metaproteomics analysis of PAH-treated sludge indicated the proteins derived from Burkholderiales population exhibiting differential protein expression profile and involved in PAH metabolism. The protein expression profile indicated that naphthalene was more liable to degradation than anthracene in sewage by Burkholderiales.	Li et al., 2019
13.	Microbial community	Toluene	Nano-liquid chromatography coupled with electrospray mass spectroscopy (Nano-LC-ESI-MS/MS)	Metaproteomic analysis of an anaerobic microbial community identified 202 unambiguous proteins derived from 236 unique spots. These proteins are involved in toluene degradation and mainly affiliated to the members of Desulfobulbaceae and several other Deltaproteobacteria.	Jehmlich et al., 2010

Comparative analysis of 2-DE results revealed that the intensity of 10 protein spots changes identically upon exposure to these xenobiotics in strain DJ77 (Lee et al., 2016b). Among these ten, five upregulated proteins with multiple functionalities were identified as putative dihydrodiol dehydrogenase (BphB), which catalyzes the NAD^+-dependent oxidation of *trans*-dihydrodiols; 2,3-dihydrobisphenyl 1,2-dioxygenase (PhnQ), which cleaves the aromatic ring; and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD), which degrades biphenyls and polychlorinated biphenyls. A part of the initial diverse catabolism of PNB by BphB, PhnQ, and BphD converged into the same catechol degradation branch. Now, catechol is first transformed into a ring-cleaved product, i.e., 2-hydroxymuconic semialdehyde by catechol 2,3-dioxygenase (PhnE). Moreover, it is assumed that this ring-cleaved product (2-hydroxymuconic semialdehyde) would be further degraded by 2-hydroxymuconic semialdehyde hydrolase (PhnD), and acetaldehyde dehydrogenase (PhnI) into a compound that can enter into the TCA cycle. Hence, these upregulated proteins, dehydrogenase, dioxygenase, and hydrolase, are involved in the catabolic degradation pathway of xenobiotics. The detection of intermediates from 2,3-dihydroxy-biphenyl degradation to pyruvate and acetyl-CoA by LC/MS analysis showed that ring-cleavage products entered the TCA cycle and were mineralized in strain DJ77. It was also suggested that strain DJ77 could completely degrade a wide range of PAHs via multiple catabolic pathways (Lee et al., 2016b).

The biodegradation mechanism of tetrabromobis-phenol A (TBBPA) was investigated in *Phanerochaete chrysosporium* by using a proteomic approach. iTRAQ quantitative analysis identified a total of 2,724 proteins in three biological samples. Compared to control TBBPA, stress caused 148 differentially expressed proteins in *P. chrysosporium*, among which 90 proteins were upregulated and 58 proteins were downregulated. The upregulation of cytochrome p450 monooxygenase, glutathione-*S*-transferase, *O*-methyltransferase, and other oxidoreductases is responsible for the biotransformation of TBBPA via oxidative hydroxylation and reductive debromination (Chen et al., 2019).

A biodegradation study of endocrine-disrupting compound 4-*n*-nonylphenol (4-*n*-NP) by filamentous fungus *Metarhizium robertsii* was investigated by Szewczyk et al. (2014) with proteomic analysis. This suggested that the main biodegradation mechanism involves the consecutive oxidation of the alkyl chain and benzene, which consequently results in the complete decomposition of the 4-*n*-NP compound. Proteomic profiling explored the involvement of nitro-reductase-like proteins related to the oxidation–reduction and ROS defense systems, and mainly engaged group of proteins in the removal of 4-*n*-NP. Proteomic data obtained in this study could not clearly explain the mechanism of 4-*n*-NP biodegradation in the tested fungal strain, but allowed for the formulation of hypotheses that the over-expressed enzymes in the cultures with 4-*n*-NP could play a role in xenobiotic removal and the biodegradation process.

Bioremediation of decabromodiphenyl ether (BDE-209) was explored in *Microbacterium* Y2 in a polluted water-sediment system through proteomics (Yu Y. et al., 2019). Proteomic analysis showed that the overexpression of haloacid dehalogenases, glutathione s-transferase, and ATP-binding cassette (ABC) transporter might occupy important roles in BDE-209 biotransformation. Moreover, heat-shock proteins (HSPs), ribonuclease E, oligoribonuclease (Orn), and ribosomal proteins were activated to counter the BDE-209 toxicity. Thus, it is suggested that these proteins are implicated in microbial degradation, antioxidative stress, and glycolysis (Yu Y. et al., 2019).

Metabolomics

Metabolomics is a well-established recent scientific technology, attributed toward the study of naturally occurring low-molecular weight (<1,000 Da) organic metabolites (organic acids; pyruvate, lactate, malate, formate, fatty acid-like acetate, etc.) inside a tissue, cell, or biofluid (Johnson et al., 2011; Malla et al., 2018; Withers et al., 2020). Metabolomics explores the relationships between organisms and the environment, such as organismal responses to abiotic stressors, including both natural factors such as temperature, and anthropogenic factors such as pollution, to investigate biotic–biotic interactions such as infections, and metabolic responses (Lindon et al., 2006; Griffiths, 2007; Mallick et al., 2019).

The combined use of metabolomics with these applications details the authentic collection of chemical outputs and inputs, which arbitrate the exchange of resources between the community and its host (**Table 4**; Theriot et al., 2014; Wang Y. F. et al., 2018). Metabolomic studies in environmental sciences have been directed toward understanding changes in the concentration of metabolites associated with exposing model organisms to toxic compounds, such as xenobiotics (Parisi et al., 2009; Seo et al., 2009).

Metabolomics approach was utilized to investigate the degradation mechanism of carbaryl and other *N*-methyl carbamates pesticides in *Burkholderia* sp. strain C3 (Seo et al., 2013). Metabolomes are dynamic and responsive to nutrient and environmental changes. The results of this study showed the metabolic adaptation of *Burkholderia* sp. C3 to carbaryl in comparison with glucose and nutrient broth. The metabolic changes were notably associated with the biosynthesis and metabolism of amino acids, sugars, PAH lipids, and cofactors. Differential metabolome analysis in response to different substrates identified 196 polar metabolites, 10 fatty acids, and 1 macromolecule (PHA) in this strain, and confirmed up-production metabolites in the pentose phosphate pathway, cysteine metabolism, amino acids, and disaccharides (Seo et al., 2013). Thus, this metabolomic study could provide detailed insights into bacterial adaptation to different metabolic networks, and the metabolism of toxic pesticides and chemicals.

Environmental pollutants cause alterations in microbial communities, which consequently changes biochemical and metabolic functions in soil microorganisms. Microbial degradation of cyfluthrin by *Photobacterium ganghwense* was investigated via a comparative metabolic approach (Wang et al., 2019). Metabolomics explored the biotransformation pathway of cyfluthrin with the identification of 156 metabolites during the biodegradation process. Recently, on the basis of interactions of indigenous soil microorganisms to PAH-contaminated soil, Li

TABLE 4 | Microorganisms or microbial communities using metabolomic approaches in biodegradation.

S. no.	Microorganisms/ microbial communities	Xenobiotics/ pollutants	Comments/results	References
1.	*Burkholderia* sp. C3	*N*-methylcarbamates	Metabolomic analysis identified a total of 196 polar metabolites, 10 medium-to-long-chain fatty acids, and one type of macromolecule polyhydroxyalkanoates (PHA) in strain C3 in the degradation of *N*-methylcarbamate pesticides.	Seo et al., 2013
2.	*Lactobacillus plantarum*	Phorate	Metabolomic analysis identified a number of differential abundant metabolites in the presence of phorate by *L. plantarum,* and apparent alterations of metabolome profiles in cell-culture supernatant that contained phorate in comparison with the non-pesticide containing one.	Li et al., 2019
3.	*Mycobacterium* sp. DBP42 *Halomonas* sp. ATBC28	Plasticizers/phthalates (DBP/DHEP/ATBC)	Metabolomic study explored the metabolic potential of biofilm-producing marine bacterial isolates that colonize plastics, and revealed different mechanisms used for ester side chain removal from different plasticizers, i.e., DBP, DHEP, and ATBC.	Wright et al., 2020
4.	*Photobacterium ganghwense*	Cyfluthrin	Metabolomics explored the biotransformation pathway of cyfluthrin with the identification of 156 metabolites during biodegradation process.	Wang et al., 2019
5.	*Mycobacterium vanbaalenii* strain PYR-1	Benz[a]anthracene	Benz[a]anthracene was metabolized by *M. vanbaalenii* PYR-1 via four degradation pathways and produced numerous metabolites. Major metabolites identified by GC-MS and NMR spectral analysis were 3-hydrobenzo[f]isobenzofuran-1-one, 6-hydrofuran[3,4-g]chromene-2,8-dione, benzo[g]chromene-2-one, naphtha[2,1-g]chromen-10-one, 10-hydroxy-11methoxybenz[a]anthracene and 10, 11dimethoxybenz[a]anthracene.	Moody et al., 2005
6.	*Bacillus* sp. 3B6	Mesotrione	*Ex situ* NMR and LC-NMR techniques used to define the metabolic pathway involved during the biotransformation of mesotrione by *Bacillus* sp. 3B6. The complementarities of these NMR techniques identified two major metabolites (glutarate and MNBA), revealing the presence of a new metabolic pathway.	Durand et al., 2010
7.	*Drechslera* sp.	Methyl tertiary-butyl ether (MtBE)	Metabolomic analysis revealed the presence of two major bioactive metabolites, monocerin, and an alkyl substituted epoxycyclohexanone derivative that showed good antifungal activity and bioremediation.	d'Errico et al., 2020
8.	*Sinorhizobium* sp. C4	Phenanthrene	Comprehensive metabolite profiles, including polar metabolites, fatty acids, and polyhydroxyalkanoates were evaluated through untargeted metabolome analyses during phenanthrene degradation by *Sinorhizobium* sp. C4.	Keum et al., 2008
9.	*Bacillus thuringiensis* strain ZS-19	Cyhalothrin	Metabolomic analysis of strain ZS-19 through HPLC and GC-MS revealed biodegradation mechanisms of cyhalothrin.	Chen et al., 2015
11.	Soil microbial communities	Toxic pollutants	Metabolomics proposed to explore bioremediation potential, molecular changes, and metabolic pathways developed in microorganisms in contaminated environments.	Withers et al., 2020
12.	Soil microbial communities	Polycyclic aromatic hydrocarbons (PAHs)	Metabolomics with combined enzyme activity and sequencing analysis revealed the response of soil microbial communities to polycyclic aromatic hydrocarbon stress and their metabolic degradation pathway.	Li et al., 2018

et al. (2018) elucidated that the majority of microbial metabolic functions were adversely affected to cope with PAH pollution. This study includes the combined study of enzyme activity and sequencing analysis with metabolomics, which further exposed the specific inhibition of soil metabolic pathways associated with carbohydrates, amino acids, and fatty acids due to microbial-community shifting under PAH stress.

Soil metabolomics is an effective approach to reveal the complex molecular networks and metabolic pathways operating in the soil microbial community. This approach can also be used to find biomarkers of soil contamination (Jones et al., 2014). High-throughput sequencing and soil metabolomics investigated the differential structures and functions of soil bacterial communities in the pepper rhizosphere and bulk soil under plastic greenhouse vegetable cultivation (PGVC) (Song et al., 2020). A total of 245 metabolites were identified, among which 11 differential metabolites were detected between rhizosphere and bulk soil, including organic acids and sugars that were positively and negatively correlated with the relative abundances of the differential bacteria. A starch and sucrose metabolic pathway was the most differentially expressed pathway in rhizospheric soil. The main functional genes participating in this pathway were predicted to be down regulated in rhizosphere soil. Sugar and organic acids as the main plant-root exudates in the rhizosphere, and they are also the main drivers of the shift in soil microbial community in the rhizosphere. These plant-root exudates act as an energy source to soil microbes, thus benefiting their growth (Kuzyakov and Blagodatskaya, 2015; Shi et al., 2015). Linear discriminant analysis (LDA) effect size (LEfSe) analysis showed that bacterial phyla of *Proteobacteria*

and *Bacteroidetes* were significantly higher in rhizosphere soil, benefitting plant growth. Thus, the relationship between soil metabolites and microbial communities guides the regulation of plant rhizoprocesses through soil amendments to increase plant growth.

Durand et al. (2010) conducted metabolic analysis of *Bacillus* sp. to characterize the metabolic pathway for the biodegradation of mesotrione, a herbicide. Analysis was carried out by using LC-NMR and LC-MS, and the result of these instrumental analyses was the identification of six metabolites, of which the structures of four metabolites were suggested. Szewczyk et al. (2017) performed metabolic analysis of fungal strain *Metarhizium brunneum* ARSEF 2017 to predict a biodegradation-pathway metabolic background for the removal of ametryn, an *s*-triazene herbicide. Qualitative LC-MS/MS metabolomic analysis of ametryn biodegradation resulted in the generation of four metabolites, i.e., 2-hydroxy atrazine, ethyl hydroxylated ametryn, *S*-demethylated ametryn, and diethyl ametryn.

Wright et al. (2020) evaluated the metabolomic characterization of two potent marine bacterial isolates, *Mycobacterium* sp. DBP42 and *Halomonas* sp. ATBC28, capable of the degradation of phthalate and plasticizers such as ATBC, DBP, and DEHP. That study presented the molecular analysis of metabolites generated during biodegradation. A metabolomic study confirmed that DBP and ATBC were degraded through the sequential removal of ester side chains, and generated monobutyl phthalate and phthalate in the case of DBP degradation, and citrate in the case of ATBC degradation in *Mycobacterium* sp. However, DEHP degradation did not follow the same pathway as that observed for DBP and ATBC. It was suggested that DEHP degradation is initiated through hydroxylation of the ester side chain by monooxygenase, and may occur via the β-oxidation of fatty acid side chains directly on the DEHP molecule. Moreover, in comparison with *Mycobacterium* sp., *Halomonas* sp. did not confirm any detectable degradation intermediates for the degradation of plasticizers and phthalate, but it harbored an array of enzymes suggested to be responsible for the degradation of other aromatic compounds. Therefore, metabolomic analyses demonstrated changes that occur in the composition of metabolites, aiding to fully understand the shifts mechanisms of metabolites during the microbial degradation or mineralization of environmental pollutants (Lindon et al., 2006; Keum et al., 2008; d'Errico et al., 2020).

BIOINFORMATICS

Bioinformatic technology developed a new array of computational technologies that uses both information technology and biological sciences. This modern technology seeks information from multiple high-throughput biological techniques, and keeps all biological data, helping to investigate and decide the relationship among organic molecules, including macromolecular sequences, biochemical and metabolic pathways, protein expressions, metabolites, and structures (Fulekar and Geetha, 2008; Cooper et al., 2018; Dangi et al., 2018; Greene, 2018; Shekhar et al., 2020). Enormous amounts of data are generated from DNA, RNA, and protein sequences that need to be accurately executed; thus, bioinformatics has led to finding the best possible way to analyze such huge amounts of biological data via specific computational tools (Aora and Bar, 2014; Bhatt et al., 2021). Therefore, bioinformatic-associated tools are very important to understand the bioremediation of toxic pollutants. Bioinformatics provides superior information regarding the cellular, molecular, and genetical bases of xenobiotic degradation and detoxification (Kumar et al., 2016; Huang et al., 2020). There are a number of bioinformatic tools and applications that are available to use for biodegradation studies, as listed in **Table 5**.

MetaRouter is one of such application that is freely open and a modular architecture to a variety of consumers from any place in a safe and secure manner, just by connecting to an Internet server (Pazos et al., 2005). For the analysis of biodegradation studies, many bioinformatic resources are exclusively available. The University of Minnesota Biocatalysts/Biodegradation Database (UM-BBD) was introduced in 1995 and contains information regarding microbial catabolism and related biotransformation, biodegradation reactions, catabolic enzymes, and pathways for xenobiotics and other hazardous pollutants of various microorganisms. This database is connected to several other databases, such as BRENDA, ENZYME, ExPASy, and NCBI, to collect and store information related to gene structure and enzymes that take part in the biodegradation of environmental contaminants (Ellis et al., 2006). Genomic sequences of microorganisms with competent and efficient degradation abilities could be easily investigated via another widely used database, the National Center for Biotechnology Information (NCBI). It gives a detailed and complete pipeline for annotations, and comprehensive analysis of more than 6,000 microbial genomes (Brown et al., 2015). PRIDE (Vizcaino et al., 2016) is the world's largest data repository of mass-spectroscopy-based proteomic data, and MetaboLights (Kale et al., 2016) is a database for metabolomic experiments and derived information. The GenBank database is freely available in NCBI, and it provides most up-to-date and comprehensive DNA sequence information (Benson et al., 2013). Recently, there have been a number of databases such as CAMERA, MG-RAST, and IMG/M that were developed and employed for the analysis and in-depth understanding of diverse microbial populations, metabolic reconstruction, taxonomic affiliations, and their inter- and intra-relationship networks. Another database, Bionemo, developed by the structural computational-biology group at the Spanish National Cancer Research Center, gives information related to specific genes and proteins that take part in biodegradation reactions and metabolic pathways (Carbajosa et al., 2009). It provides insights into sequences, domains, protein structures, and regulatory elements, and transcription factors for their respective genes. Integrated bioinformatic approaches are employed for the metagenomic characterization of the soil microbial communities of different soil sites by using MetaPhlAn, KEGG, XLSX, and LEfSe bioinformatic databases to reveal the ancestral and functional characterization of diverse soil microbial populations (Arora et al., 2009; Xu et al., 2014; Kumar et al., 2016). The degradation or detoxification of xenobiotic pollutants through microbial communities is a highly considered and proficient remediation technology, and there is no single resource accessible that provides all the information with reference to environmental contaminants, microorganisms, and their

TABLE 5 | Bioinformatic databases and software tools used in biodegradation studies.

Database/software tools	Features/functions	Web address/URL	References
University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD)	Information about microbial catabolism and related biotransformation, and biodegradation pathways for xenobiotics and other hazardous pollutants.	https://umbbd.ethz.ch/	Ellis et al., 2006
Biodegradation Network-Molecular Biology (Bionemo)	Molecular knowledge about the structure and function of biodegradative genes and proteins.	https://bionemo.bioinfo.cnio.es	Carbajosa et al., 2009
Kyoto Encyclopedia of Genes and Genomes (KEGG)	Highly recommended for information regarding genetic, metabolic, enzymatic, and cellular progressions of microorganisms.	http://genome.ad.jp/kegg/	Kanehisa et al., 2017
National Center for Biotechnology Information (NCBI)	Public databases and software tools for storing, disseminating, and analyzing genome data.	http://www.ncbi.nih.gov/	Brown et al., 2015
OxDBase	Enzymatic database that contains all literature-cited information related to oxygenases.	www.imtech.res.in/raghava/oxdbase/	Arora et al., 2009
PathPred	Used to predict microbial-degradation pathways for pollutants.	http://genome.jp/tools.pathpred/	Moriya et al., 2010
MetaRouter	Maintains varied information regarding biodegradation networks, predicting biodegradative pathways for chemical compounds.	http://pdg.cnb.uam.es/MetaRouter	Pazos et al., 2005
MetaCyc	Provides information about enzymatic and metabolic mechanism/pathways.	http://metacyc.org	Capsi et al., 2016
Biocyc	Stores information related to organism-specific genome databases/pathways.	http://biocyc.org	Capsi et al., 2016
Molecular Evolutionary Genetic Analysis (MEGA 7.0)	Used for sequence alignment, hierarchical classification, and constructing phylogenetic tress.	www.megasoftware.net	Kumar et al., 2016
Biodegradative Strain Database (BSD)	Web-based database that provides detailed information about biodegradative bacteria and the hazardous chemicals that they degrade.	http://www.bsd.cme.msu.edu/	Urbance et al., 2003
KBase	Large-scale bioinformatic database that predicts and manages genomic data of plants and diverse microbial populations.	http://kbase.us/	Arkin et al., 2016
PAHbase	Functional PAH database that contains significant information on PAH-degrading bacteria, their occurrence phylogeny, metabolic pathways, and the genetic basis of their biodegradation capability.	http://www.pahbase.in	Kessner et al., 2008
ProteoWizard	Used for rapid proteomic analysis.	http://proteowizard.sourceforge.net/	Surani et al., 2011
BioRadBase	First comprehensive knowledge database that provides detailed information about the bioremediation of radioactive waste through microorganisms.	http://biorad.igib.res.in.	Reena et al., 2012
BiofOmics	Novel, systematic, and large-scale database for management and analysis of biofilm data from high-throughput experiment studies of microorganisms.	www.biofomics.org	Lourenco et al., 2012
Proteomics Identifications (PRIDE)	World's largest database for analysis of mass-spectrometry-based proteomic data. Includes generic standard-based format that can be annotated to capture data generated using any proteomic pipeline.	http://www.ebi.ac.uk/pride/	Vizcaino et al., 2016
MetaboLights	Database for metabolomic studies that provide primary research data and metadata for cross-platform and -species metabolomic studies.	http://www.ebi.ac.uk	Kale et al., 2016

bioremediation potentialities. Thus, these databases coalescing the detailed information about the nature of pollutants, their metabolic pathways, bioremediation microorganisms, catabolic genes, enzymes, and protein-expression profiles would be a significant tool to open up a new vista and enlighten future research science in the field of bioremediation.

CONCLUSIONS

Microbial communities have great potential to mediate the successful biodegradation process of xenobiotic-contaminated soil/water environments. However, the greater part of mainstream microorganisms involved in bioremediation are still undefined because not all organisms in nature could be cultured under *in vitro* environments, but reside in viable-but-non-culturable (VBNC) environments. Thus, to explore the hidden knowledge of these VBNC organisms, recent advanced practices and sophisticated up-to-date technologies are highly desirable to understand the genetic and molecular biology of microorganisms. Newly developed molecular techniques offer promising approaches to address the in-depth characterization of microbial communities from molecule to gene. Recent omics technologies such as metagenomics, transcriptomics, and proteomics are helpful in obtaining information about nucleic acids, enzymes, catabolic genes, plasmids, and metabolic machineries and metabolites generated during

the biodegradation process. However, the solitary employment of any individual omics technology is not sufficient to explore or illustrate secret information regarding microbial-remediation practices. Therefore, an interdisciplinary application of multiple omics studies highlights the perspectives of system biology for providing an integrative understanding between genes, proteins, and environmental factors responsible for the whole microbial-degradation process, and gives a new array of novel technologies, such as genome-editing and next-generation-sequencing tools CRISPR-Cas9, TALEN, and ZFNs, which are potent gene-editing tools that design microbes with specific degradation-function genes and provide unique insights into microbial remediation. Moreover, the successful execution of omics technologies could not be possible without the use of bioinformatic tools. The establishment of informative genomic and proteomic databases has been revolutionized by bioinformatics, which facilitates broad information about cellular- and metabolic-mechanism pathways for environmental pollutants. Hence, the involvement of these advanced technologies in the biological sciences shows the way to next-level research in the bioremediation potential of microorganisms, and exploits their capability to remove xenobiotic contamination.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

Conceptualization and writing—original draft preparation: SM. Writing—review and editing: ZL, SP, WZ, PB, and SC. Supervision, funding acquisition, and project administration: SC. All authors contributed to the article and approved the submitted version.

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A Combinational Strategy Mitigated Old-Aged Petroleum Contaminants: Ineffectiveness of Biostimulation as a Bioremediation Technique

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Hydrocarbon contamination emerging from the crude oil industrial-related activities has led to severe environmental issues. Prolonged contamination with the constant infiltration of crude oil into the soil is a severe problem in remediating contaminated soils. Hence, the current study focuses on comparing various bioremediation strategies, thereby isolating native bacteria competent to reduce TPH in both liquid and microcosm environments in an old-aged petroleum hydrocarbon contaminated soil. Assays in the modified 6SW-Vit medium after 7 days of incubation revealed that *Bacillus altitudinis* strain HRG-1 was highly hydrophobic and had a suitable ability to decrease surface tension (40.98%) and TPH (73.3%). The results of biodegradation in the microcosm proved that among the designated treatments, including bio-stimulated microcosm (SM), bacterialized microcosm (BM), a combined bio-stimulated microcosm and bacterialized microcosm (SB), and natural attenuation (NA), the SB treatment was the most effective in mitigating TPH (38.2%). However, the SM treatment indicated the lowest TPH biodegradation (18%). Pearson correlation coefficient among microcosm biological indicators under investigation revealed that soil basal respiration had the highest correlation with the amount of residual TPH ($r = -0.73915$, $P < 0.0001$), followed by the microbial population ($r = -0.65218$, $P < 0.0001$), catalase activity ($r = 0.48323$, $P = 0.0028$), polyphenol oxidase activity ($r = -0.43842$, $P = 0.0075$), and dehydrogenase activity ($r = -0.34990$, $P = 0.0364$), respectively. Nevertheless, considering the capability of strain HRG-1 and the higher efficiency of the combined technique, their use is recommended to diminish the concentration of petroleum hydrocarbons in hot and dry contaminated areas.

Keywords: total petroleum hydrocarbons, aged-contaminated soil, bioremediation, soil biological factors, correlation coefficient

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INTRODUCTION

Total petroleum hydrocarbons are one of the most momentous contaminants in the environment. Considering the harmful effects of crude oil leakage on the region's ecosystem, it is necessary to remove them from the environment. One of the most effective, low-cost, and applicable techniques to remove contaminants is called bioremediation. Bioremediation denotes the use of

living indigenous or exogenous microorganisms with the property of degradation of contaminants or improvement of various physical-chemical soil conditions to stimulate the growth of efficient microorganisms (Wilson and Jones, 1993; MarGesin et al., 2000). Solving the contamination problem has attracted many researchers' attention in recent years; however, the aging of organic contaminants and the successive entry of petroleum hydrocarbons into the soil have been less discussed and investigated.

Numerous studies have reported using different bioremediation strategies in petroleum-contaminated soils, each of which presenting different results. For instance, Liu et al. (2011) said bioaugmentation as a superior strategy, while in another study, Wu et al. (2019) considered biostimulation a preferable approach. In other researchers' works, the combined bioaugmentation and biostimulation has been proposed as the most efficient strategy (Varjani and Upasani, 2019).

The core problem facing bioremediation is the aging phenomenon of organic contaminants in the soil. As time passes, it becomes laborious to separate the hydrocarbon contaminant from the soil matrix. With these contaminants' infiltration into soil organic matter and soil pores, their extraction ability and accessibility for microorganisms and other environmental receptors are reduced (Tang et al., 2012). Due to the complexity of old-contaminants' behavior in the soil, more robust strategies are needed for the bioremediation of these contaminants. Many studies have been conducted on artificial soil contamination (Kang et al., 2010; Xu et al., 2018; Baoune et al., 2019), and soils with extremely old and natural contamination have not been investigated enough, and if so, the duration of the contamination has not been clearly mentioned (Dandie et al., 2010; Dong et al., 2013; Lominchar et al., 2018; Liu et al., 2019; Gou et al., 2020; Wang et al., 2020). However, there are many areas suffering from long-term contamination of crude oil and its products, and failure to address this issue can exert irreversible effects. Therefore, the simultaneous use of different bioremediation strategies can be more effective in solving this problem.

Another problem of contaminated soils in hot-dry areas is the salinity effect. High salinity inhibits the growth of microorganisms, increases the adaptation time of microorganisms to new conditions, and reduces the intensity and amount of biodegradation (Qin et al., 2012). Nonetheless, to analyze soil contaminants' changes resulting from the use of single or combined bioremediation techniques, various biological indicators such as soil basal respiration, microbial population, and enzymatic activity have been examined (Abed et al., 2015; Wu et al., 2017). Numerous studies have reported a strong correlation (positive or negative) between the amount of petroleum hydrocarbons and soil enzymes (Ma et al., 2014; Khan et al., 2015; Ebadi et al., 2017). However, the quantity of the correlation coefficient between soil enzymes and the amount of hydrocarbon contaminants, especially in old-aged contaminated soils, has been less discussed. To evaluate the result of soil bioremediation, it is necessary to consider the amount of residual hydrocarbons, and to determine the effect of contaminants on the biological activity of soil microorganisms. This elaborates an insight into the health condition of soil microorganisms (MarGesin et al., 2000).

The present study aimed to reduce TPH in both microcosm and culture media by a capable bacterial strain and add various supplements to increase biodegradation of contaminants in an old-aged petroleum contaminated saline-sodic soil (more than 60 years of contamination). Additionally, various soil biological indicators such as several enzyme activities, soil basal respiration, and microbial population were investigated to achieve the most appropriate one in showing TPH variations in an old-aged petroleum contaminated soil. To that end, the relationship between the soil biological characteristics and the amount of residual TPH was examined using the Pearson product-moment correlation.

This study was conducted to answer fundamental questions, including (a) which bioremediation strategy has a more influential role in reducing TPH in a highly contaminated aged soil? (b) could this soil with such a long history of contamination be remediated by only one strain or not?

MATERIALS AND METHODS

Soil Sampling and Characterization

Sampling was carried out in crude oil-contaminated saline and sodic soil with more than 60 years of contamination (ranging from 0 to 20 cm depth) around one of the oil exploitation wells located in Ahvaz oilfield (southwestern Iran). The samples were transferred to the laboratory and stored at 4°C until the experiments were performed. The main physical-chemical parameters of the soil were investigated using standard methods (Sparks, 1996). **Table 1** presents the properties of the contaminated soil.

Culture Medium and Materials

The modified 6SW-Vit medium (McGenity and Gramain, 2010) containing three solutions was used for biodegradation assays. Solution A contained (g 800 ml^{-1}) 30 NaCl, 10 $MgCl_2.6H_2O$, 2.53 KCl, 2.18 Na_2SO_4, 20 ml Tris–HCl (1 M; pH 7.6), 0.5 yeast extract. Solution B contained (ml 100 ml^{-1}) 0.6 H_3BO_3 (400 mM), 0.7 $SrCl_2$ (400 mM), 0.7 NaF (70 mM), 1 NH_4Cl (500 mM), 1 KH_2PO_4 (100 mM), two trace element solution SL-10, and solution C contained (ml 100 ml^{-1}) 10 $CaCl_2.2H_2O$ (1 M). First, the solutions were separately autoclaved and thereafter mixed, when they were cooled down. Trace element solution SL-10 contained (per L) 10 ml HCl (25%; 7.7 M), 1.5 g $FeCl_2.4H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2.4H_2O$, 6 mg H_3BO_3, 190 mg $CoCl_2.6H_2O$, 2 mg $CuCl_2.2H_2O$, 24 mg $NiCl_2.6H_2O$, 36 mg $Na_2MoO_4.2H_2O$. Finally, pH was adjusted to the range of 7–7.2. All solvents, acids, inorganic salts and Tween 80 nonionic surfactant were purchased from Merck Co., and the heavy Persian crude oil was used in this study.

Enrichment and Isolation Process

During the enrichment process, 10 g of crude oil-contaminated soil was transferred to a 500-ml flask containing 100 ml of the modified 6SW-Vit medium with 1% crude oil as a sole source

TABLE 1 | Physicochemical and biological petroleum-contaminated soil characteristics.

Characteristics	Unit	Amount
Sand	%	66 ± 3.26
Silt	%	24 ± 2.94
Clay	%	10 ± 3.74
Electrical conductivity (EC)	dS/m	80.6 ± 0.94
pH		7.52 ± 0.08
Field capacity (FC)	%	26.22 ± 1.59
Sodium adsorption ratio (SAR)	$(mmol/l)^{1/2}$	258.92 ± 1.63
Total nitrogen	%	0.261 ± 0.02
Organic carbon	%	8.775 ± 0.33
Total phosphorus	%	0.0247 ± 0.001
C/N/P		356/10/1
Na^+	mg/kg	32660 ± 254.32
K^+	mg/kg	19.154 ± 1.25
Ca^{2+}	mg/kg	1200 ± 48.98
Mg^{2+}	mg/kg	729 ± 29.76
Fe^{2+}	mg/kg	7.43 ± 0.17
Mn^{2+}	mg/kg	19.79 ± 0.59
Cu^{2+}	mg/kg	0.67 ± 0.05
Zn^{2+}	mg/kg	0.68 ± 0.03
B	mg/kg	1.354 ± 0.03
SO_4^{2-}	meq/l	56.02 ± 6.89
Cl^-	meq/l	817 ± 25.93
CO_3^{2-}	meq/l	
HCO_3^-	meq/l	6.4 ± 0.82
Bacterial population	CFU/g	$1.4 \pm 0.368 \times 10^4$
TPH	mg/kg	82895 ± 5330.13

Data are mean of three replicates (±SD).

of carbon. The samples were incubated for 7 days in a shaker incubator (150 rpm and 40°C). To complete the enrichment process, 10 ml of the enriched medium was transferred to 90 ml of the fresh saline medium under all previous conditions, and this operation was repeated four times. Upon completion of the enrichment process, a serial dilution was prepared from the final enriched culture medium and transferred to plates containing the modified 6SW-Vit agar culture medium. To select the superior isolate, the ability to degrade crude oil was assessed by pure isolates isolated from the enrichment process using the turbidity method (spectrophotometer JENWAY 6705 UV/Vis) in the 6SW-Vit culture medium (Kumar et al., 2008).

Biochemical and 16S rRNA Characterization

For primary identification and classification of the bacterial strain, various biochemical analyzes were conducted (Krieg and Manual, 1984). Qiagen kit Cat. No. 51504 was used to extract the total genomic DNA of the bacterial strain. The 16S rRNA gene of pure isolates was amplified, and then the purified PCR products were sequenced by Microsynth Company (Balgach, Switzerland). EzBioCloud and BLAST databases were used to determine the similarity of the sequence to other sequences of known bacteria.

Analyses in Culture Medium

Evaluation of Crude Oil Biodegradation

To select the highest biodegraded concentration of crude oil, four crude oil concentrations [0.25, 0.5, 1, and 2 (V / V)] were designated, and according to the spectrophotometric method (Rahman et al., 2002), the optimal concentration was determined. To more accurately investigate biodegradation, the selected optimal concentration of crude oil was poured in a 250-ml Erlenmeyer flask containing 50 ml of a modified 6SW-Vit medium, and the superior isolate (4% V) was inoculated in it with the initial population of 4.1×10^7 CFU/ml. The specimens were incubated in a shaker incubator (150 rpm and 40°C) for 7 days. After the incubation period, to evaluate the reduction in TPH, the specimens were dehydrated and deasphaltenated by anhydrous sodium sulfate and a PTFE syringe filter (0.45 μm, membrane solution), respectively. A GC-FID (Gas chromatography flame ionization detector) device (AGILENT, 6890N, HP5 column, United States) was used to measure the residual concentration of TPH.

Cell Surface Hydrophobicity Assays

Bacterial adhesion to hydrocarbons (BATH) test was used to measure cells' hydrophobicity with two different types of solvents (hexane as a nonpolar and dichloromethane as a polar solvent). The trend of changes in the hydrophobicity of the cell surface was examined every 24 h over a week. Bacterial cells were precipitated by centrifuging (TOMY SEIKO CO., LTD, RS-20IV) at 6,000 g for 20 min and washed twice with saline solution. The cells were kept in a buffer saline solution (pH = 7) containing 16.9 g L^{-1} K_2HPO_4, 7.3 g L^{-1} KH_2PO_4, 1.8 g L^{-1} urea, and 0.2 g L^{-1} $MgSO_4.7H_2O$ to achieve OD = 0.3 at 600 nm wavelength. Furthermore, 3 ml of the prepared culture medium and the same amount of hydrocarbons (n-hexane and dichloromethane) were poured into the test tubes, and after 5 min, they were mixed with a vortex at high speed for 60 s. After keeping the test tubes at room temperature (15 min) and measuring their turbidity at 600 nm, the percentage of hydrophobicity was calculated using the following equation (Rosenberg et al., 1980).

$$\text{Percentage of hydrophobicity} = (1 - (X / X_0)) \times 100$$

X is the optical density of the aqueous phase after addition of n-hexane and dichloromethane. X_0 is the optical density of the primary aqueous phase.

The percentage of the cells moved to the hydrocarbon phase indicates cell-surface hydrophobicity.

Biosurfactant Production Analyses and Surface Tension Measurement

The oil displacement analysis was conducted by first pouring 40 ml of distilled water into a petri dish (150 mm diameter) and then pouring 40 μl of crude oil onto it to form a thin layer of oil on the surface of the distilled water. Additionally, 10 μl of the cell-free modified 6SW-Vit medium were gently poured from a suitable height onto a Petri dish's center. At the end, the diameter of the clear halo zone was measured as a qualitative indicator of biosurfactant production (Morikawa et al., 2000).

To investigate the hemolytic activity of the cells, the blood agar medium (5% sheep's blood) was used to culture the bacterial strain. Then, it was incubated at 30°C for 48 h. The clear zone formed around the colony represents the production of biosurfactant (Carrillo et al., 1996).

The Wilhelmy plate method (KRUSS tesiometer) was also used to determine surface tension according to (Rosenberg et al., 1980).

Analyses in Microcosm

Microcosm Design

Four treatments were designed in this study including 1: bio-stimulated microcosm (SM) with substances such as Tween 80 surfactant (1% V/W), NH_4NO_3 as a source of nitrogen and KH_2PO_4 as a source of phosphorus, 2: bacterialized microcosm (BM) (10% V/W) with an initial population of 5.4×10^8 CFU/ml, 3: combined bio-stimulated microcosm and bacterialized microcosm (SB) and 4: natural attenuation (NA). To prepare the designed treatments, 100 g of the contaminated soil was poured into each jar, and the humidity in all treatments was considered 65% field capacity (FC). The C/N/P ratio of the soil was adjusted to 100/10/2 in the bio-SM. All treatments were incubated in a dark environment at 40°C for 60 days, and every 20 days, the activity of soil enzymes (dehydrogenase, polyphenol oxidase, and catalase), soil basal respiration, soil bacterial population, and biodegradation rate of TPH were examined. The treatments were exposed to the open air for 10 min every 7 days to provide oxygen, and the moisture content was adjusted to the desired amount every 7 days.

Enzymatic Analyses

To determine dehydrogenase activity, 3 g of the moist soil sample was thoroughly mixed with 30 mg $CaCO_3$, and then 0.5 ml of 3% triphenyl tetrazolium chloride (TTC) and 1.25 ml distilled water were added to each sample and incubated for 24 h at 37°C. Afterward, 10 ml of methanol was added to each sample and thoroughly mixed by Vortex, and then centrifuged at 4,000 rpm (15 min) for the precipitation process. Absorbance in the extract was measured at 485 nm (Casida et al., 1964). Eventually, dehydrogenase activity was determined as μg TPF g^{-1} moist soil h^{-1}.

To evaluate polyphenol oxidase activity, 1 g of the moist soil was poured into 50-ml glass falcons. Then 10 ml of the pyrogalic acid solution (1%) was added to it, mixed well and incubated at 30°C for 1 h. Subsequently, 2.5 ml of HCl (0.5 M) was gently added to it, and the resulting solution was extracted by ether with a separatory funnel. The organic phase was collected and spectrophotometrically read at 430 nm. The control had all the above conditions, and only 10 ml of distilled water was used instead of 10 ml of pyrogalic acid (Ma et al., 2003). Polyphenol oxidase activity was expressed as mg purpurogallin g^{-1} moist soil h^{-1}.

To assay catalase activity, 2 g of the moist soil with 40 ml of distilled water, and 5 ml of H_2O_2 (0.3%) were mixed and shaken (150 rpm, 20 min) and immediately after shaking, 5 ml of H_2SO_4 (3 M) was added to prevent further decomposition of H_2O_2. Finally, the resulting product was filtered, and 25 ml of the filtered solution was titrated with $KMnO_4$ (0.1 N) (Lin et al., 2009). Catalase activity was conveyed as ml $KMnO_4$ g^{-1} moist soil h^{-1}.

Soil Basal Respiration and Bacterial Population

100 g of the contaminated soil was poured in sterile glass jars closed with lids and then, NaOH (0.2 mol/l) was used to trap the CO_2 evolved from the soil incubation experiment. Finally, The NaOH solution was titrated by 0.1 M HCl, and it was presented as mg CO_2/kg soil/h (Schinner et al., 2012).

Soil microbial population was evaluated using the standard plate count (SPC) method with plates containing the modified 6SW-Vit agar culture medium.

TPH Biodegradation

Soil treatments were extracted every 20 days by ultrasound (Hielscher, UP400S, 400 W, 24 KHz) to measure biodegradation of TPH. Briefly, 4 g of the contaminated soil was mixed with a mixture of dichloromethane and acetone solvents (10:10 ml). For the dehydration process, 2 g of anhydrous sodium sulfate was added to the samples. The samples were then extracted (20 min, twice) and centrifuged (4,000 rpm, 10 min). The organic phase was transferred to other glass Falcons and spectrophotometrically read at a wavelength of 420 nm (Rahman et al., 2002).

Statistical Analyses

This study was conducted as a factorial experiment in a completely randomized design (CRD) with three replications. Before analysing variance, normality of distribution of the data (Jarque-Bera test), and homogeneity of variances (Levene's test) were analyzed using XLSTAT 2019, and SPSS 21, respectively. Two-way analysis of variance (ANOVA) was conducted to evaluate the interaction between microcosm treatments and different times (SAS 9.4). Duncan's post hoc test was applied to compare among multiple groups (SAS 9.4). Pearson product-moment correlation was conducted to determine the relationships between the studied microcosm biological characteristics and the amount of residual TPH (SAS 9.4). Probability levels of 0.01 were considered statistically significant. Microsoft Excel software 2013 was used to draw graphs.

RESULTS AND DISCUSSION

Selection and Identification of Bacterial Strain

The enrichment process only yielded two isolates after 5 weeks. The turbidity results indicated that merely one of the two isolates was efficient enough to create turbidity so that it was selected as the superior isolate for further studies. The 16S rRNA gene sequencing results signified our strain's highest affinity (100%) with *Bacillus altitudinis* strain 41KF2b$^{(T)}$ under the accession number of ASJC01000029. The strain was registered in the gene bank under *B. altitudinis* strain HRG-1$^{(T)}$ under the accession number of MN590432. This strain was seamlessly nurtured in the pH range of 5.5 to 8.5 (optimum pH = 7), NaCl concentrations of 0 to 5% (optimum 3%) and temperature range of 20 to

55°C (optimum 30 °C). **Table 2** shows some morphological and biochemical characteristics of the strain HRG-1.

Biodegradation of TPH in the Modified 6SW-Vit Medium

Among the four selected crude oil concentrations, 0.25% displayed the highest amount of degradation in the liquid medium; hence, it was selected as the optimal concentration (**Supplementary Figure 1**). The GC-FID results revealed that the HRG-1 strain could degrade 73.3% ± 6.58 of TPH in the modified 6SW-Vit medium after 7 days (**Figure 1**). In one study conducted under optimal conditions, Liu et al. (2016), reported that after 5 days, *Bacillus licheniformis* strain Y-1 degraded 60.2% of crude oil. In another study, after 12 days of incubation, *Dietzia* sp. Strain CN-3 efficiently degraded 91.6% of crude oil (0.5% w/v) in the liquid medium (180 rpm, 30°C) (Chen et al., 2017).

Bacterial Adhesion to Hydrocarbons Test

Biosurfactants can be adsorbed by the cell surface, thereby increasing cell surface hydrophobicity (Ahimou et al., 2000). Cell surface hydrophobicity test is one of the exciting experiments in environmental engineering and other microbial disciplines (Saini, 2010). The results showed that the highest and lowest hydrophobicity levels were related to dichloromethane solvent (88.44%) on the 7 day, and (52.55%) on the 5 day of the experiment, respectively (**Figure 2A**). No significant difference between dichloromethane and n-hexane was observed, except on the 5th, 6th, and 7th days. The reason that such changes in target hydrocarbons result in changes in hydrophobicity is probably the different viscosity of hydrocarbons as well as the size of the droplets formed during the mixing process (Rosenberg, 1984).

TABLE 2 | Some morphological and biochemical characteristics of HRG-1 strain.

Test	Result
Shape	Rod-shaped
Color	Creamy
Gram reaction	+
Spore	+
Motility	+
Fermentations	
Glucose	+
Maltose	–
Mannitol	+
Fructose	+
Arabinose	–
Lactose	–
Glycerol	–
Casein hydrolysis	–
Nitrate reduction	–
Citrate utilization	+
Oxidative/Fermentative	O/F
Catalase	+
Oxidase	+
Lipase	+
Urease	–
Lecithinase	–
α-amylase	–

Evaluation of Oil Displacement, Hemolytic Activity and Changes in Surface Tension

The oil displacement test is a qualitative study of the presence of biosurfactants in solution. The diameter of the clear zone on the surface is directly related to the presence of biosurfactants (Ibrahim et al., 2013). Our study indicated that the diameter of the clear zone on the thin layer of oil was 31 mm, suggesting the production of biosurfactants during the biodegradation of crude oil (**Supplementary Figure 2a**).

Another hint for biosurfactant production is the lysis of red blood cells; HRG-1 strain did it best and caused hemoglobin breakdown (**Supplementary Figure 2b**). Consistent with the classifications proposed by Carrillo et al. (1996) and creating a white and clear zone around the colony, this strain was placed in the type of β-hemolysis.

One of the core indicators of biosurfactants' production is a decrease in surface tension (Diniz Rufino et al., 2014). Biosurfactants boost hydrocarbon compounds' surface area, thereby allowing further degradation (Batista et al., 2006; Hassanshahian et al., 2014). In this investigation, changes in surface tension in the modified 6SW-Vit medium during 7 days of biodegradation exhibited the highest surface tension related to the initial time (47.81 mN/m), while the lowest one to the 7th day (28.22 mN/m). However, after 7 days, the surface tension dropped by 40.98% (**Figure 2B**). In a study, Goswami and Deka (2019) reported that after 48 h of incubation, biosurfactant producing bacterium (*B. altitudinis* strain MS16) reduced the surface tension of the culture medium from 72.8 to 32.3 mN/m.

Biological Assays in Crude Oil-Contaminated Microcosm

In some cases, when the issue of oxidation of soil organic matter is raised, dehydrogenase activity is a worthy indicator for organic matter changes, particularly in petroleum-contaminated soils, and it is part of the respiratory process of soil microorganisms (Das and Varma, 2010). The results of this study displayed that the amount of dehydrogenase activity (in all treatments) at the end of the first 20 days was higher than that at the beginning of the experiment (99.6 ± 2.82 μg TPF g^{-1} moist soil h^{-1}). Nevertheless, in all treatments, the highest and lowest dehydrogenase activity was observed in the first and third 20 days, respectively. Changes in dehydrogenase activity throughout the experiment had a decreasing trend, being consistent with the notions reported by Chen et al. (2019). The highest dehydrogenase activity during the experimental period was related to the BM treatment in the first 20 days (276.5 μg TPF g^{-1} moist soil h^{-1}). The lowest was related to the SM treatment in the third 20 days (50.26 μg TPF g^{-1} moist soil h^{-1}). The results clearly revealed that the application of biostimulation prevented the increase of dehydrogenase activity during the incubation period (**Figure 3A**).

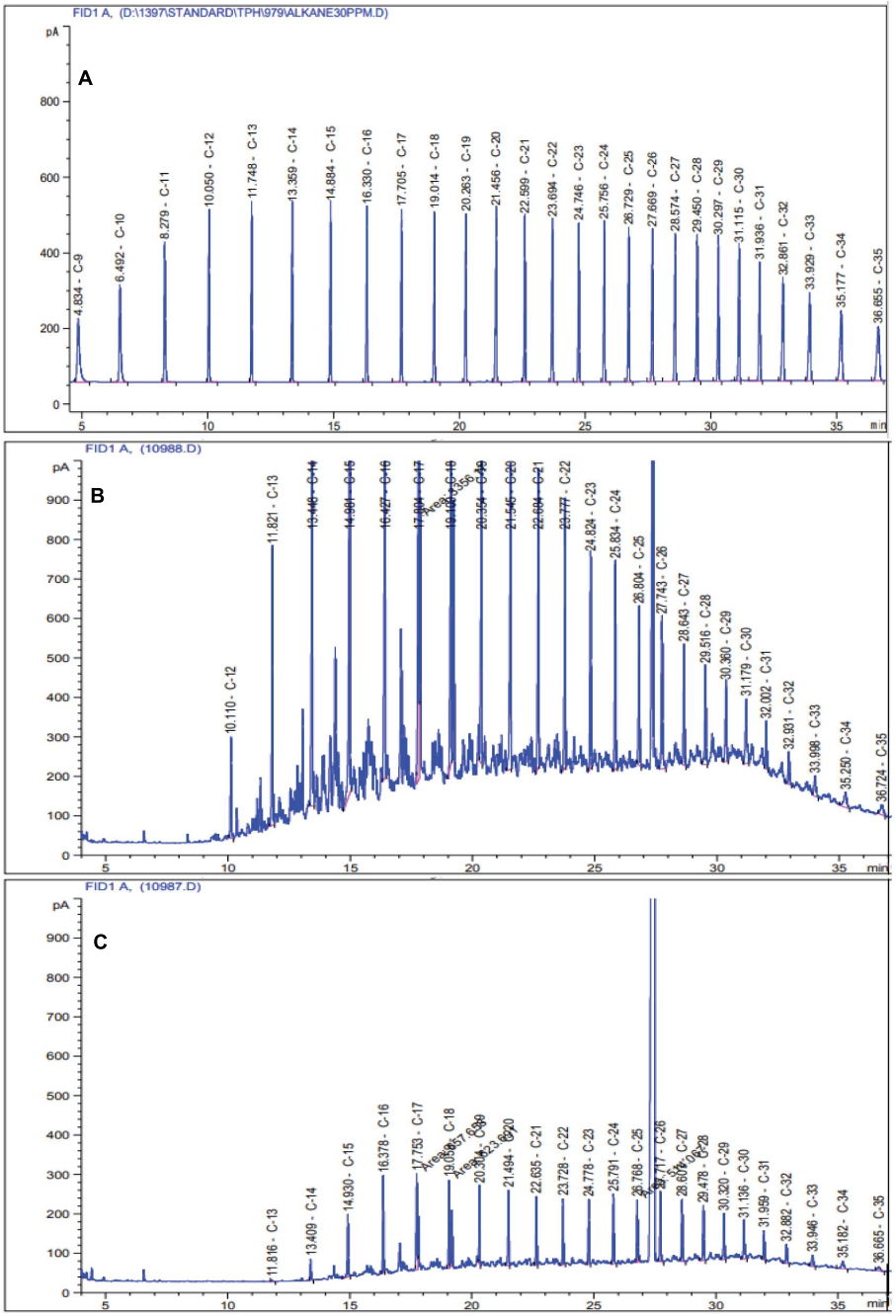

FIGURE 1 | Chromatograms of residual TPH in the modified 6SW-Vit medium after 7 days of incubation at 40 °C temperature. **(A)** standard mix solution of TPH, **(B)** non-biodegraded control, and **(C)** biodegraded treatment.

Polyphenol oxidase plays a crucial role in the degradation of aromatic hydrocarbons (phenolic compounds); it is an essential indicator of microbial activity, particularly in contaminated soils (Dindar et al., 2015). The results of the present study revealed that the polyphenol oxidase activity in all treatments (except SB) in the first 20 days of incubation was increased compared to its initial time (0.256 ± 0.019 mg purpurogallin g^{-1} moist soil h^{-1}). During 60 days of incubation, the lowest and the highest polyphenol oxidase activity was associated with the SM treatment (0.212 mg purpurogallin g^{-1} moist soil h^{-1}) in the first 20 days and the BM treatment (0.662 mg purpurogallin g^{-1} moist soil h^{-1}) in the second 20 days, respectively (**Figure 3C**). In their study, Wu et al. (2017) found that polyphenol oxidase activity in all treatments reached its maximum on the 7th day. Then, it rapidly dropped toward the end of the experiment, being in line with the polyphenol oxidase activity changes in our

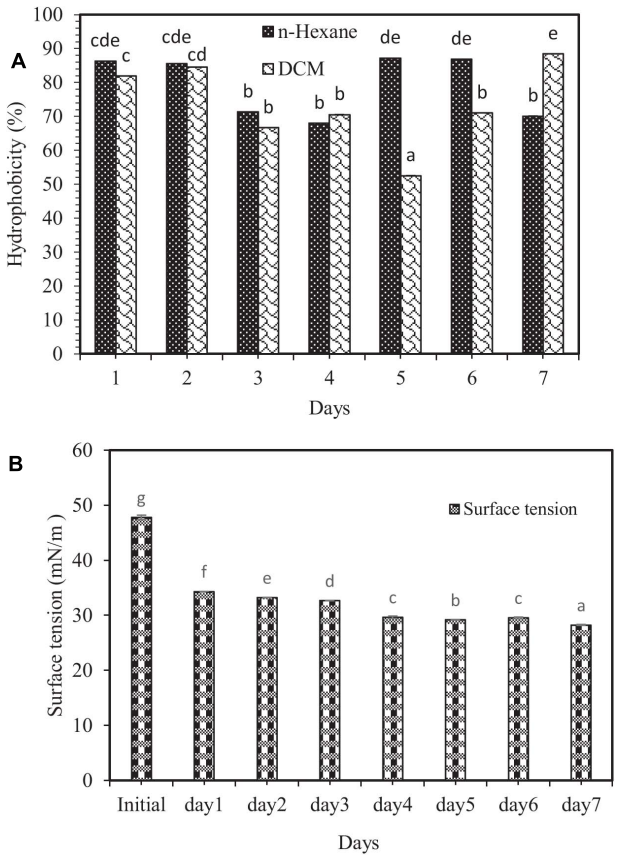

FIGURE 2 | (A) Changes in the percentage of cell surface hydrophobicity, and **(B)** surface tension variations in the modified 6SW-Vit medium amended with crude oil at 7 days. Means with the same letters are not significantly different at ($P \leq 0.01$). Error bars are not clearly visible because they are shorter than the symbol size and represent the standard deviation ($n = 3$). DCM and n-hexane are the abbreviations of dichloromethane and normal-hexane, respectively.

study. It is noteworthy that similar to dehydrogenase activity, the application of biostimulation prohibited the increase of polyphenol oxidase activity during the incubation period.

Catalase diminishes the harmful effects of heavy metals and hydrocarbons by performing its catalytic function in the degradation of H_2O_2; thus, it is interesting to study it in contaminated soils (Achuba and Okoh, 2014). Its unique characteristics such as time-consuming performance, low cost and ease of use have made it highly useful to study catalase activity (Chelikani et al., 2004). The results of this study presented that the activity of catalase was decreased from the starting point of the experiment (1.6 ± 0.081 ml $KMnO_4$ g $^{-1}$ moist soil h^{-1}) to the end of the second 20 days of incubation. However, in the third 20 days, a slight increase in this enzyme activity was detected compared to the second 20 days. The highest and lowest catalase activity during the experimental period was related to the NA treatment in the third 20 days (1.783 ml $KMnO_4$ g $^{-1}$ moist soil h^{-1}), and the SB treatment in the second 20 days (0.3 ml $KMnO_4$ g^{-1} moist soil h^{-1}), respectively (**Figure 3C**). Wu et al. (2016), reported that the higher the concentration of petroleum compounds is, the lower the catalase activity is. Instead, other investigation proved that the higher the concentration of petroleum compounds was, the higher the catalase activity was (Borowik et al., 2017).

Soil basal respiration, and microbial structure and population are acceptable criteria providing practical information on living microorganisms and demonstrating the long-term or short-term effects of contaminants on microorganisms (Schaefer et al., 2005). To provide a comprehensive definition of soil basal respiration, one should refer to all metabolic reactions obtained from the degradation of soil organic matter as a considerable indicator of microorganisms' activity in the soil, especially in contaminated ones (Dos Santos et al., 2012). The results indicated that soil respiration was gradually increased from the beginning of the experiment (0.16 ± 0.008 mg CO_2/kg soil/h) to the end of the 60th days of incubation in all treatments. Nonetheless, in the SB treatment, the upward trend continued as far as the end of the second 20 days, and then it was decreased. The maximum and the minimum amount of soil respiration was related to the BM treatment in the third 20 days (0.693 mg CO_2/kg soil/h) and the SM treatment in the first 20 days (0.226 mg CO_2/kg soil/h), respectively (**Figure 3D**). However, no significant difference in the respiration rate was observed between BM and SB treatments during the whole incubation period.

Toxic substances (petroleum-based hydrocarbons) can dramatically decrease the activities and the efficiency of degrader microorganisms, particularly when the organisms are inoculated into contaminated soil or water (Mariano et al., 2007). Therefore, an examination of the viability of the microorganisms provides us with pivotal information during biodegradation. Consistent with the initial soil bacterial population ($1.4 \pm 0.368 \times 10^4$ CFU / g), the experiments showed that the largest and smallest bacterial population was recorded in the BM treatment in the second 20 days (4.3×10^6 CFU / g) and the SM treatment in the third 20 days (2×10^3 CFU / g), respectively (**Figure 3E**). During the incubation period, no significant difference was observed between BM and SB treatments. However, the SM treatment presented all the smaller microbial population than the NA treatment did. Margesin (2000) revealed that biostimulation had a significant adverse effect on the population of degrading microorganisms, being consistent with the results obtained in our study.

Biodegradation of TPH in Crude Oil-Contaminated Microcosm

The older the soil organic contaminants are, the greater their pernicious lingering in the soil is, and the lower their degradability is (Hatzinger and Alexander, 1995). Under other conditions, in saline soils, the diversity and population of microorganisms remain low, and the solubility of petroleum compounds and oxygen reduces, which in turn can reduce the biodegradation of petroleum contaminants (Whitehouse, 1984). Moreover, considering the fact that this soil has been contaminated with crude oil for more than 60 years and has saline-sodic nature, removing the contamination from it is highly

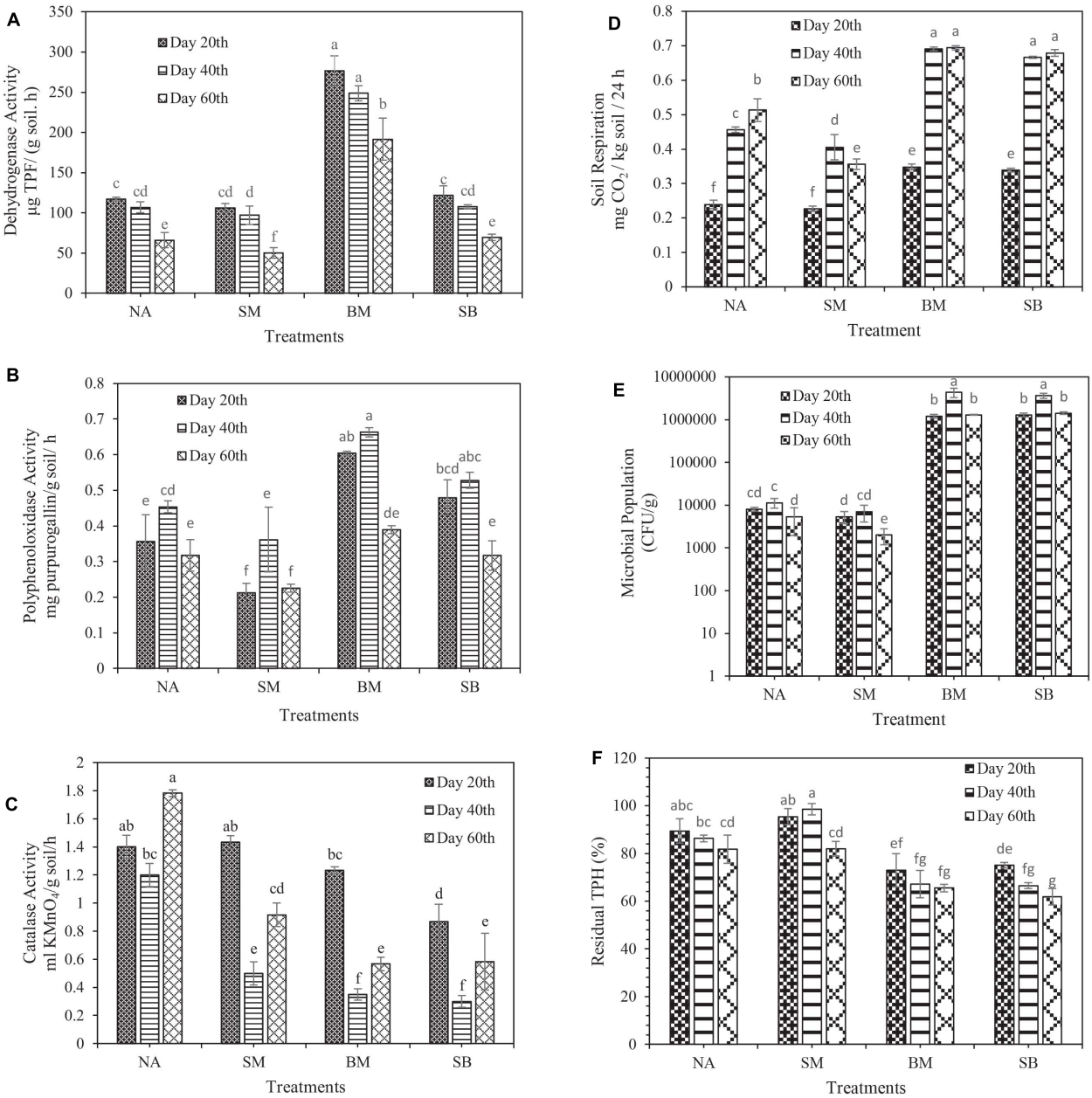

FIGURE 3 | Evaluation of changes in biological characteristics of crude oil-contaminated soil and biodegradation of TPH during 60 days of incubation. **(A)** Dehydrogenase activity, **(B)** polyphenol oxidase activity, **(C)** catalase activity, **(D)** soil basal respiration, **(E)** microbial population, and **(F)** biodegradation of TPH. (NA, natural attenuation; SM, bio-stimulated microcosm; BM, bacterialized microcosm, and SB, combined bio-stimulated microcosm and bacterialized microcosm). Means with the same letters are not significantly different at ($P \leq 0.01$). Error bars indicate the standard deviation ($n = 3$).

challenging and requires the adoption of various biological approaches. The results indicated that at the end of the 60th days of incubation, SB, BM, NA, and SM treatments reduced TPH by 38.2% ± 3.43, 34.4% ± 1.6, 18.2% ± 5.99, and 18% ± 3.11, respectively (**Figure 3F**). Notably, no significant difference was observed between SM and NA treatments at the end of the incubation period, and our results remained skeptical about the application of biostimulation alone as one of the bioremediation strategies. In this study, contrary to other reports by Kauppi et al. (2011), Wu et al. (2016), the biostimulation strategy had a negligible effect on the biodegradation of TPH even lower than NA had, which is due to the effect of surfactant (tween 80) on increasing the availability of contaminants (Cheng et al., 2008, 2017) and probably exposure of negligible soil microorganisms to excessive toxicity of these contaminants. Another point is that the preferential degradation of surfactants can also prevent contaminants' biodegradation (Li and Chen, 2009). For instance, in an investigation reported by Bruheim et al. (1999), the use of

the Corexit 9,527 reduced the biodegradation of alkanes in crude oil. Some reports demonstrate the opposite effect of Tween 80 on the biodegradation of contaminants (Pathak et al., 2009). Another assumption is that due to the large size of soil particles in this investigation (sandy-loam), contaminants are separated much easier from soil particles (Abdel-Moghny et al., 2012). However, the effect of the aforementioned and the surfactant usage might all the more increase the availability of contaminants, thereby exposing the small number of soil microorganisms to excessive toxicity. Interestingly, the results showed that in soils with a low population of native microorganisms, the use of biostimulation alone is not appropriate and capable microorganisms must be used for bioremediation process.

This study, as in some other studies, Garg et al. (2016), Roy et al. (2018), Varjani and Upasani (2019), Zeneli et al. (2019), shows that a combined strategy is the best technique to degrade hydrocarbon contaminants from the environment, especially in soils with old as well as excessive contamination. However, to achieve more significant biodegradation in old petroleum-contaminated soils, the use of potent microorganisms must be considered. Considering the limited reports on the performance of *B. altitudinis* in the bioremediation of hydrocarbon compounds (Yetti et al., 2016; Shahzad et al., 2020), this is the first report on the effectiveness of this strain in reducing TPH in highly contaminated aged soils with a high amount of salinity.

Correlation and Analysis of Variance

The results of a correlation between soil biological indicators (enzymatic activities, soil basal respiration, and microbial population) and the amount of residual TPH in the microcosm environment showed a weak negative correlation for dehydrogenase activity ($r = -0.34990$, $P = 0.0364$), a moderate negative correlation for polyphenol oxidase activity ($r = -0.43842$, $P = 0.0075$), a moderate positive correlation for catalase activity ($r = 0.48323$, $P = 0.0028$), a strong negative correlation for soil respiration ($r = -0.73915$, $P < 0.0001$), and a moderate negative correlation for bacterial population ($r = -0.65218$, $P < 0.0001$). However, among the studied enzymes, dehydrogenase activity demonstrated the lowest correlation with the soil residual TPH content. Contrary to dehydrogenase and polyphenol oxidase activities with a weaker and negative correlation, catalase exhibited a stronger and positive correlation than the other two enzymes did. However, the results proved that the most reliable biological indicator in correlation with the soil residual TPH is the soil basal respiration.

The results of analysis of variance showed that the effects of treatment and time were individually significant for all microcosm indicators under investigation during the experiment period at ($P \leq 0.0001$). The results of the interaction between treatments and times showed that catalase ($F = 6.3$, $P = 0.0004$) and soil basal respiration ($F = 6.2$, $P = 0.0005$) were significant at the 1% probability level ($P \leq 0.01$), but the other biological indicators were not significant (dehydrogenase ($F = 2.06$, $P = 0.096$), polyphenol oxidase ($F = 2.48$, $P = 0.052$), microbial population ($F = 2.08$, $P = 0.093$), and biodegradation of TPH ($F = 1.4$, $P = 0.255$).

CONCLUSION

In the present study, due to the long aging of soil contaminants, SB was the most effective and robust strategy to reduce TPH (38.2%) over a 60-day period. In heavily old aged petroleum-contaminated soils, considering the small number of native soil microorganisms as the main limiting factor for the bioremediation process, the biostimulation strategy alone was insufficient and not recommended. However, no significant difference in alleviating TPH was observed between SM and NA treatments at the end of the experiment. Regarding the vast difference in mitigating TPH between bacterial inoculated treatments and bio-stimulated treatment alone, it is concluded that the use of suitable microorganisms is necessary to reduce contamination in such soils. Therefore, the results indicated that the hydrophobic and biosurfactant-producing strain HRG-1 could play a pivotal role to remediate highly contaminated aged soils. The statistical analysis revealed that catalase had the highest correlation (positive) with the amount of soil residual TPH only in the studied enzymes. However, soil basal respiration indicated that it was more reliable than the other biological indicators were regarding its correlation with the amount of residual TPH in this soil with such a long history in terms of contamination.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

HG: conception and design of study, analysis and/or interpretation of data, writing and drafting the manuscript, and revising the manuscript critically for final version. AP: conception and design of study, analysis and/or interpretation of data, and revising the manuscript critically for final version. HA: interpretation of soil analysis. NY: interpretation of chemical analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Microbial Degradation of Naphthalene and Substituted Naphthalenes: Metabolic Diversity and Genomic Insight for Bioremediation

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Low molecular weight polycyclic aromatic hydrocarbons (PAHs) like naphthalene and substituted naphthalenes (methylnaphthalene, naphthoic acids, 1-naphthyl *N*-methylcarbamate, etc.) are used in various industries and exhibit genotoxic, mutagenic, and/or carcinogenic effects on living organisms. These synthetic organic compounds (SOCs) or xenobiotics are considered as priority pollutants that pose a critical environmental and public health concern worldwide. The extent of anthropogenic activities like emissions from coal gasification, petroleum refining, motor vehicle exhaust, and agricultural applications determine the concentration, fate, and transport of these ubiquitous and recalcitrant compounds. Besides physicochemical methods for cleanup/removal, a green and eco-friendly technology like bioremediation, using microbes with the ability to degrade SOCs completely or convert to non-toxic by-products, has been a safe, cost-effective, and promising alternative. Various bacterial species from soil flora belonging to *Proteobacteria* (*Pseudomonas*, *Pseudoxanthomonas*, *Comamonas*, *Burkholderia*, and *Novosphingobium*), *Firmicutes* (*Bacillus* and *Paenibacillus*), and *Actinobacteria* (*Rhodococcus* and *Arthrobacter*) displayed the ability to degrade various SOCs. Metabolic studies, genomic and metagenomics analyses have aided our understanding of the catabolic complexity and diversity present in these simple life forms which can be further applied for efficient biodegradation. The prolonged persistence of PAHs has led to the evolution of new degradative phenotypes through horizontal gene transfer using genetic elements like plasmids, transposons, phages, genomic islands, and integrative conjugative elements. Systems biology and genetic engineering of either specific isolates or mock community (consortia) might achieve complete, rapid, and efficient bioremediation of these PAHs through synergistic actions. In this review, we highlight various metabolic routes and diversity, genetic makeup and diversity, and cellular responses/adaptations by naphthalene and substituted naphthalene-degrading bacteria. This will provide insights into the ecological aspects of field application and strain optimization for efficient bioremediation.

Keywords: biodegradation, metabolic pathways, genetic diversity, gene transfer mechanisms, cellular responses and evolution, bioremediation, naphthalene, substituted naphthalenes

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INTRODUCTION

The rapid expansion of industries (petrochemical, agricultural, pharmaceutical, textile and dyes, cosmetic, etc.) has led to global economic prosperity and better living standards. This exponential development has resulted in the generation of a huge volume of synthetic organic compounds (SOCs) which are used in the manufacturing of various products. These xenobiotics or SOCs include polycyclic aromatic hydrocarbons (PAHs), pesticides, herbicides, plasticizers, dyes, pharmaceutical products, organophosphates, flame retardants, volatile organic solvents, etc. Their release into atmospheric, aquatic, and terrestrial ecosystems exerts multidimensional effects by altering physicochemical properties and community structure and wielding deleterious effects on various living forms (Petrie et al., 2015; Bernhardt et al., 2017; Sarkar et al., 2020). Many aromatic pollutants have shown critical and deteriorating effects on a number of pristine ecosystems/biodiversity hot spots like coral reefs, Arctic/Antarctic ice caps, high-altitude lakes, deep sea sediment, etc. (Jones, 2010; Beyer et al., 2020; Nordborg et al., 2020). Recent geomicrobiological studies have shown that the deposition of synthetic organics (like aromatic pollutants) and its derivatives on the surface of human-built structures (built environment), i.e., cultural heritages and granitic/stone/wooden/metal monuments, is accelerating its decay (Gadd, 2017; Liu et al., 2018). Human activities can enhance and intensify the biodeterioration of monuments and structures through atmospheric pollution and climate change (Liu et al., 2020). These organic pollutants react with each other in the presence of atmospheric water vapor and get deposited on to the structures leading to physical and chemical deterioration of the material. Biodeterioration has been widely recognized as biologically induced undesirable change in the appearance and properties of a material, affecting its preservation (Pochon and Jaton, 1967). Further microbial influences (metabolism) of these compounds diminish structural integrity, preservation, and cultural importance (Gadd, 2017; Liu et al., 2018). On the other hand, in a few cases, microbial adaptation on these structures and their response were found to be beneficial due to the formation of biofilm and other protective encrustations, lowering the decay/decomposition rate (Martino, 2016). Therefore, effective strategies for the long-term sustainable conservation of stone/metal/wood monuments require an in-depth understanding of the key processes involved. Compared with natural processes (geological processes, wild forest fires, volcanic eruptions, plant and bacterial reactions), anthropogenic activity results in the release of large quantities of PAHs and other SOCs into the ecosystems. Many of the PAHs used in agriculture (insecticides and pesticides like DDT, atrazine, carbaryl, pentachlorophenols, etc.), industries (crude oil, oil sludge/waste, petroleum-derived plastics, polychlorinated biphenyls, plasticizers, detergents, disinfectants, fumigants, fragrances, and preservatives), personal care products (sunscreens, antiseptics, insect repellent, and polycyclic musks), and ammunition (explosives such as 2,4,6-TNT) are potential xenobiotics and impact the planetary health (Srogi, 2007; Vamsee-Krishna and Phale, 2008; Petrie et al., 2015). This list can be further broadened with compounds derived from petroleum products (fuel oil, lubricants, asphaltene), high molecular weight bioplastics, and ionic liquids (Amde et al., 2015). The list of a wide spectrum of aromatic pollutants and its use in various industries is depicted in **Table 1**. The recent past has also witnessed the onset of ramped-up levels of anthropogenic emissions of volatile organics along with CO_2 and other greenhouse gases (Dvorak et al., 2017). Nevertheless, the anthropogenic input far exceeds the natural sources. In addition, a spectrum of SOCs is found to be present persistently in many ecological compartments and designated as emerging contaminants which have shown adverse negative effects on living community (**Figure 1**). Environmental authorities like the United States Environmental Protection Agency (USEPA) have recognized many of them as priority pollutants due to cytotoxic, genotoxic, mutagenic, and carcinogenic activities. Thus, strict guidelines for their disposal and effective strategies for their cleanup/removal from polluted ecosystems are demanded. Various physical and chemical cleanup methods like pyrolysis, oxidative thermal treatment, air-sparging, land-filling, incineration, etc., which are ineffective and expensive, have led to the generation of corrosive, toxic, and recalcitrant by-products. With the increase in global environmental awareness, microbes with the ability to degrade these pollutants and their derivatives (like halo-, nitro-, alkyl-, and/or methyl-) have received an increasing attention (Fennell et al., 2004; Haritash and Kaushik, 2009; Phale et al., 2020; Sarkar et al., 2020; Schwanemann et al., 2020). The use of these indigenous microbial candidates either alone or as mixed culture (consortia) for the removal of aromatic pollutants has been advantageous in terms of environmental safety, cost, efficiency, effectiveness, and sustainability. Researchers are also exploring the combined application of microbiological processes and electrochemical oxido-reduction methods, termed as bioelectrochemical systems (BESs), as a promising technology for pollutant treatment/removal (Huang et al., 2011). Due to its high efficiency, low cost, environmental safety, ambient operating temperatures with biologically compatible materials, and the recovery of by-products of value (e.g., electricity, fuels, and chemicals), BESs are gaining attention (Pant et al., 2012; Nazari et al., 2020). The advent of high-throughput genome sequencing and omics tools/techniques are significantly adding new information on genetic regulation and proteomic and fluxomic responses of several degrading microbes. The combination of such tools with system biology is further expanding our knowledge on the selection and fine-tuning of target catabolic pathways (as metabolic designing) of microbes for effective and efficient biodegradation. In order to develop effective bioremediation strategies by suitable microbial candidate(s), one needs to understand the biochemical potential, metabolic diversity, and genetic makeup as well as the organism's ecology (auto-/syn-ecology).

In this review, we attempt to summarize the degradation of simple PAHs like naphthalene and substituted naphthalenes by various bacterial isolates with respect to metabolic pathways and diversity, enzymes involved in the degradation, the genetic makeup/content and diversity, cellular responses, and various bioremediation aspects. Understanding at the biochemical and

TABLE 1 | Aromatic compounds used in various industries.

Industries	Compounds	Products
Pharmaceuticals	Lincomycin, sulfathiazole	Antibiotics, antiparasitic agents, ionophores
	Amphetamine, bezafibrate, codeine, carbamazepine, diazepam, ephedrine, ibuprofen, fluoxetine, metformin, methandone, propranolol, valsartan, tramadol, morphine, phthalates, phthalate esters, tamoxifen, warfarin	Anti-inflammatory, anticoagulants, hallucinogens, analgesics, antidepressants, lipid regulators, flexible tubings, blood bags, plastic wares
	Estrone, estriol, mestranol, cholesterol	Synthetic estrogens, androgens
Agricultural, household products, various industries and their wastewater	Naphthalene, creosote, mothballs, methyl naphthalene, carbaryl, chlorpyrifos, diethyl phthalate, tri(2-chloroethyl) phosphate, anthracene, 2,6-di-*tert*-butylphenol, 1,2,3-trichloropropane, phenol, dichlorobenzene, acetophenone, asphalt, coal tar	Insecticides, plasticizers, detergents, flame retardants, feedstock, disinfectants, greasing agents, fumigants, fragrances, food preservatives, waste sludge, crude oil
Personal care products	Bisphenol A, 1-benzophenone, methyl naphthalene, methylparaben, triclosan, phthalates	Odor repellents, polycyclic musks, sunscreen agents, fragrances, antiseptics, emulsifier, preservatives
Arms and ammunition	TNT, nitro-aromatics	Explosive, fire retardants

molecular levels will help in identifying a suitable host strain which can be further genetically engineered for effective and efficient bioremediation of such priority pollutants. This will further aid in developing strategies to formulate site-specific consortia for effective bioremediation.

AROMATIC COMPOUNDS: SOURCE, FATE, AND IMPACT ON THE BIOSPHERE

The preponderance of toxic and hazardous aromatic compounds (satisfying Huckel's rule of $4n + 2$ π electrons, $n = 1, 2, 3, \ldots$) is posing an alarming threat to various ecological compartments like air, soil, sediment, and surface and groundwater (Puglisi et al., 2007). With either single- (monocyclic) or multiple-benzene rings (polycyclic) in linear, angular, or cluster arrangement, these compounds have shown stability (persistence/recalcitrance) in the environment due to higher negative resonance energy and inertness (unreactive), which can be attributed to their hydrophobicity and reduced state. Further substitution of aromatic ring(s) by methyl ($-CH_3$), carboxyl (–COOH), hydroxyl (–OH), or sulfonate ($-HSO_3$) groups renders them more stable, with higher affinity toward macromolecules and bioaccumulation property in the biological systems (Seo et al., 2009; Phale et al., 2020). Several of such low molecular weight polycyclic aromatic hydrocarbons (LMW-PAHs), viz. naphthalene and its derivatives [methyl naphthalenes, naphthoic acids, naphthalene sulfonates and 1-naphthyl *N*-methylcarbamate (carbaryl)], have been considered as priority organic pollutants by the USEPA with genotoxic, mutagenic, and/or carcinogenic effects (Cerniglia, 1984). The release of such LMW-PAHs in the environment results in the bioaccumulation of these compounds in the food chain at various levels, thus affecting the health of the ecosystem (Binkova et al., 2000; Srogi, 2007; Quinn et al., 2009).

The sources and routes of exposure of PAHs to biota primarily occur through transport and interaction among various components of the ecosystem like soil, groundwater, surface water, crops, and atmosphere (Arey and Atkinson, 2003). The interplay and partitioning of various LMW-PAHs in the ecosystem and its exposure routes to biota/human are depicted in **Figure 1**. Atmospheric contamination and transport (drifting) through vehicular emission, industrial exhaust (coal gasification, combustion, and coke production), and its precipitation leads to PAH deposition on surfaces. Industrial activities like the production of synthetic textiles, dyes, and paint; wood preservation; rubber processing; cement manufacturing activity; pesticide production; and application in agriculture are the main contributors of PAHs in terrestrial and aquatic systems (Bamforth and Singleton, 2005; Wick et al., 2011). It has been shown that soils of peri-urban to urban areas, near highway sites, and larger cities are more exposed to PAHs because of emissions from power plants, residential heating, airway/road traffic burden, and construction activities (Suman et al., 2016). Wang et al. (2008) have shown a maximum of 7,189 μg/kg PAHs in soils close to the roads in comparison to open spaces (2,404 μg/kg) in New Orleans, LA, United States. Similarly, as high as 300 g/kg PAHs have been reported from areas near coal gasification sites in various cities of the United States (Kanaly and Harayama, 2000; Bamforth and Singleton, 2005). Soils from various cities of India like Delhi (Sharma et al., 2008), Agra (Dubey et al., 2014), Mumbai (Kulkarni and Venkataraman, 2000), and Visakhapatnam (Kulkarni et al., 2014) were reported to contain higher concentrations of PAHs. Aromatic compounds have a higher tendency to adsorb onto soil particles, organic matter, and clay minerals, thus acting as a major sink in the ecosystem (Srogi, 2007; Peng et al., 2008). Atmospheric deposition (wet/dry deposition and vapors), runoff from urban sites, wastewater discharges, groundwater recharge, etc. are the major contributors for PAHs into aqueous ecosystems (Srogi, 2007). It has been estimated that around 80% of total PAHs in the marine ecosystem have originated from atmospheric precipitation, deposition, and dumping of waste (Motelay-Massei et al., 2006; Srogi, 2007). The higher levels of PAHs in surface water or leachates from solid waste disposal sites ultimately channel to groundwater, thus creating more vulnerability to the community's health, as groundwater is consumed by over 70% of populations of Southern and South-Eastern Asia (Duttagupta et al., 2019). A recent study by Duttagupta et al. (2020) involving samples from river (32) and groundwater (235) locations of West Bengal, India, has shown

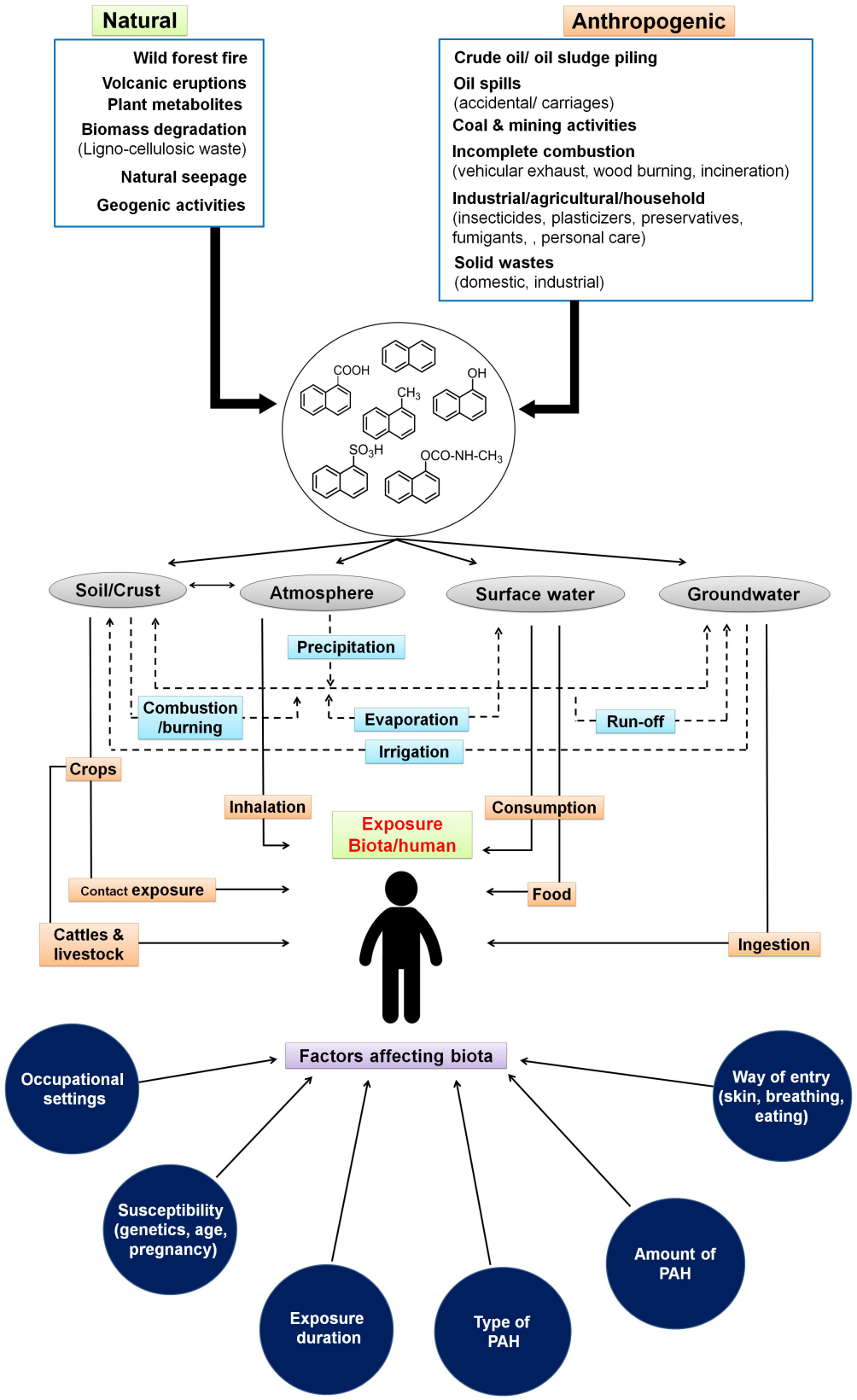


FIGURE 1 | Sources and routes for entry of low molecular weight polycyclic aromatic hydrocarbons through various ecological compartments and various factors affecting biota. Dotted lines indicate the interaction among various compartments of the ecosystem.

that an estimated 53% of urban and 44% of rural residents (total of 20 million residents) are potentially exposed to naphthalene (4.9–10.6 μg/L) and its derivatives. Differential land-use pattern and increased groundwater pumping/abstraction have been suggested as main controlling factors for vertical transport (advection) of LMW-PAHs in subsurface regimes. River basins and subsurface sediments are found to be impacted by PAHs due to agricultural runoff and domestic and industrial wastewater discharge as well as solid waste/garbage dumping. The loadings are further enhanced through atmospheric precipitation. Higher concentrations of PAHs and its alkyl derivatives (total of 51 types) have been reported from various rivers/river basins across the globe like Fraser, Luanhe, Densu, Missouri, Anacostia, Ebro, Delaware, etc. (Yunker et al., 2002; Motelay-Massei et al., 2006; Li et al., 2010; Amoako et al., 2011; Kim et al., 2018). Naphthalene and phenanthrene are found to be the most predominant (detected in 70% of the samples) in the sediments of the Ganga River basin (Duttagupta et al., 2019). It has been also observed that chlorination of drinking water may lead to the formation of more toxic oxygenated and chlorinated PAHs (Manoli and Samara, 1999). Accumulation of PAHs into grains, fruits, and vegetables occurs through uptake by plants from contaminated soil, groundwater, and atmospheric deposition (Fismes et al., 2002). Many aquatic biota like fish, mussels, shellfish, and shrimp are found to be contaminated with PAHs through ingestion of contaminated food, marine water, and absorption in the tissues and skin (Mackay and Fraser, 2000). Food cooking/processing methods like grilling, barbecuing, smoking, frying, roasting, drying, baking, and charbroiling also contribute a significant amount of PAHs into foods. This is highly dependent on the choice of smoking material, phenolic/aromatic content, cooking procedures, heater type, moisture content, oxygen availability, and combustion temperature (Guillén et al., 2000; Gomes et al., 2013). PAHs have also been detected in milk at varying levels (0.75–2.1 mg/L) (Girelli et al., 2014). Accumulation of these PAHs in food also depends on the physicochemical properties of food, whereas its toxicity effects are linked to the organism's physiology, metabolic activity, uptake, distribution, and partitioning in the body (Menichini et al., 2011).

The toxicity and the hazardous impact of PAHs were known a long time ago (Cerniglia, 1984). The LMW-PAHs (two to three rings) can bind covalently to various macromolecules like DNA, RNA, and proteins and exert carcinogenicity (Santarelli et al., 2008). Due to their hydrophobic nature, they get partitioned into the lipid membranes. In humans, cytochrome-P_{450} monooxygenase oxidizes PAHs to epoxides, some of which are highly reactive (such as bay-region diol epoxides) and responsible for the transformation of normal cells to malignant ones (Marston et al., 2001). In addition, the PAH transformation products like quinones, phenolics, epoxides, diols, etc. are more toxic than the parent compounds. Several PAHs and their metabolic intermediates have the ability to interfere with hormones and various enzymes in metabolism leading to adverse effects on growth, the central nervous system, and the reproductive and immune systems (Swetha and Phale, 2005; Vamsee-Krishna et al., 2006; Oostingh et al., 2008). Short-term exposure to LMW-PAHs has been reported to cause impaired lung function in asthmatic patients with thrombotic effects and increased risk of skin, lung, bladder, and gastrointestinal cancers (Olsson et al., 2010; Diggs et al., 2011). Animal studies have also shown adverse reproductive and developmental effects from PAH exposure and may further induce cataracts and cause kidney and liver damage and jaundice. Various biotransformation products of PAHs like diols, epoxides, quinones, and free radicals (cations) have shown to form DNA adducts. The stable adducts have shown to alter DNA replication machineries, while unstable adducts depurinate DNA (mostly adenine, but sometimes guanine); both generate errors leading to mutations (Schweigert et al., 2001). In addition, quinones (benzo-/ubi-) can generate reactive oxygen species (ROS) that confer lethal damage to DNA and other macromolecules, thus affecting function/viability of the tissue (Ewa and Danuta, 2017). Long-term exposure to low concentrations of pyrene, biphenyl, and naphthalene has been reported to cause cancer in laboratory animals (Diggs et al., 2012). Owing to their lethal and toxic effects, the cleanup/removal of these PAHs from the impacted/polluted sites is a priority.

Various physical and chemical methods to remove PAHs from contaminated sites/environments have been employed. Processes like incineration, dechlorination, UV oxidation, fixation, solvent extraction, etc. have several drawbacks like toxic by-product formation, complex process, safety and regulatory issues, inefficiency, and high cost. However, microbe-mediated biodegradation, referred to as bioremediation, which involves the application of microbe(s) either as a pure culture or as consortia, is a promising alternative. This process is eco-friendly, non-invasive, cost effective, and sustainable as compared with physical and chemical methods. Bioremediation can be carried out at the impacted site (*in situ*) or in a specially prepared place (*ex situ*) and hence considered as a sustainable cleanup alternative than the conventional physical and chemical methods (Juhasz and Naidu, 2000; Andreoni and Gianfreda, 2007; Megharaj et al., 2011; Phale et al., 2020; Sarkar et al., 2020).

From the ecological and environmental sustainability viewpoint, understanding the microbial metabolic steps involved in the degradation of aromatic pollutants is of the highest scientific and economic value. It has been estimated that a net 2.1×10^{18} g carbon (C) has been preserved in the form of sediment rocks and organic compounds, viz. oil, natural gas, and coal (fossil fuels), significantly contributing to the global C cycle. However, rapid industrialization, fossil fuel exploitation, and human activities are exploring such lithospheric carbon pool and adding $\sim 5.5 \times 10^{15}$ g of organic carbon (in the form of pollutants) to the atmosphere each year (Gonzalez-Gaya et al., 2019). The majority of this organic carbon is contributed to the terrestrial and marine ecosystems through deposition, transport, and runoff. Further, fossil fuel-derived emerging synthetic contaminants, i.e., plastics, plasticizers, and plastic stabilizers (phthalates, its isomers), are predominantly contaminating marine, soil, and water ecosystems and its biota critically, thus contributing to a global climate risk. A range of polyethylene terephthalate (PET)-derived micro- and nano-plastics, plastic debris, and their toxic monomeric products have been assembled together in the Pacific region in between North America and South-East Asia, forming "great Pacific garbage patch" leading

to the destruction of marine flora and fauna (Newell et al., 2020). Scientific expeditions have proved the non-feasibility of removing such pollutants/garbage by any physicochemical means. In this context, microbes with oxidative metabolism of pollutants to CO_2, chemical energy, and other non-toxic by-products, which ultimately get influx to other nutrient cycling processes (H, O, N, S, P, Fe, etc.), are most beneficial. Thus, understanding the microbial ecophysiology of aromatic pollutant mineralization and its ecological controls is critical for estimating microbial C cycling, net C balance, and future climate risks. Considering as high priority the removal of such compounds from the environment, various eco-industries focusing on cleanup technologies are developed. Alternatively, valorization of industrial waste/waste chemicals (i.e., waste-to-wealth approach) accumulating in the ecosystem is recognized to be one of the pillars of the circular economy and sustainable development goal (Close et al., 2012). So, for efficient removal and bioremediation of such aromatic pollutants, it is important to understand the metabolic, enzymatic, and genetic aspects of these potential degrading candidates.

NAPHTHALENE AND SUBSTITUTED NAPHTHALENES AS MODEL COMPOUNDS

Among several aromatic pollutants, we intended to focus on low molecular weight PAHs like naphthalene and substituted naphthalenes. These compounds are found to be the main components of petroleum-derived fuels, textile dyes, consumer products, pesticides (mothballs and insect repellents), plasticizers, and tanning agents, hence ubiquitously present in many ecosystems (Preuss et al., 2003). Recent reports have highlighted the accumulation of higher concentrations of naphthalene in aquifer sediment, groundwater and subsurface soil, vadose zone, and river beds, signifying its bioaccumulation in the environment (Duttagupta et al., 2019, 2020). The physicochemical properties, applications, and health effects of naphthalene and naphthalene-based derivatives are summarized in **Table 2**. Compared with other higher molecular weight PAHs, naphthalene and its derivatives are often used as model substrates to study PAH metabolism, genetics, and metabolic diversity as they are less hydrophobic, more soluble in water, and abundant in the ecosystem. A large number of microbes have the ability to metabolize them with comprehensive information on metabolic pathways, enzymes, and regulatory features (Mallick et al., 2011; Phale et al., 2019, 2020). In addition, because of higher abundance and bioavailability, these compounds are designated as prototypic (signature) compounds to assess the pollution in the environment. The USEPA estimated an average of 5.19 μg naphthalene/m^3 derived primarily from the incomplete combustion of fuels, 0.3–4 μg from cigarette smoke, 7.8–46 μg from sidestream smoke, and 100- to 10,000-fold higher exposure through creosote and mothball manufacturing (Preuss et al., 2003). Particularly, naphthalene is found to have species-, regional-, and sex-selective respiratory toxicity and carcinogenic effects. Based on evidences from animal studies, the International Agency for Research on Cancer (IARC) has classified naphthalene to be a "likely human carcinogen" (group 2B)[1]. Exposure to substituted naphthalenes is predominantly through inhalation or parenteral administration (oral consumption) leading to damage of lung tissues with increased incidences of pulmonary tumors in rats and mice (National Toxicology Program[2]). Nausea, vomiting, abdominal pain, diarrhea, headache, confusion, profuse sweating, fever, tachycardia, etc. are the consequences of acute exposure. On the other hand, carbaryl (1-naphthyl *N*-methylcarbamate), a broad-spectrum carbamate insecticide, has been reported to be toxic to aquatic invertebrates, amphibians, bees, and humans and has been shown to inhibit acetylcholine esterase leading to paralysis (Smulders et al., 2003; Bulen and Distel, 2011). Thus, it is imperative to understand the microbial degradation mechanisms, genetic regulations, enzymes, and cellular response to strategize its bioremediation from a contaminated environment.

MICROBIAL DEGRADATION OF NAPHTHALENE AND SUBSTITUTED NAPHTHALENES

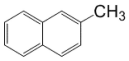
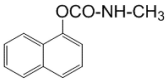
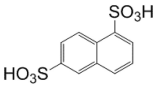
Taxonomic Diversity of Degrading Microbes

In contaminated niches, hydrophobic and lipophilic aromatic pollutants exert varied cellular effects on environmental microbiome (communities), e.g., altering the membrane fluidity, permeabilization of membranes, swelling of lipid bilayers, disruption in energy transduction (electron transport chain/proton motive force), and the activity of membrane-associated proteins (Sikkema et al., 1995). Additionally, some soluble intermediates like catechols and quinones produce ROS and form adducts with DNA and proteins (Penning et al., 1999). Thus, the abundance of such compounds in the ecosystem acts as a selection pressure on the microbial community to evolve as an efficient degrader at various physiological levels such as uptake/transport, intracellular transformation, assimilation/utilization, and compartmentalization.

The Ribosomal Database Project-II (RDP-II) search shows that a total of 926 bacteria have been isolated from the environment contaminated with naphthalene or its derivatives or enrichment cultures. *Proteobacteria* members represented the maximum ($n = 755$), followed by *Firmicutes* (52), *Bacteroidetes* (43), *Actinobacteria* (39), *Tenericutes* (10), and unclassified bacteria (8) (**Figure 2**). Members of γ-*proteobacteria* (*Pseudomonadales* and *Xanthomonadales*) are predominantly (54%) reported among all Gram-negative, high G + C content groups, whereas *Clostridiales* and *Bacillales* (30%) are among the Gram-positive low G + C content groups. *Pseudomonas* (a total of 338 spp., being the highest) are reported for the degradation of naphthalene and its methyl derivatives from many contaminated

[1]http://www.inchem.org/documents/iarc/vol82/82-06.html
[2]https://ntp.niehs.nih.gov/

TABLE 2 | Details of the physicochemical properties of naphthalene and its derivatives, its application, identification methods, and associated diseases.

Structure and physicochemical properties	Naphthalene	1-Methyl naphthalene	2-Methyl naphthalene	1-Naphthoic acid	1-Naphthyl *N*-methylcarbamate (Carbaryl)	Naphthalene monosulfonate	Naphthalene disulfonate
							
Mol. wt. (Da)	128.17	142.2	142.2	172.18	201.22	207.23	202.23
Density (mmHg at 77°F)	1.15	1.01	1.01	1.3	1.23	0.38	0.4
Vapor pressure	0.05	23	0.06	0.06	1.36×10^{-6}	7.6×10^{-8}	7.5×10^{-8}
O/W coefficient (Log*P*)	3.3	3.87	3.86	2.54	2.36	−1.78	−1.85
Autoignition temperature (°)	979	984	–	–	Not flammable	Not flammable	Not flammable
Chemical safety	Irritant, health hazard	Irritant, health hazard	Irritant	Irritant	Irritant, health hazard	Irritant	Irritant
Toxic/lethal dose	5–15 g	0.03–0.5 ml	–	–	500 mg	1,390 mg	2,300 mg
Applications	Insecticide repellent Fuels-additives Lubricants Paint Abrasives Adhesives	Adjuvant Scrubber oil Feedstock Beverages	Insecticide Dye carrier Feedstock Textile surfactants	Pesticide Metabolite-intermediate	Pesticide Fungicide Growth regulator Cleaning agent Cosmetics	Dye carrier Dry-cleaning agent Degreaser	Dye carrier Degreaser Liposome preparation
Identification methods	GC-MS	GC-MS RP-HPLC	GC-MS	GC-MS	RP-HPLC	HPLC	HPLC
Associated diseases	Kidney damage Anemia Cataract Hyperplasia Hepatomegaly	Skin–eye irritation Respiratory depression	Eye irritation Respiratory congestion Pyretic	Respiratory congestion Behavioral convulsion	Asthma, bronchitis Agranulocytosis Catalepsy Melanoma	Dermal rashes Corneal damage Kidney malfunction	Dermal rashes Corneal damage Kidney malfunction

(coal tar, petroleum, crude oil, oil sludge, spillage, wastewater, organic waste, and dumpsites) and pristine (soil, river, sediment, and groundwater) ecosystems (**Figure 2**). Besides, enrichment studies and metagenomic analysis of some of these sites have indicated the probable degradation capacity of uncultivable *Legionella* and *Clostridium* members, indicating the need of cultivating these bacteria to understand novel pathways and metabolic diversity.

Metabolic Pathways for Complete Utilization/Mineralization

Naphthalene

Among various reported microbes for the degradation of aromatics, the majority of them have the ability to degrade naphthalene as the sole source of carbon and energy. The sequence of events involved in naphthalene metabolism has been reported from *Pseudomonas putida* (strains: NCIB 9816-4, G7, AK-5, PMD-1, and CSV86), *Pseudomonas stutzeri* AN10, *Pseudomonas fluorescens* PC20, and other spp. (ND6 and AS1) (Mahajan et al., 1994; Resnick et al., 1996; Annweiler et al., 2000; Basu et al., 2003; Dennis and Zylstra, 2004; Sota et al., 2006; Izmalkova et al., 2013). The metabolism is initiated by a multicomponent dioxygenase enzyme [naphthalene dioxygenase (NDO), ring-hydroxylating dioxygenase] which catalyzes the oxidation of one of the aromatic rings of naphthalene using molecular oxygen as another substrate to convert naphthalene to *cis*-naphthalene dihydrodiol (**Figure 3**). *cis*-Dihydrodiol is converted to 1,2-dihydroxynaphthalene by dehydrogenase. Ring-cleaving dioxygenase, 1,2-dihydroxynaphthalene dioxygenase (12DHNDO), converts 1,2-dihydroxynaphthalene to 2-hydroxychromene-2-carboxylic acid. An enzymatic *cis–trans* isomerization forms *trans-o*-hydroxybenzylidene pyruvate, which gets cleaved by a hydratase-aldolase to salicylaldehyde and pyruvate. The organic acid pyruvate is the first C3 compound derived from naphthalene carbon skeleton and channelized to central carbon pathway. Further, NAD^+-dependent salicylaldehyde dehydrogenase converts salicylaldehyde to salicylate. Metabolism up to this step is referred to as the "upper pathway" for naphthalene degradation. This route is very common in most of the naphthalene degraders. However, there are few exceptions; for example, in *Bacillus thermoleovorans* Hamburg 2, naphthalene degradation is initiated by naphthalene 2,3-dioxygenase to yield 2,3-dihydroxy-naphthalene (Annweiler et al., 2000).

Depending on the organism and its genetic makeup, the generated salicylate is further metabolized either *via* the catechol route using salicylate 1-hydroxylase (S1H) or the gentisate route employing salicylate 5-hydroxyalse (S5H) (**Figure 3**). As salicylate is designated to be a major intermediate of naphthalene metabolism (upper pathway), the steps from salicylate to TCA intermediates often termed as lower pathway and genes are organized as a single operon. It is often seen that both the upper pathway (*nah*) operon and lower pathway (*sal* operon) genes

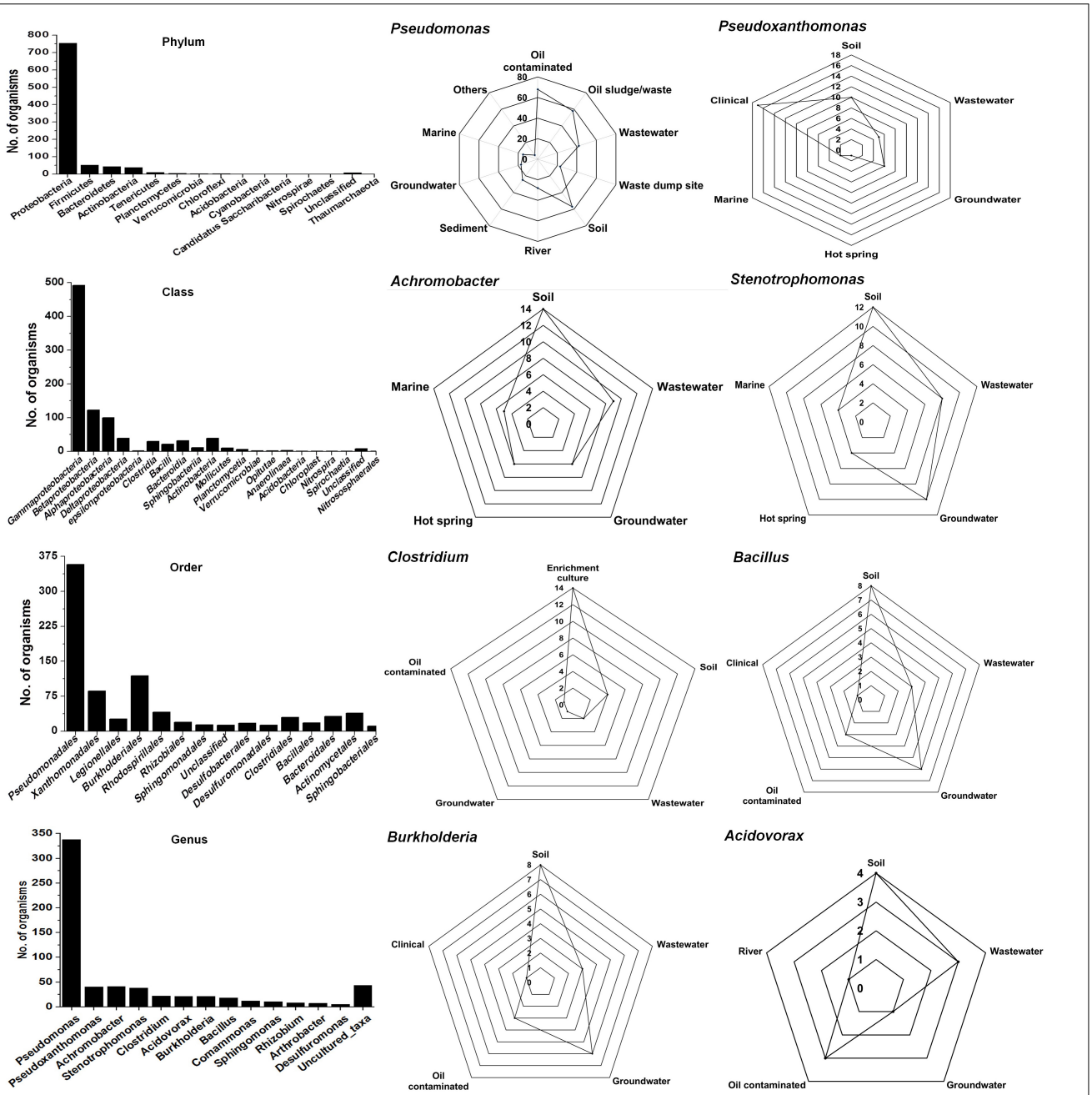


FIGURE 2 | Taxonomic diversity and ecological distribution of bacterial members from the environment contaminated with naphthalene and naphthalene derivatives.

are regulated through a common regulator; for example, NahR and salicylate act as an inducer for both operons to metabolize naphthalene completely (Phale et al., 2019, 2020).

Further, catechol is ring-cleaved through the *meta* route by catechol 2,3-dioxygenase (C23DO) to 2-hydroxymuconic semialdehyde (Yen et al., 1988), which is further hydrolyzed by 2-hydroxymuconic semialdehyde hydrolase to yield 2-hydroxypenta-2,4-dienoate. Subsequent actions of hydratase (2-oxopent-4-enoate hydratase) and aldolase (4-hydroxy-2-oxovalerate aldolase) convert 2-hydroxypenta-2,4-dienoate into pyruvic acid and acetaldehyde which are then funneled into the central carbon pathway (**Figure 3**). Alternatively, catechol is ring-cleaved *via* the *ortho* route by catechol 1,2-oxygenase (C12DO) to yield *cis,cis*-muconic acid. Muconate cycloisomerase, muconolactone isomerase, and β-ketoadipate enol-lactone hydrolase convert *cis,cis*-muconic acid to 3-oxoadipate which enters central carbon pathways *via* succinyl-CoA and acetyl-CoA (Nozaki et al., 1968) (**Figure 3**).

In the gentisate (2,5-dihydroxybenzoate) pathway, the aromatic ring is cleaved by gentisate 1,2-dioxygenase (GDO) to

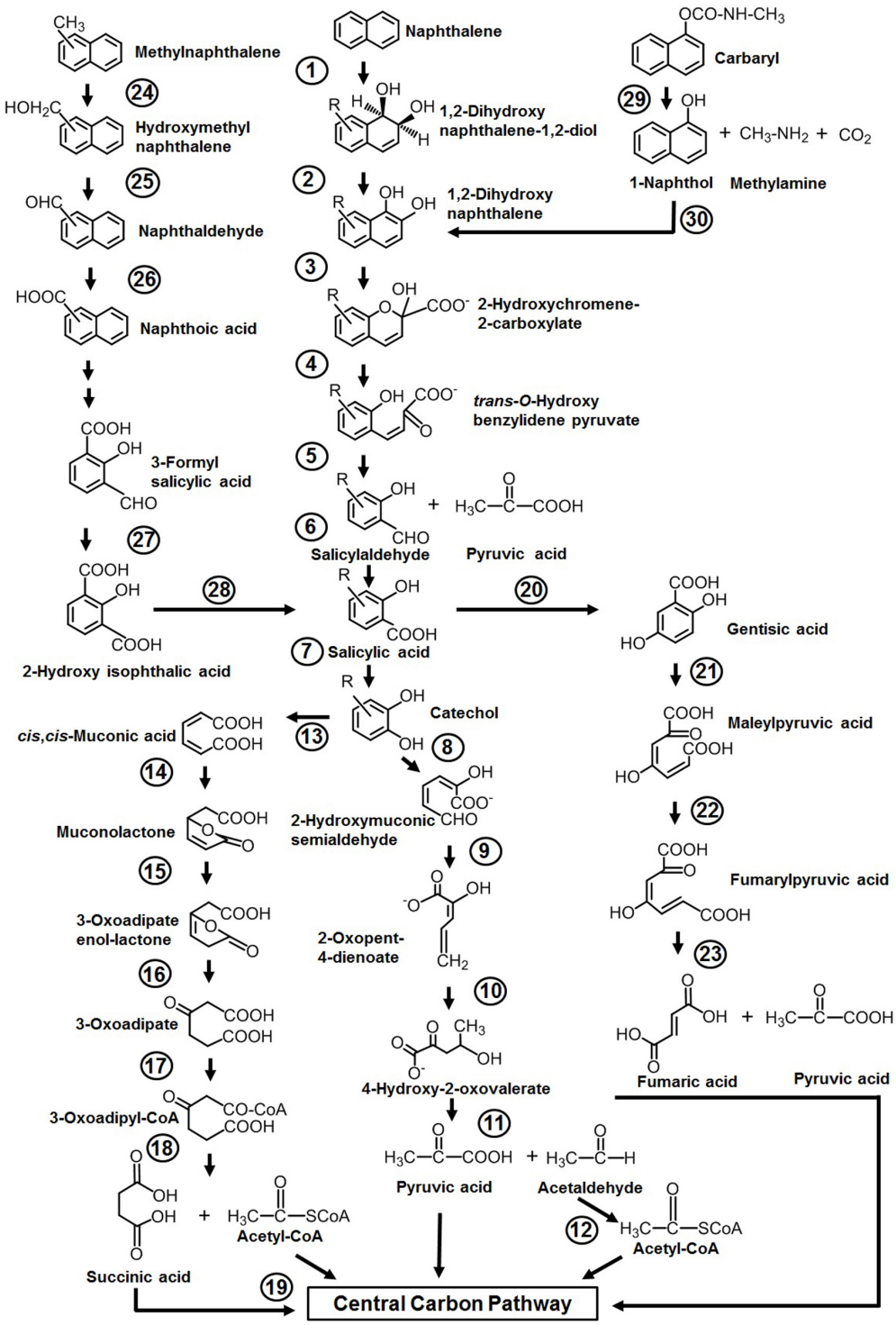


FIGURE 3 | Pathways for the degradation of naphthalene, methylnaphthalenes, naphthoic acid, and Carbaryl. Numbers within circles represent enzymes responsible for sequential conversion of naphthalene and its derivatives to its subsequent products. 1, Naphthalene dioxygenase (NDO); 2, *cis*-dihydrodiol dehydrogenase; 3, 1,2-dihydroxynaphthalene dioxygenase; 4, 2-hydroxychromene-2-carboxylate isomerase; 5, *trans-O*-hydroxybenzylidene pyruvate hydratase-aldolase; 6, salicylaldehyde dehydrogenase; 7, salicylate 1-hydroxylase; 8, catechol 2,3-dioxygenase (C23DO); 9, 2-hydroxymuconic semialdehyde dehydrogenase; 10, 2-oxopent-4-enoate hydratase; 11, 4-hydroxy-2-oxovalerate aldolase; 12, acetaldehyde dehydrogenase; 13, catechol 1,2-dioxygenase (C12DO); 14, muconate cycloisomerase; 15, muconolactone delta-isomerase; 16, β-ketoadipate enol-lactone hydrolase; 17, β-ketoadipate succinyl-CoA transferase; 18, β-ketoadipyl-CoA thiolase; 19, succinyl-CoA:acetyl-CoA succinyl transferase; 20, salicylate 5-hydroxylase; 21, gentisate 1,2-dioxygenase (GDO); 22, maleylpyruvate isomerase; 23, fumarylpyruvate hydrolase; 24, methylnaphthalene hydroxylase (NDO); 25, hydroxymethylnaphthalene dehydrogenase; 26, naphthaldehyde dehydrogenase; 27, 3-formylsalicylate oxidase; 28, hydroxyisophthalate decarboxylase; 29, carbaryl hydrolase (CH); 30, 1-naphthol 2-hydroxylase.

produce maleylpyruvate. This product can directly hydrolyze to pyruvate and malate or may undergo into isomerization to yield fumarylpyruvate which is hydrolyzed to pyruvate and fumarate (Larkin and Day, 1986). At the biochemical and genetic levels, alternative pathway selection has been noticed in Gram-negative and Gram-positive bacteria (Morawski et al., 1997; Whyte et al., 1997). Gram-negative members (*Pseudomonas*) prefer salicylate for decarboxylation by salicylate 1-hydroxylase to yield catechol, where salicylate acts as an inducer for naphthalene metabolism (Gibson and Subramanian, 1984). On the other hand, in Gram-positive bacteria (*Rhodococcus*), salicylate 5-hydroxylase is employed to convert salicylate to gentisate, where salicylate has no induction effects on the transcription of naphthalene genes (Grund et al., 1992) (**Figure 3**).

Methylnaphthalenes

Organisms like *P. putida* CSV86, *Marinobacter* sp. NCE312, *Neptunomonas naphthovorans*, *Sphingomonas paucimobilis* 2322, *Vibrio cyclotrophicus*, *P. fluorescens* LP6a, *Pseudomonas* spp., and *Mycobacterium* have been reported to degrade mono- or di-methylnaphthalenes (Dean-Raymond and Bartha, 1975; Cane and Williams, 1982; Mahajan et al., 1994; Dutta et al., 1998; Hedlund et al., 1999). Among these, the degradation pathway for 1- and 2-methylnaphthalene from *P. putida* CSV86 has been well elucidated at the biochemical and enzymatic levels (Mahajan et al., 1994). 1-Methylnaphthalene is metabolized by two routes, firstly by aromatic ring-hydroxylation (of the unsubstituted ring of methylnaphthalene) to yield *cis*-1,2-dihydroxy-1,2-dihydro-8-methylnaphthalene, which is further oxidized to methylsalicylate and methylcatechol, which upon ring-cleavage channeled to the central carbon pathway (**Figure 3**). This pathway is referred to as the "carbon source pathway." In the second, "detoxification pathway," the methyl group is hydroxylated, probably by NDO, to form 1-hydroxy methylnaphthalene which is further oxidized to 1-naphthoic acid and excreted into the medium as a dead-end product. It has been shown that strain CSV86 failed to grow on 1- and 2-naphthoic acid as the sole source of carbon and energy, confirming the detoxification pathway (Mahajan et al., 1994; Basu et al., 2003). In case of 2-methylnaphthalene, the methyl group is hydroxylated by hydroxylase, resulting in the formation of 2-hydroxymethyl naphthalene. Further, it undergoes ring-hydroxylation of unsubstituted ring to yield dihydrodiol, which gets oxidized by a series of enzyme-catalyzed reactions to form 4-hydroxymethyl catechol and follows the *meta* ring-cleavage route to enter the central carbon pathway. Similarly, *S. paucimobilis* 2322 has been reported to hydroxylate 2-methyl naphthalene by employing NDO, and further oxidation yields methylsalicylate and methylcatechol (Dutta et al., 1998).

Naphthoic Acid

Naphthoic acids (substituted/unsubstituted) are formed as detoxification/biotransformation by-products during degradation of methylnaphthalenes, phenanthrene, and anthracene and are excreted into the spent medium. The soil isolate *Stenotrophomonas maltophilia* CSV89 has been reported to metabolize 1-naphthoic acid as a carbon source (Phale et al., 1995). The metabolism is initiated by double hydroxylation of the aromatic ring to yield 1,2-dihydroxy-8-carboxynaphthalene. The resultant diol gets oxidized *via* 2-hydroxy-3-carboxybenzal pyruvate, 3-formylsalicylate, 2-hydroxyisophthalate, and salicylate to catechol and enters *via* the *meta* ring-cleavage route to the central carbon pathway (**Figure 3**).

1-Naphthyl *N*-methylcarbamate (Carbaryl)

Carbaryl is a naphthalene-based carbamate pesticide. With the onset of the Green Revolution in the 1970s in India, the use of chemical fertilizers and pesticides has increased the PAH load through non-point source agricultural outflow (Pingali, 2012; Duttagupta et al., 2020). An estimated 55% (85,722,000 hectares) of total cultivated farmland are under the use of chemical pesticides. In the last 5 years (2015–2020, till date), an average of 55,000–60,000 metric tons of pesticides/year are used in the Indian agricultural field (Department of Agriculture, cooperation, farmers welfare, Govt. of India[3], August 2020). The crops grown over the northern and middle Gangetic plain areas (states with the highest population and density) are pervasively using pesticides, among which insecticides are predominant. Carbaryl (1-naphthyl *N*-methylcarbamate) is a wide-spectrum, moderate to very toxic carbamate insecticide with an average use of 100–110 metric tons in Indian agriculture. It is generally sold under the trade name Sevin for controlling insects (aphids, fire ants, fleas, ticks, spiders, and many other outdoor pests) infesting various crops (corn, soybean, cotton, fruits, and vegetables). Few microbes like *Pseudomonas* spp. (NCIB 12042, 12043, C4, C5, C6, C7, *P. putida* XWY-1), *Rhodococcus* sp. (NCIB 12038), *Sphingobium qiguonii* (CF06), *Burkholderia* sp. (C3), *Micrococcus*, and *Arthrobacter* sp. (RC100) are reported to degrade carbaryl (Larkin and Day, 1986; Chapalamadugu and Chaudhry, 1991; Hayatsu et al., 1999; Swetha and Phale, 2005; Trivedi et al., 2017). The carbaryl degradation pathway is studied in detail at the biochemical and enzymatic as well as at the genetic level from the soil isolates *Pseudomonas* sp. strains C4, C5, and C6 (Swetha and Phale, 2005; Trivedi et al., 2016) (**Figure 3**). The metabolic pathway starts with the hydrolysis of ester bond by carbaryl hydrolase (CH) to form 1-naphthol, methylamine, and CO_2. The subsequent action of 1-naphthol hydroxylase (1-NH) converts 1-naphthol to 1,2-dihydroxynaphthalene, which is further metabolized to the central carbon pathway *via* salicylic acid and gentisic acid. Few carbaryl degraders were also reported to metabolize it through salicylic acid *via* the catechol *ortho* ring-cleavage route (Larkin and Day, 1986; Chapalamadugu and Chaudhry, 1991). It is interesting to note that, in naphthalene degraders, salicylic acid is predominantly metabolized through catechol, whereas Carbaryl degraders prefer the gentisic acid route for salicylate metabolism.

Naphthalene Sulfonates

Naphthalene sulfonic/disulfonic acids and naphthylaminesulfonic acid derivatives are used as intermediates in the production of azo dyes, wetting agents, dispersants, etc. Although the compounds are less toxic to humans, cytotoxicity assessment has shown its lethal effects on fish, water fleas

[3]http://ppqs.gov.in/statistical-database

(*Daphnia*), and water algae (Greim et al., 1994). Members of *Pseudomonas* spp. (strains A3, C22) have been reported to initiate metabolism through double hydroxylation of the aromatic ring bearing sulfonate group to yield dihydro diol, which is further converted to 1,2-dihydroxynaphthalene by spontaneous elimination of the sulfite group (Brilon et al., 1981). The generated 1,2-dihydroxynaphthalene is catabolized through the classical naphthalene pathway either *via* the catechol or gentisate route (**Figure 4**). It has also been demonstrated that amino- and hydroxy-naphthalene sulfonic acids are completely degraded by mixed bacterial consortium harboring complementary catabolic pathways (Nortemann et al., 1986). One member of this community was shown to desulfonate amino- or hydroxy-naphthalene sulfonic acids by 1,2-dioxygenation, and amino- or hydroxysalicylates were excreted as dead-end metabolites into the medium, which are subsequently assimilated by other members of the consortium. Naphthalene disulfonic acids, being comparatively more polar but resistant for biodegradation, are metabolized by different pathways. The first desulfonation occurs by regioselective dihydroxylation of the aromatic ring with the sulfonate group, while the second one occurs during hydroxylation of 5-sulfosalicylic acid by salicylate 5-hydroxylase to generate gentisic acid, which enters the central carbon pathway (Brilon et al., 1981) (**Figure 4**). The enzymes responsible for naphthalene degradation are also responsible for naphthalene sulfonate metabolism (Brilon et al., 1981; Keck et al., 2006).

Enzymes

The LMW-PAHs are reducing, hydrophobic, and sparingly soluble, thus resistant to natural attenuation/degradation. However, aerobic microorganisms have the ability to oxidize them by incorporating molecular oxygen (O_2). These enzymes mainly belong to oxido-reductase class and perform various reactions like aromatic ring-hydroxylation (mono- or di-), dehydrogenation, and aromatic ring-cleavage. These reactions yield products with increased oxidation status and are more susceptible for further metabolism to the central carbon pathway (Phale et al., 2020). The degradation pathway enzymes are reported to be inducible. The activities of these enzymes were found to be very low or negligible when cells are grown on a simple carbon source like glucose or organic acids. Various enzymes (oxygenase, hydrolase, dehydrogenase, oxidase, etc.) involved in the metabolism of naphthalene and its derivatives are summarized in **Table 3**.

Radio-isotopic studies ($^{18}O_2$) have demonstrated that incorporation of molecular O_2 by oxygenases to the aromatic ring is the most crucial step in activating the compound for further biodegradation (Hayaishi et al., 1955; Mason et al., 1955). Incorporation of a single oxygen atom (O) from the molecular oxygen (O_2) into the substrate is initiated by either internal or external monooxygenase (also referred to as hydroxylase). The other oxygen atom is reduced to water. External mono-oxygenases use NADH or NADPH to reduce flavin, while in internal mono-oxygenases, flavin is reduced by the substrate. The position of hydroxylation results in the diversity of product formation. For example, salicylate 1-hydroxylase hydroxylates salicylate at C1 position to form catechol. On the other hand, multicomponent salicylate 5-hydroxylase (with reductase, ferredoxin, and oxygenase subunit) performs hydroxylation at the C5 position of salicylate to yield gentisate (Yamamoto et al., 1965).

Dioxygenases incorporate both atoms of O_2 into the substrate. Based on the product formed, they are grouped either as ring-hydroxylating or ring-cleaving dioxygenase. The ring-hydroxylating dioxygenase converts aromatic substrate to yield *cis*-dihydrodiols (like naphthalene) and is found to be prevalent in bacteria. To date, ring-hydroxylating dioxygenase-containing organisms have been shown to grow on a range of aromatic carbon sources, and these enzymes have been classified as NDO (for naphthalene), toluene dioxygenase (TDO for toluene), and biphenyl dioxygenase (BPDO for biphenyl). Both NDO and BPDO are capable of catalyzing dioxygenation and side-chain hydroxylation of various PAHs (toluene, nitrotoluene, xylene, ethylbenzene, naphthalene, biphenyl, fluorene, indole, methylnaphthalene, naphthalene sulfonates, phenanthrene, anthracene, acetophenones, etc.) (Boyd and Sheldrake, 1998; Phale et al., 2020). The NDO is a multicomponent system that includes oxido-reductase, a ferredoxin, and an oxygenase component containing the active site (Gibson and Subramanian, 1984; Resnick et al., 1996). The catalytic unit of the NDO is composed of large and small subunits (α and β), respectively, arranged in α3β3 configuration. NDO is a member of a large family of oxygenases with α subunits containing a Rieske [2Fe-2S] center and mononuclear non-heme iron, which determines the substrate specificity of NDO (Parales et al., 1998). Generally, two electrons from the reduced pyridine nucleotide are transferred *via* reductase, ferredoxin, and Rieske center to Fe(II) ion at the active site during a catalytic cycle. The reducing equivalents allow the activation of molecular oxygen, which is a prerequisite to dihydroxylation of the substrate (Ferraro et al., 2005). So far, few NDOs have been purified and extensively characterized from different strains of bacteria, and the genetic control of the pathways involved in naphthalene degradation has been studied in detail (Resnick et al., 1996; Parales et al., 1998; Karlsson et al., 2003). The ring-cleaving dioxygenases (intradiol or *ortho* ring-cleavage and extradiol or *meta* ring-cleavage) act on hydroxylated aromatic compounds. For example, the *ortho* ring-cleaving dioxygenase is catechol 1,2-dioxygenase, while the *meta* ring-cleaving is catechol 2,3-dioxygenase (Kojima et al., 1961; Nozaki et al., 1968). Besides various oxygenases, various dehydrogenases are responsible for dehydrogenation of aromatic dihydrodiols, alcohols, and aldehydes and use NAD^+/$NADP^+$ as an electron acceptor and are one of the important enzymes involved in metabolism (Gibson and Subramanian, 1984; Shaw and Harayama, 1990; Phale et al., 2020).

Enzymes like hydrolases (esterases, amidases) are the second important classes of enzymes that utilize water to cleave the covalent bond and display a broad substrate specificity. Carbaryl hydrolase and other hydrolases are proposed to be periplasmic in Gram-negative members as an integral (transmembrane) component (Kamini et al., 2018). Carbaryl possesses an amide as well as an ester linkage; hence, hydrolysis can be catalyzed either by esterase or amidase to yield 1-naphthol. The CH

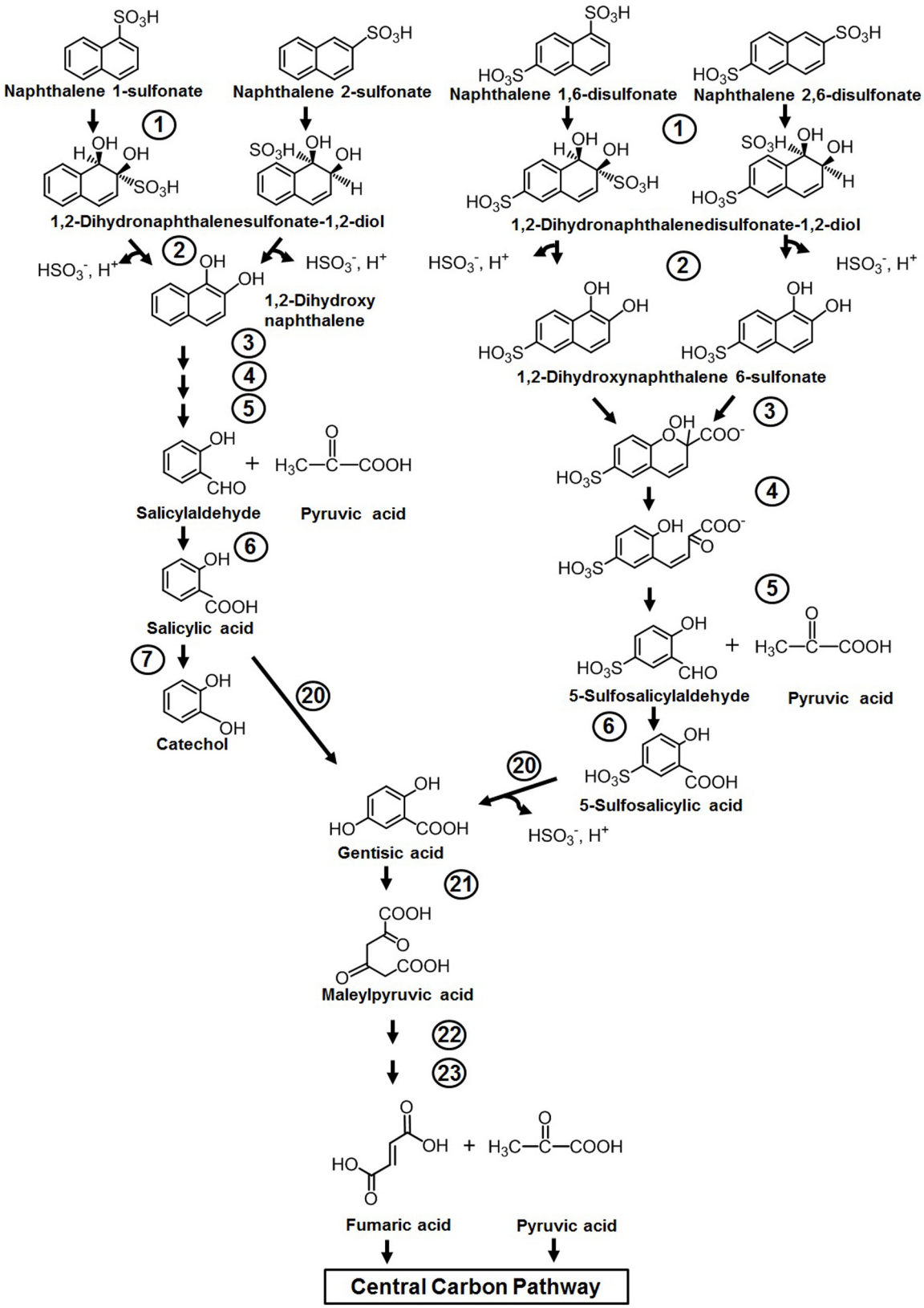


FIGURE 4 | Metabolic pathway for the degradation of naphthalene sulfonates. Numbers within circles represent enzymes responsible for the metabolism of naphthalene sulfonates and are similar/the same as described in **Figure 3**.

TABLE 3 | Biochemical details of the enzymes responsible for degradation of naphthalene and its derivatives.

Enzymes	Genes	Mol. wt. (in kDa)* and organization[$]	Co-factors	Gene Ontology function
Naphthalene 1,2-dioxygenase reductase (ferredoxin reductase) EC: 1.14.12.12	*nah*Aa	35.5 Trimer	2Fe-2S FAD FMN NADH	Two iron, two sulfur cluster binding Dioxygenase activity Electron transfer activity Metal ion binding
Naphthalene 1,2-dioxygenase (ferredoxin component) EC: 1.14.12.12	*nah*Ab	11.4 Trimer	2Fe-2S NADH	Two iron, two sulfur cluster binding Dioxygenase activity
Naphthalene 1,2-dioxygenase (large subunit) EC: 1.14.12.12	*nah*Ac	55.0 Dimer	2Fe-2S Fe^{2+} NADH	Two iron, two sulfur cluster binding Dioxygenase activity Ion binding Cellular metabolic process
Naphthalene 1,2-dioxygenase (small subunit) EC: 1.14.12.12	*nah*Ad	20.0 Dimer	Fe-S NADH	Dioxygenase activity Cellular metabolic process
cis-Dihydrodiol dehydrogenase EC: 1.3.1.29	*nah*B	27.5 Tetramer	$NADP^+$ NAD^+	Cellular metabolic process
1,2-Dihydroxynaphthalene dioxygenase EC: 1.13.11.56	*nah*C	33.9 Monomer	Fe^{2+}	Dioxygenase activity Cellular metabolic process
2-Hydroxychromene-2-carboxylate isomerase EC: 5.99.1.4	*nah*D	23.1 Dimer	Glutathione	Isomerase Protein disulfide oxido-reductase Metabolic process
Trans-*O*-hydroxybenzylidene pyruvate hydratase-aldolase EC: 4.1.2.45	*nah*E	36.9 Monomer	–	Aldehyde-lyase activity Hydratase-aldolase activity
Salicylaldehyde dehydrogenase EC: 1.2.1.65	*nah*F	52.0 Monomer	NAD^+	Dehydrogenase activity Cellular metabolic process
Salicylate hydroxylase EC: 1.14.13.1	*nah*G	46.83 Monomer	NADH	FAD binding Monooxygenase Metabolic process
Catechol 2,3-dioxygenase EC: 1.13.11.2	*nah*H (*xyl*E)	35.2 Tetramer	Fe^{2+}	Dioxygenase Ferrous ion binding
2-Hydroxymuconic semialdehyde dehydrogenase EC: 1.2.1.85	*nah*I (*xyl*G)	51.8 Dimer	NAD^+	Hydroxylation Oxido-reductase
2-Hydroxymuconic semialdehyde hydrolase EC: 3.7.1.9	*nah*N (*xyl*F)	30.6 Monomer	–	Hydrolase
2-Oxopent-4-enoate hydratase EC: 4.2.1.80	*nah*L (*xyl*J)	23.9 Dimer	–	Hydrolase
4-Hydroxy-2-oxovalerate aldolase EC: 4.1.3.39	*nah*M (*xyl*K)	37.4 –	Mn^{2+}	Aldolase Metal ion binding
Acetaldehyde dehydrogenase EC: 1.2.1.10	*nah*O (*dmp*F	33.1 Dimer	CoA NAD^+	Dehydrogenase activity NAD binding
4-Oxalocrotonate decarboxylase EC: 4.1.1.77	*nah*K (*xyl*L)	27.456 Dimer	NAD^+	Catalytic activity Metal ion binding
4-Oxalocrotonate tautomerase EC: 5.3.2.6	*nah*J (*xyl*H)	6.8 Hexamer	–	Isomerase Metabolic processes
Catechol 1,2-dioxygenase EC: 1.13.11.1	*cat*A	34.2 Dimer	Fe^{3+}	Oxido-reductase Metal binding
Muconate cycloisomerase EC: 5.5.1.1	*cat*B	41.1 Octamer	Mn^{2+}	Cycloisomerase Metabolic process Metal binding
Muconolactone Delta-isomerase EC: 5.3.3.4	*cat*C	10.6 Decamer	–	Delta-isomerase Metabolic process
β-Ketoadipate enol-lactone hydrolase EC: 3.1.1.24	*pca*D	28.6 Trimer	–	Enol-lactonase
β-Ketoadipate succinyl-CoA transferase (α subunit) EC: 2.8.3.6	*pca*I	24.4 Dimer	–	CoA transferase (binding) Metabolic process

(Continued)

TABLE 3 | Continued

Enzymes	Genes	Mol. wt. (in kDa)* and organization[$]	Co-factors	Gene Ontology function
β-Ketoadipate succinyl-CoA transferase (β subunit) EC: 2.8.3.6	*pca*J	22.5 Dimer	–	CoA transferase (binding) Metabolic process
β-Ketoadipyl-CoA thiolase EC: 2.3.1.174	*pca*F (*paa*J)	42.27 Tetramer	–	Thiolase Metabolic process Catalytic activity DNA damage stimulus
Salicylate-5-hydroxylase (large subunit) EC: 1.14.13.172	*nag*G	48.8 Monomer	2Fe-2S Fe^{2+} NAD^{+}	Oxido-reductase Metabolic process Metal binding
Salicylate-5-hydroxylase (small subunit) EC: 1.14.13.172	*nag*H	18.8 Monomer	NADH	Oxido-reductase Metabolic process Metal binding
Gentisate 1,2-dioxygenase EC: 1.13.11.4	*nag*I	39.7 –	Fe^{2+}	Dioxygenase Metal binding
Maleylpyruvate isomerase EC: 5.2.1.4	*nag*L	23.5 –	Glutathione	Isomerase Amino acid metabolic process
Fumarylpyruvate hydrolase EC: 3.7.1.20	*nag*K	20.9 –	Mg^{2+} Mn^{2+}	Hydrolase Metal ion binding
Carbaryl hydrolase EC: 3.1.1.aj	*mcb*A (CH)	85.4 Monomer	–	Integral membrane component
1-Naphthol 2-hydroxylase EC: 1.14.13.M78	*mcb*C (1-NH)	64.7 Dimer	FAD NADPH	Cellular metabolic process
Formylsalicylate oxidase EC: 1.2.3.-	–	– Monomer	–	Cellular metabolic process

**Molecular weight deduced from the nucleotide sequence of the gene.*
[$]Determined experimentally.

from *Rhizobium* sp. strain AC10023 and *Arthrobacter* sp. strain RC100 was reported to act as esterase and amidase, respectively. CH from *Arthrobacter* sp. RC100 has shown hydrolysis of four *N*-methylcarbamate insecticides, e.g., Carbaryl, xylylcarb, metolcarb, and XMC (Hayatsu et al., 2001). CH from *Pseudomonas* sp. C5pp was reported to act on Carbaryl (100% activity) and 1-naphthylacetate (36%) but not on 1-naphthalene acetamide, suggesting it to be an esterase (Trivedi et al., 2016).

Genetic Organization and Regulatory Features

Biochemical studies, enzyme regulation pattern, and genetic analyses have shown that the naphthalene degradation genes are arranged as two inducible regulatory units, "operons": *nah* ("upper pathway," naphthalene to salicylate) and *sal* ("lower pathway," salicylate to central carbon pathway *via* catechol). Salicylic acid and its analogs act as inducers (Shamsuzzaman and Barnsley, 1974). The operons are suppressed in the presence of glucose or organic acids. The complete genetic organization of naphthalene degradation (as operons) is summarized in **Figure 5**. Several nomenclatural variations/forms of *nah* genes (*ndo*/*pah*/*dox*) are described and found to share high sequence homology (90%) in all *Pseudomonas* spp. (Abbasian et al., 2016). The naphthalene "upper pathway" genes are often arranged as *en bloc* in consensus order as shown in **Figure 5A**. An additional gene *nah*Q has also been reported to be involved in naphthalene metabolism and is often found in between *nah*C and *nah*E, but its actual function is yet to be examined. Similarly, the *nah*Y gene responsible for naphthalene-responsive chemotaxis is found to be present at the distal end of the *nah* operon in some of the members. In *Ralstonia* sp. U2, genes encoding glutathione-*S*-transferase (*gsh*) were found to be present in between *nah*Aa and *nah*Ab without affecting naphthalene utilization trait (Zylstra et al., 1997).

The "lower pathway" (*sal* operon) often consists of *nah*GTHINLMOKJ and converts salicylate to pyruvate and acetaldehyde *via* the catechol *meta* ring-cleavage pathway. Gene *nah*G (encoding salicylate hydroxylase) was found to be conserved at the proximal end of the operon (**Figure 5B**). As compared with other strains of naphthalene degradation, in *P. putida* CSV86, the *nah* and *sal* operons are tandem and in close proximity (∼7.5 kb). In some Gram-negative members like *Ralstonia* sp. U2, *Polaromonas naphthalenivorans* CJ2, and *P. putida* AK5, naphthalene is metabolized to central carbon metabolites *via* the gentisate route (as *sgp*/*nag* operon). The genetic cassette is often present as *nag*AaGHAbAcAdBFCQEDJI with *nag*R (encoding LysR type regulator) located at the upstream end (**Figure 5C**).

Carbaryl is metabolized to the central carbon cycle *via* 1-naphthol, 1,2-dihydroxynaphthalene, salicylate, and gentisate (**Figure 3**). Based on genetic and metabolic studies, the pathway has been proposed to be organized into "upper" (carbaryl to salicylate), "middle" (salicylate to gentisate), and "lower" pathways (gentisate to central carbon pathway intermediates)

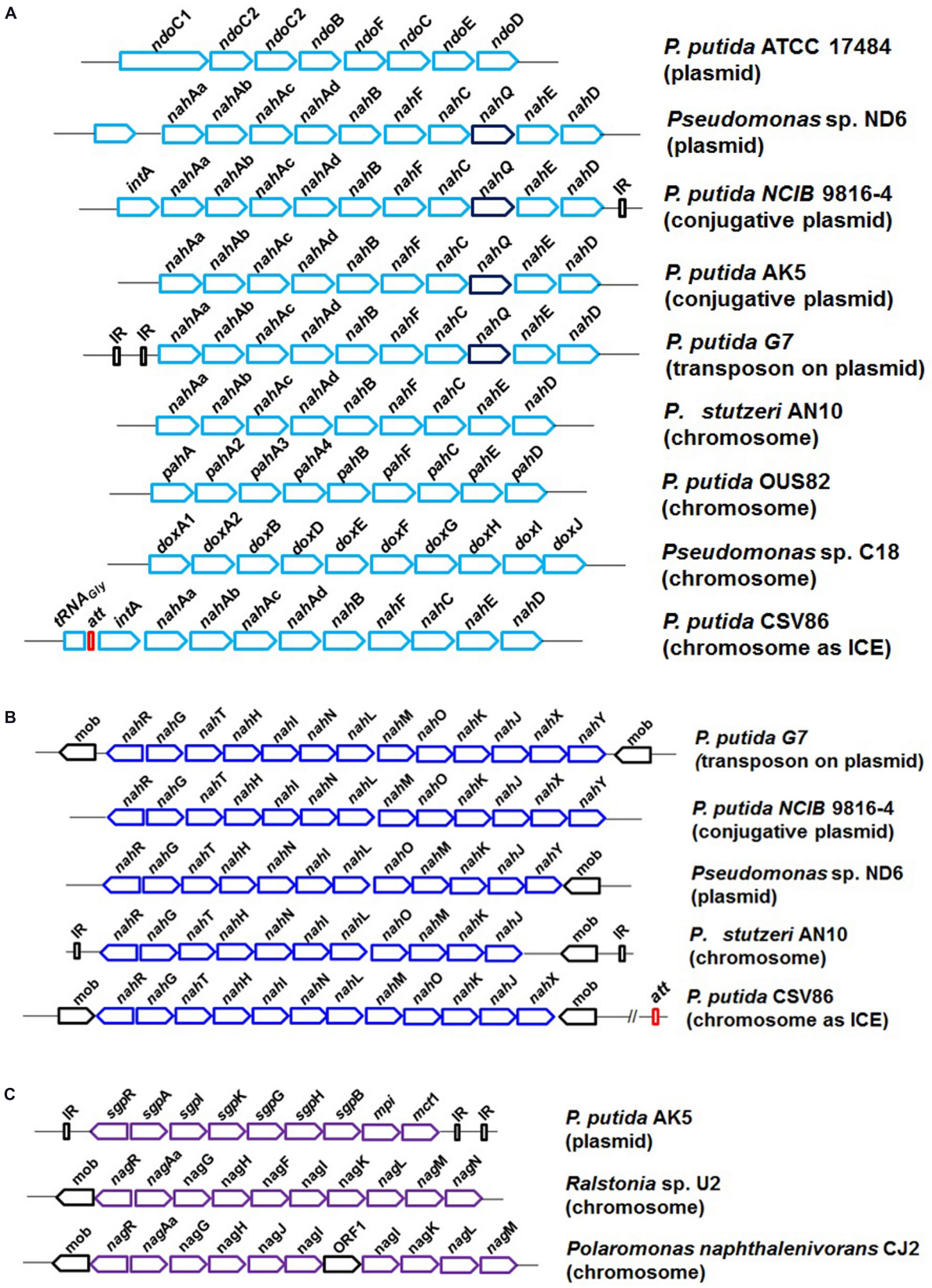


FIGURE 5 | The genetic organization and diversity observed in the degradation of naphthalene in bacterial members; **(A)** naphthalene upper pathway, naphthalene to salicylate metabolism; **(B)** naphthalene lower pathway, salicylate to central carbon pathway *via* catechol; and **(C)** salicylate to central carbon pathway via gentisate.

(Singh et al., 2013). Genomic analysis of C5pp (as supercontig-A, 76.3 kb) has shown that genes *mcb*ACBDEF are involved in converting Carbaryl to salicylate, followed by *mcb*IJKL for salicylate to gentisate, and *mcb*OQP for gentisate to central carbon intermediates (as fumarate and pyruvate, Trivedi et al., 2016) (**Figure 6**).

Enzymes involved in the degradation of aromatics including naphthalene and salicylate are reported to be inducible by the respective compound and suppressed by simple carbon source like glucose or organic acids (Shingler, 2003; Phale et al., 2019, 2020). Among various pathways for the metabolism of naphthalene and its derivatives, regulatory features are studied to some extent for naphthalene and carbaryl. In the case of naphthalene, both "upper" and "lower" pathway genes are regulated through a NahR, which is a LysR-type *trans*-acting positive regulator. It is required for the induction of *nah* genes by salicylate and their subsequent high-level expression (Yen and Gunsalus, 1982). It has been also found that the integration host factor (IHF) along with XylR (sigma-54-dependent transcriptional regulator) are also important for transcription activation of genes in naphthalene metabolism (Ramos et al., 1997). The enzymes of the catechol *meta* ring-cleavage route, i.e., catechol 2,3-dioxygenase and others, were found to be induced in the presence of naphthalene and/or salicylate (Basu et al., 2006). The catechol *ortho* ring-cleavage route enzymes, i.e., catechol 1,2-dioxygenase and others, were found to be induced by benzoate as well as by *cis*,*cis*-muconate (Parsek et al., 1994; Tover et al., 2001).

In strain C5pp, five genes, i.e., *mcb*G, *mcb*H, *mcb*N, *mcb*R, and *mcb*S, code for regulators belonging to the LysR/TetR family transcriptional regulator for Carbaryl degradation. The closest homolog of *mcb*G was found to be LysR-type PhnS (58% amino acid identity) regulator involved in phenanthrene metabolism in *Burkholderia* sp. strain RP00725 (Trivedi et al., 2016). Gene *mcb*H was found to be a part of the middle pathway (salicylate to gentisate) and belongs to the NagR/DntR/NahR-type LysR transcriptional regulators from *Pseudomonas* sp. and *Burkholderia* sp. Members of this family are reported to recognize salicylate as the specific effector molecule to induce the degradation genes. On the other hand, three genes *mcb*N, *mcb*R, and *mcb*S belonging to LysR- and TetR-type transcriptional regulators were identified in the lower pathway (gentisate to central carbon pathway metabolites).

Genetic Diversity

In prokaryotes, the horizontal gene transfer process (acquisition, exchange, or transmission) through plasmids, transposons, prophages, genomic islands, and integrative conjugative elements (ICEs) is the major reason for the plasticity in bacterial genomes leading to gain or loss of a specific function/property. It provides fast-forward adaptation to various environments, conferring potential adaptive metabolic benefits, viz. aromatic compound degradation, to the bacterial host. The metabolic variations are generally made through the fine-tuning of degradative operons, its regulation, and specificity of the enzymes, aiding in the degradation of a wider range of aromatics (Nojiri et al., 2004; Phale et al., 2019, 2020). The genetic cassette for naphthalene degradation has been found to be located on various mobile elements like plasmids (conjugative and non-conjugative), transposons, genome, ICE, and combinations of different bacterial members (**Figure 5**). In *P. putida* G7, plasmid NAH7 has *nah* and *sal* operons transcribed in the same direction and is part of a defective transposon that requires Tn4653 transposase for its mobilization (Sota et al., 2006). In *P. putida* strain NCIB9816-4, genes were found to be present on a conjugative plasmid pDTG1 as two operons (∼15 kb apart) which are transcribed in opposite direction (Dennis and Zylstra, 2004). In *P. putida* strain AK5, a non-conjugative plasmid pAK5 codes for enzymes responsible for the degradation of naphthalene *via* the gentisate pathway (Izmalkova et al., 2013). In *P. putida* strain PMD-1, the *nah* operon was found to be located on the chromosome, while the *sal* operon was present on conjugative plasmid pMWD-1 (Zuniga et al., 1981). However, in *P. stutzeri* AN10, all naphthalene degradation genes (*nah* and *sal* operons) were found to be located on the chromosome and hypothesized to be recruited through transposition, recombination, and rearrangement events (Bosch et al., 2000). In *P. putida* CSV86, *nah* and *sal* operons are located on the genome as ICE (ICE_{CSV86}). The structure is found to be guarded by $tRNA_{Gly}$ followed by direct repeats denoting sites for recombination/attachment (*att*R *and att*L) at both ends and phage-like integrase, hence structurally similar to the ICE*clc* element (ICE*clc*B13 of *Pseudomonas knackmusii for* chlorocatechol degradation). It has been reported that the genes on the ICE are transferrable through conjugation at a very low (10^{-8}) frequency, thus disseminating degradation traits to the recipient (Basu and Phale, 2008; Phale et al., 2019).

Genes responsible for Carbaryl degradation are mostly located on plasmids. *Arthrobacter* sp. RC100 harbors three plasmids (pRC1, pRC2, and pRC300) of which two conjugative plasmids, pRC1 and pRC2, encode enzymes for the conversion of carbaryl to gentisic acid. On the other hand, enzymes involved in the conversion of gentisic acid to central carbon metabolites are located on the chromosome (Hayatsu et al., 1999). A *Rhizobium* sp. strain AC100, which transforms carbaryl to 1-naphthol, harbors a plasmid pAC200 carrying the gene *ceh*A encoding CH as part of the *Tnceh* transposon flanked by insertion element-like sequence (*ist*A and *ist*B) (Hashimoto et al., 2002). In *Sphingomonas* sp. strain CF06, Carbaryl degradation genes were proposed to present on five plasmids: pCF01, pCF02, pCF03, pCF04, and pCF05 with high inter-DNA homology, indicating gene duplication events (Feng et al., 1997). In one of the Carbaryl-degrading consortia of two *Pseudomonas* spp., the strain 50581 harbors a conjugative plasmid, pCD1 (50 kb), which encodes the *mcd* gene for Carbaryl hydrolase, while the chromosomal counterpart in 50552 encodes enzymes for 1-naphthol degradation (Chapalamadugu and Chaudhry, 1991). The *mcd* gene for carbofuran hydrolase is found to be located on a 100-kb plasmid (pPDL11) in *Achromobacter* sp. strain WM111. This gene was shown to be present on various plasmids (100, 105, 115, or 124 kb) in many bacteria from geographically distant areas (Parekh et al., 1995). In *Pseudomonas* sp. C5pp, all genes responsible for Carbaryl degradation were found to be located on the genome spanning 76.3 kb sequence (Trivedi et al., 2016).

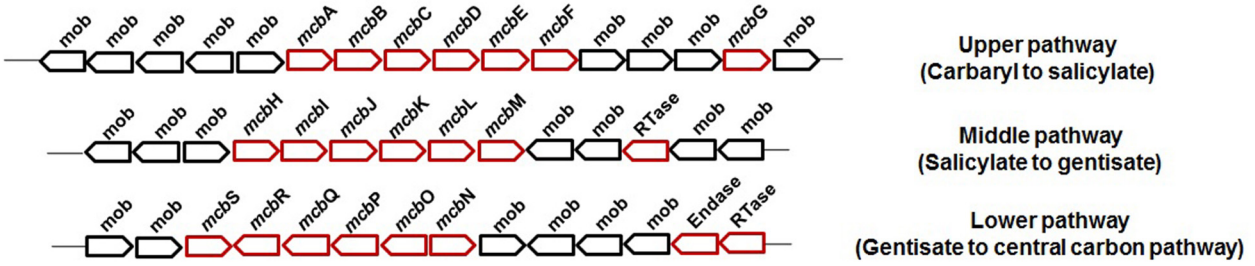


FIGURE 6 | The genetic organization involved in the degradation of Carbaryl by *Pseudomonas* sp. C5pp.

The genome (6.15 Mb) analysis revealed the presence of 42 MGEs and 36 GEIs, out of which 17 MGEs were located in supercontig-A (76.3 kb) with mean skewing of G + C content (54–60 mol%), indicating the possible occurrence of HGT events (Trivedi et al., 2016). *P. putida* XWY-1 has shown a similar gene arrangement for Carbaryl degradation, but the genes are located on the plasmid (Zhu et al., 2019).

Microbial Response(s) for Assisting Efficient Degradation

Besides metabolic efficiency at the biochemical and genomic levels, microbes do show additional properties or responses like chemotaxis, cell surface alteration properties, compartmentalization, preferential utilization, biosurfactant production, etc. which help them to metabolize these aromatic pollutants from the contaminated niche more efficiently (**Figure 7**).

Chemotaxis

Chemotactic responses have been ascribed to enhance the degradation of organic pollutants in heterogeneous contaminated ecosystems. Pedit et al. (2002) demonstrated that chemotaxis to naphthalene by *P. putida* G7 has increased the rate of naphthalene degradation in an aqueous system. The wild-type strain G7 degraded naphthalene at a much faster rate than chemotaxis-deficient mutant strains. The protein NahY (538 amino acids with a membrane topology) was found to be co-transcribed with *meta*-cleavage pathway genes from the NAH7 plasmid and resembled a chemotaxis transducer protein, which appears to function as a chemoreceptor for naphthalene degradation (Grimm and Harwood, 1997). Another study by Hanzel et al. (2011) showed the chemotactic response of *P. putida* to vapor-phase naphthalene, where gas-phase diffusion resulted in steady naphthalene flux to the cells controlling the chemotactic response of cells. Researchers have taken advantage of such chemotactic behavior to engineer microbes to enhance the degradation rate. It has been shown that chemosensory pathways also regulate other cellular functions like cell division, cell cycle regulation, and biofilm formation, thus contribute in controlling degradation rate. However, the exploration of such trait (chemotaxis) for effective degradation is impeded by certain bottlenecks. The major obstacles are as follows: (a) the same compound/ligand recognition by different paralogous receptors; (b) the presence of other receptors, i.e., energy taxis; (c) the significant sequence divergence in the sensor domain of the same receptor family; and (d) the lack of information on major bacterial sensor proteins (Ortega et al., 2017; Martin-Mora et al., 2018). Sometimes, biodegradation of aromatics results in several metabolites/intermediates that might be chemotactic to one population but repellent to others and, hence, creates further complexity. For the identification of ligand (aromatic)–chemoreceptor interactions, hybrid sensor proteins (PcaY, McfR, and NahY) were constructed by fusing sensor domains and signaling domains of *P. putida* and *Escherichia coli* targeting receptors for aromatic acids, TCA intermediates, and naphthalene (Luu et al., 2019).

Cell Surface/Membrane Fluidity

Crucial changes in the structure and integrity of the bacterial membrane were observed in microbes in response to naphthalene and other PAHs. Naphthalene is found to interact through hydrophobic interaction and disturbs the acyl chain interaction, thus increasing the swelling of the membrane and fluidity (Sikkema et al., 1995). To counter the deleterious effect, bacteria regulates membrane fluidity by changing the proportion and composition of fatty acid between *iso/ante-iso* branched chain fatty acids and isomerization of *cis* unsaturated fatty acids to the corresponding *trans* isomers (Heipieper and de Bont, 1994). In *P. stutzeri* when grown under naphthalene amendments, the ratio of saturated to unsaturated fatty acids was found to increase from 1.1 to 2.1, while in *Pseudomonas* sp. JS150, it increased from 7.5 to 12.0 (Mrozik et al., 2004). When grown on naphthalene, *Achromobacter* sp. KAs 3–5 cells showed cellular aggregation around naphthalene crystals with decreased cell surface charge (from −22.5 to −2.5 mV) with cytoplasmic condensation and vacuolation, suggesting changes in the cell structure and cell surface properties (Mohapatra et al., 2019). Although cellular/surface changes are directly linked to better uptake of the aromatic pollutants, no concurrent bioengineering strategies have been thoroughly optimized. The manipulation of cell shapes has been rarely exploited to optimize bioprocesses (Volke and Nikel, 2018). Gene deletions affecting the cell division induce morphological changes in cells. Gene deletions affecting cell division induce morphological changes in cells. In *Bacillus subtilis*, the cell septation protein SepF has shown to be involved in the septum formation and is required for a later step in cell division but does not represent an essential gene. Deletion of

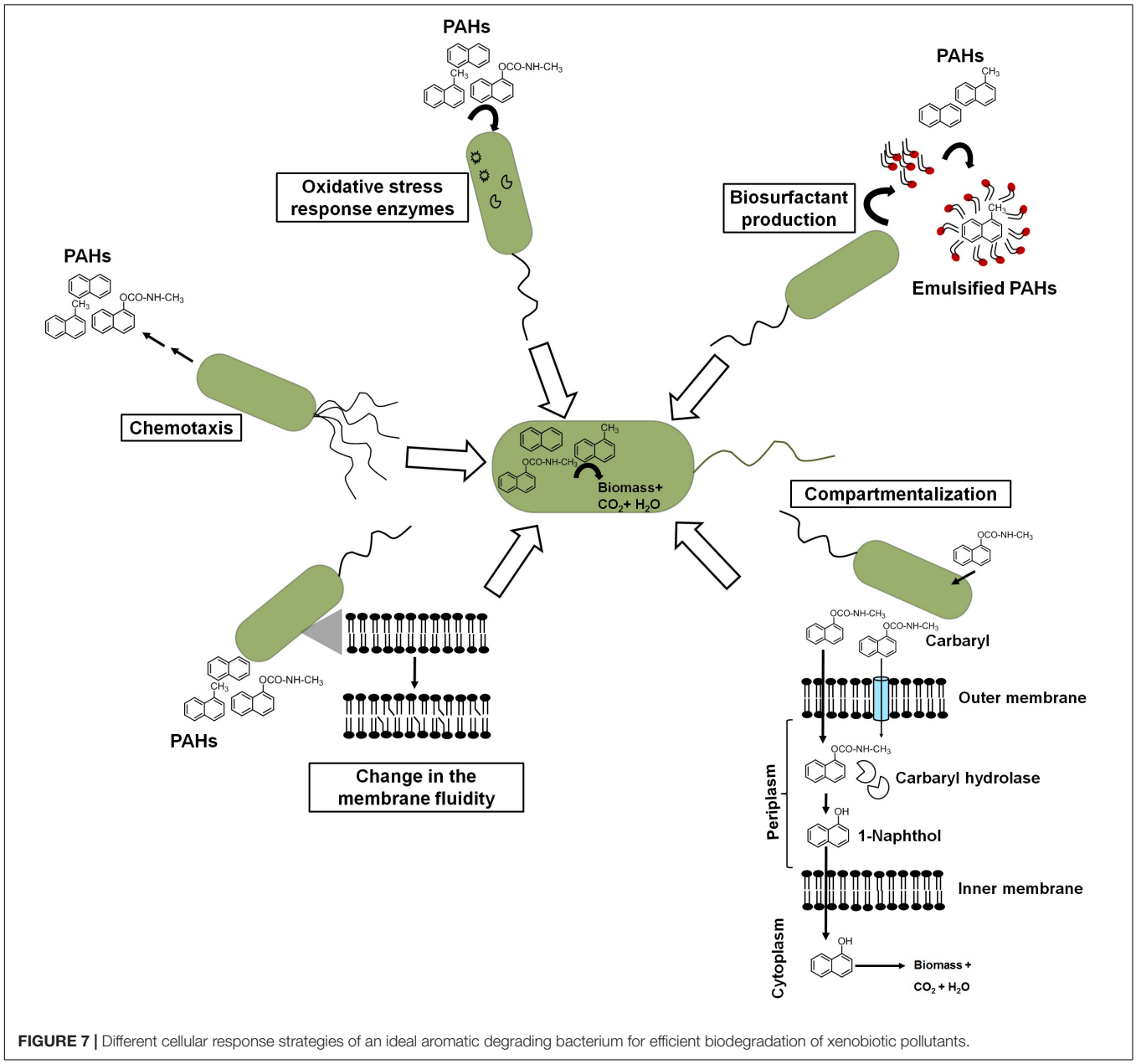


FIGURE 7 | Different cellular response strategies of an ideal aromatic degrading bacterium for efficient biodegradation of xenobiotic pollutants.

genes encoding peptidoglycan hydrolases in *B. subtilis* resulted in elongated cells with increased specific growth rate and improved enzyme production capacities (Cui et al., 2018).

Compartmentalization of the Pathway

Compartmentalization of the carbaryl degradation pathway has been proposed for efficient degradation in *Pseudomonas* sp. strains C5pp and C7 (Kamini et al., 2018). Carbaryl is proposed to be transported *via* partitioning in the outer membrane and/or through diffusion porins into the periplasmic space. CH, a periplasmic enzyme, catalyzes the hydrolysis of Carbaryl to 1-naphthol, which is more recalcitrant, hydrophobic, and toxic. Localization of CH in the periplasm with lower affinity for Carbaryl provides controlled formation of 1-naphthol, thus preventing its accumulation and subsequent toxicity to the cells (Kamini et al., 2018). The generated 1-naphthol gets transported *via* partition and/or diffusion across the inner membrane to the cytosol which gets hydroxylated by high-affinity 1NH enzyme to 1,2-dihydroxynaphthalene, which is further metabolized to the central carbon pathway.

Preferential Utilization of Aromatic Compounds

Though microbes possess genetic and metabolic abilities to degrade xenobiotics as the carbon source, the hierarchy in utilization, i.e., preferential utilization of simple carbon sources over the complex ones, has been a major barrier for biodegradation. The presence and utilization of simple carbon sources represses genes encoding enzymes for the degradation

of complex/non-preferred carbon sources like PAHs. A well-studied example is the utilization of glucose prior to lactose when both were given together to *E. coli* (Jacob and Monod, 1965). *Pseudomonas* are ubiquitously reported to degrade various PAHs and xenobiotic compounds as the carbon source. The carbon source utilization hierarchy in pseudomonads is organic acids > glucose > aromatic compounds (Hylemon and Phibbs, 1972; Collier et al., 1996). However, there is an exception to this. Interestingly, *P. putida* CSV86 displays a unique hierarchy with preferential utilization of aromatics (benzoate, naphthalene, etc.) over glucose and co-metabolism of aromatics with organic acids (Basu et al., 2006). In this bacterium, aromatic degradation and transport genes are not suppressed even in the presence of a second carbon source like glucose or organic acid. The glucose transport and metabolic genes are observed to be repressed when grown on glucose + aromatics, where aromatics get utilized in the first log phase followed by glucose in the second log phase (Basu et al., 2006; Choudhary et al., 2017). On the other hand, the presence of organic acids did not affect the expression of aromatics metabolism, thus making this bacterium a promising candidate for biodegradation studies (Phale et al., 2020).

Oxidative Stress Management

It is well established that hydrocarbon biotransformation leads to oxidative stress and upregulation of antioxidant enzymes in microbes. Inefficient naphthalene biodegradation, either by stationary-phase cells or in the presence of toxic compounds, leads to the generation of ROS (Kang et al., 2006). As naphthalene degradative enzymes harbor Fe-S clusters, under oxidative stress, the Fe from haem and Fe-S proteins can be oxidized, which may lead to protein inactivation. Along with superoxide dismutase (SOD), ferredoxin-$NADP^+$ reductase (Fpr) mediates reversible redox reactions between $NADP^+$/NADPH and two molecules of ferredoxin or flavodoxin, thus scavenging the ROS and repairing Fe-S centers under oxidative stress (Lee et al., 2006). It has been reported that both Fpr and SodA (SOD) are inducible under oxidative stress in *Pseudomonas*, whereas increased activity of SOD and catalase has been observed during growth of four *Pseudomonas* spp. (O1, W1, As1, and G1) in naphthalene-amended condition (Kang et al., 2006). The addition of antioxidants like ascorbate or ferrous iron (Fe^{2+}) has been found to confer higher growth rates on naphthalene. In *Rhodococcus erythropolis*, growth on naphthalene showed increased transcription of oxidative stress-related genes of cytochrome P450: *sodA* (Fe/Mn superoxide dismutase), *sod*C (Cu/Zn superoxide dismutase), and *rec*A (Sazykin et al., 2019). Comparative quantitative proteomic analysis of naphthalene-grown cells of *Pseudomonas* sp. showed upregulation of various oxidative stress response-related proteins as a strategy to tackle the stress (Herbst et al., 2013).

Biosurfactant Production

In response to hydrophobic carbon sources, microbes have been reported to produce biosurfactants which are amphiphilic, surface-active compounds and assemble at the oil–water or air–water interface. This aids in pseudo-solubilization and facilitates the uptake of aromatics for efficient biodegradation (Rahman et al., 2002). Due to these properties, biosurfactants have a wide application in various industries. The addition of chemical surfactant or biosurfactants to bacterial culture displayed increased efficiency and rate of degradation of hydrocarbons. Among biosurfactants, rhamnolipids produced by *Pseudomonas aeruginosa* are well studied and characterized (Hisatsuka et al., 1971; Rahman et al., 2002). Besides, other types of biosurfactants include lipopeptide (viscosin from *P. fluorescens*), Emulsan 378 (*P. fluorescens*) (Rosenberg and Ron, 1999), trehalose dimycolipids from *Rhodococcus* sp. (Ramdahl, 1985), lichenysins from *Bacillus* sp. (Saraswathy and Hallberg, 2002), and surfactin from *B. subtilis* (Siegmund and Wagner, 1991) and *B. amyloliquefaciens* (Zhi et al., 2017). These potent surfactants are shown to reduce surface tension from 72 to <30 dyn/cm, thus resulting in better uptake of hydrocarbons. The production of various rhamnolipid- and glycolipid-based biosurfactants has been reported for species members of *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Burkholderia*, etc. while growing under naphthalene and methylnaphthalenes (Kanga et al., 1997; Puntus et al., 2005). *P. maltophilia* CSV89 when grown on aromatic compounds like naphthoic acid produced an extracellular biosurfactant, Biosur-Pm (Phale et al., 1995). The kinetics of Biosur-Pm production suggested its synthesis to be a growth- and pH-dependent process. Cells were found to produce a higher amount of Biosur-Pm at neutral pH as compared with pH 8.5. Cells grown at pH 8.5 were more hydrophobic with higher affinity toward aromatic and aliphatic compounds as compared with cells grown at pH 7.0. In *Rhodococcus* sp. N6, a higher carbon to nitrogen ratio (C:N) and iron-limiting conditions are responsible for the optimum production of extracellular biosurfactants (Mutalik et al., 2008). Attempts have been made for the enhanced biosynthesis of biosurfactant (surfactin) through the optimization of bacterial strains and fermentation. However, the low surfactant titers (1.0 g/L) in the medium are a challenge to produce at a large scale (Jiao et al., 2017; Wu et al., 2019). As a result, genetic engineering methods have been used to enhance its biosynthesis. However, due to a large-size operon (∼25 kb) and complex biosynthetic regulation of quorum sensing system, it has shown to be difficult to engineer (Jiao et al., 2017; Wu et al., 2019). *Bacillus* spp. have been engineered to improve surfactin production mainly through promoter exchanges (*srf*A operon), overexpression of the surfactin exporter YerP, and the regulators ComX and PhrC (Jiao et al., 2017). However, these genetic engineering methods all resulted in a single or a few gene modifications, but the commercial production has still not been achieved. Hence, further knowledge-based optimizations need to be explored.

FACTORS AFFECTING THE BIODEGRADATION OF PAHS

The PAH biodegradation studies have been mainly investigated under standard laboratory conditions. However, at the contaminated site or environment, numerous abiotic and biotic factors (temperature, pH, oxygen, nutrient availability, substrate bioavailability, other xenobiotics, end-product

inhibition, etc.) are shown to vary and influence the degradation ability of microorganisms.

Temperature has a profound effect on the biodegradation of PAHs. With increasing temperature, the concentration of dissolved oxygen decreases, which impacts the metabolism of aerobic microbes, as they require molecular O_2 as one of the substrates for oxygenases which perform ring-hydroxylating or ring-cleaving reaction. It is often noted that the increase in temperature transforms parent PAHs into more toxic compounds, which inhibits biodegradation (Muller et al., 1998).

It has been observed that many of the PAH-contaminated sites have extreme pH conditions, for example, acidic mine drainage-impacted sites (pH 1–4) and alkaline leachate-impacted gas/coal gasification sites (pH 8–12). These conditions are found to impact biodegradation processes severely. Hence, the addition of suitable chemicals (having mild to minimum oxido-reductive potential) is recommended to adjust the pH before application of microbes for bioremediation like ammonium sulfate or ammonium nitrate for alkaline soil and liming with calcium or magnesium carbonate for acidic sites (Bowlen et al., 1995; Gupta and Sar, 2020).

The availability of oxygen at impacted sites is a rate-limiting factor for biodegradation of PAHs. Owing to the oxido-reductive environmental condition, oxygen is often introduced from an external source (tilling, air-sparging, and addition of chemicals) for the *in situ* bioremediation process (Pardieck et al., 1992). Odencrantz et al. (1996) have shown the effective bioremediation of BTEX compounds in a contaminated aquifer after the addition of magnesium peroxide (O_2-releasing compound). Another study involving *in situ* degradation of phenols and BTEX in a contaminated aquifer has used sodium nitrate through injection and construction of abstraction wells for effective bioremediation (Bewley and Webb, 2001).

The availability of nutrients like N, P, K, and Fe is reported to be essential for effective bioremediation. Thus, supplementation of these nutrients (referred to as biostimulation) in limiting concentration is required to enhance the growth of indigenous microorganisms for effective bioremediation of PAHs (Sarkar et al., 2020). On the other hand, high/excess nutrient levels were found to affect the rate of biodegradation of PAHs.

The bioavailability of PAHs in the environment is often limited due to their low aqueous solubility (hydrophobicity) and strong tendency to adsorb onto minerals and organic matter of the matrix (Cornelissen et al., 2005; Rein et al., 2016). The aqueous solubility of PAHs decreases with increasing molecular weight, which in turn reduces the bioavailability in groundwater and surface water. It has also been shown that aging (retention for a longer time in the environment) of PAHs renders them more difficult to extract/degrade from a polluted ecosystem, thus significantly impacting the rate of bioremediation (Luo et al., 2012).

Contaminated environments are usually burdened with dynamic concentrations of a particular PAH or combination/mixtures of PAHs, which often limits the degradation efficiency (Penning et al., 1999; Juhasz and Naidu, 2000). The presence of a high concentration of naphthalene has shown inhibitory effects on the degradation of other PAHs by bacterial co-cultures (Bouchez et al., 1995). Phenanthrene biodegradation is reported to be inhibited due to the presence of a higher concentration of naphthalene and methylnaphthalene (Stringfellow and Aitken, 1995). In some environments, the physicochemical transformation processes (photo-oxidation, chemical oxido-reduction) generate more toxic by-products, which limit the metabolism of PAHs. Hence, both the amount and composition (concentration) of PAHs as well as the toxic (by-)products generated during treatment at the contaminated sites should be monitored before strategizing the effective *in situ* bioremediation plans.

Although much information on pollutant-degrading bacterial strains, metabolic steps, and enzymes is involved, the field-scale application and bioremediation attempts have yielded poor results. Such failures can be attributed due to lack of insights into important factors such as (i) the thermodynamic feasibility of assembled catabolic networks, (ii) kinetic properties and regulatory feature of the enzymes, (iii) physicochemical properties of metabolites, (iv) extent of induction of enzymes, (v) suppression of degradation pathways by a simple carbon source like carbohydrates and organic acids, (vi) cross-talk between metabolic routes, and (vii) stress responses and changes in overall cell physiology (de Lorenzo, 2009; Ramos et al., 2011; Phale et al., 2020). In this context, the selection of suitable candidate(s) for biodegradation is a crucial initial step. Over the years, several microbes have been considered for application in biodegradation processes, but no single naturally isolated bacterial strain was found to possess all the desired characteristics. Hence, metabolic engineering strategies for optimizing genetic and regulatory processes are becoming suitable alternatives to improve overall cell robustness and biodegradation rate (Nielsen et al., 2014). Recent advancement in the use of systems biology and "omics" techniques has helped to gain a deeper insight into the genetic and physiological background of microbes to model enzymatic reactions and determine the constraints for efficient elimination. To overcome environmental constraints (factors), computational tools/databases are assisting in the fine-tuning of pathways to maximize performance in a cost-effective manner (Dvorak et al., 2017). In addition, researchers are developing effective consortia which are shown to degrade various pollutants based on co-metabolism/synergistic actions of different mutualistic/symbiotic microbes. Further research on genome-scale metabolic modeling (fluxomic and interactomic) studies enabled us to unravel the biodegradative behavior of isolates. In the last decade, BESs including microbial fuel cells and microbial electrolysis cells have become preferred options for waste treatment at the industrial scale. By integrating biological treatment, electrolytic dissociation, and electrochemical oxidation/reduction, these systems are regarded as a new sustainable strategy for the treatment of various organic pollutants with higher treatment efficiencies than conventional processes (Huang et al., 2011; Pant et al., 2012; Nazari et al., 2020). Especially, the oxidation of organic pollutants in wastewater of domestic, brewery, and paper-recycling units, food processing industry, and landfill leachate has been successful (Nazari et al., 2020). Besides, the catalytic activities of electrochemically active microorganisms lead to sustainable bioprocessing approaches, viz. electricity generation, nutrient recovery, formation of

value-added by-products, and bioremediation (dehalogenation and reduction of nitro-organics). With the knowledge of the participating microbes in biodegradation and their metabolic potential, several microbial bioremediation approaches such as biostimulation and bioaugmentation are popularized, which are elaborated further in the next section. Along with microbes, the introduction of plants to the polluted site(s) and the use of plants with microbes as well as composting and biopiling are increasingly being preferred for contaminant attenuation in polluted sites.

BIOREMEDIATION STRATEGIES

A contaminated environment is usually found to be deficient in essential nutrients to support microbial growth and metabolic processes of potential microbes. Based on the knowledge of participating microbial members (or communities) and their nutrient requirement, two major strategies are popularly used for the bioremediation of contaminated niches, i.e., biostimulation and bioaugmentation. In addition, the use of plants (phytoremediation) either alone or in combination with potential microbe(s) (phyto-bioremediation) is also preferred as an effective cleanup procedure. The strategies are depicted in **Figure 8**.

Biostimulation refers to the supplementation of one or more nutrients to the impacted sites for accelerating the growth and metabolic abilities of microorganisms (Sarkar et al., 2020). The application of water-soluble inorganic nutrients (NO_2^-, NO_3^-, Fe^{3+}, SO_4^{2-}, and CO_2), slow-release fertilizers, and oxygenation resulted in the increase of the rate of degradation (Sarkar et al., 2016, 2020). It has been estimated that an optimal ratio of 100:10:1 for C:N:P is required for effective biodegradation of hydrocarbon pollutants (Dibble and Bartha, 1979; Dias et al., 2012). Several laboratory studies have shown that excess carbon (as in PAHs) compared with other nutrients critically affects the microbial metabolism of hydrocarbons (Sarkar et al., 2020). Several low-cost nutrients like biochar, sawdust, crop residues, leaf litter, compost, etc. have been successfully applied to enhance the growth of microorganisms and remediate contaminated sites (Simons et al., 2013; Qin et al., 2013). Among all nutrients, nitrate amendment has been the most effective biostimulating agent for bioremediation of hydrocarbon pollutants (Roy et al., 2018). Effective biodegradation (80%) of petroleum sludge containing 400 g/kg total petroleum hydrocarbons (TPH) was achieved with nitrate amendment. Increased activity (stimulation) of nitrate-reducing *Azovibrio*, *Pseudoxanthomonas*, *Commamonas*, and *Bacillus* was evident in this study (Sarkar et al., 2016; Roy et al., 2018). Other studies have also shown a positive effect of either N or P on the biodegradation of pesticides (Sharma et al., 2019).

Bioaugmentation aims to supplement microbes externally, either as a single pure culture or mixed culture (consortium), to enhance the overall metabolic activity for complete degradation. Pure cultures of *Pseudomonas*, *Flavobacterium*, *Sphingomonas*, *Achromobacter*, *Bacillus*, and *Rhodococcus* are promising bioaugmentation agents. It is observed that the use of mixed bacterial culture is more advantageous than pure culture due to synergistic interactions among microbial species (Tyagi et al., 2011; Ghaly et al., 2013; Sarkar et al., 2020). The use of consortium harboring *Delftia*, *Bacillus cereus*, *Pseudomonas resinovorans*, *P. fluorescens*, *Exiguobacterium*, *Arthrobacter*, and *R. erythropolis* has been effective for the bioremediation of diesel-contaminated soil (Sprocati et al., 2012). A consortium of *Aeromonas*, *Alcaligenes xylosoxidans*, *Gordonia*, *P. fluorescens*, *P. putida*, *Rhodococcus equi*, *S. maltophilia*, and *Xanthomonas* has shown 89% degradation of various hydrocarbons in a soil column within a year (Szulc et al., 2014). A combinatorial approach where both biostimulation and bioaugmentation are applied led to a more effective bioremediation of PAHs and short- and long-chain alkanes for various hydrocarbon-impacted niches (Sarkar et al., 2016; Wu et al., 2016). The use of microbial consortium harboring various *Pseudomonas* spp. and other *gamma-proteobacteria* members in combination with NH_4NO_3 and K_2HPO_4 is an effective strategy for total petroleum hydrocarbon degradation (Varjani et al., 2015).

Plants (native and adapted species) are also used to remove aromatic pollutants from contaminated sites and are referred to as phytoremediation. Moreover, it particularly suits to the treatment of larger areas of surface contamination, where other methods may not be as effective. Several species of either native plants or grasses (*Agropyron*, *Bouteloua*, *Cyanodondactylon*, *Elymus*, *Festuca*, *Melilotus*, etc.) or legumes (*Alfalfa*) are popularly used to degrade various PAHs (Harvey et al., 2002; Hall et al., 2011; Truu et al., 2015). A combined use of different types of grasses was shown to be effective for phytoremediation of various PAHs and pesticides in sandy loam soils (Lalande et al., 2003; Basumatary et al., 2012). These plants have also shown to release various metabolites (amino acids, sugars, inorganic nutrients) and enzymes (dehalogenase, reductase, peroxidase, laccase, etc.) in the root exudates. This helps to enhance the growth and metabolic activities of rhizospheric microbes, or they interact directly with PAHs to biotransform. Various studies have also shown that the combined use of plants and microbes is an emerging cost-effective technique for contaminant removal for maximum efficiency with minimum environmental disturbances (**Figure 8**). The application of such techniques with agricultural management methods like integrated nutrient management, water use, crop rotation, and other agronomic practices will help in the complete removal/cleaning up of such pesticide pollutants as well as xenobiotics from the agricultural fields and contaminated site.

CONCLUSION AND FUTURE DIRECTION

The contaminated ecosystems harbor vast microbial diversity with dynamic metabolic flexibility. However, it often lacks suitable conditions, i.e., robust microbial members with metabolic pathways and its regulation for complete mineralization, nutrients required for metabolism, and other biotic/abiotic factors. Hence, designing appropriate microbial host(s) with efficient, rapid, and broad range of degradation abilities is a key factor in the scaling up of the bioremediation process. In addition, other properties like chemotaxis, cell

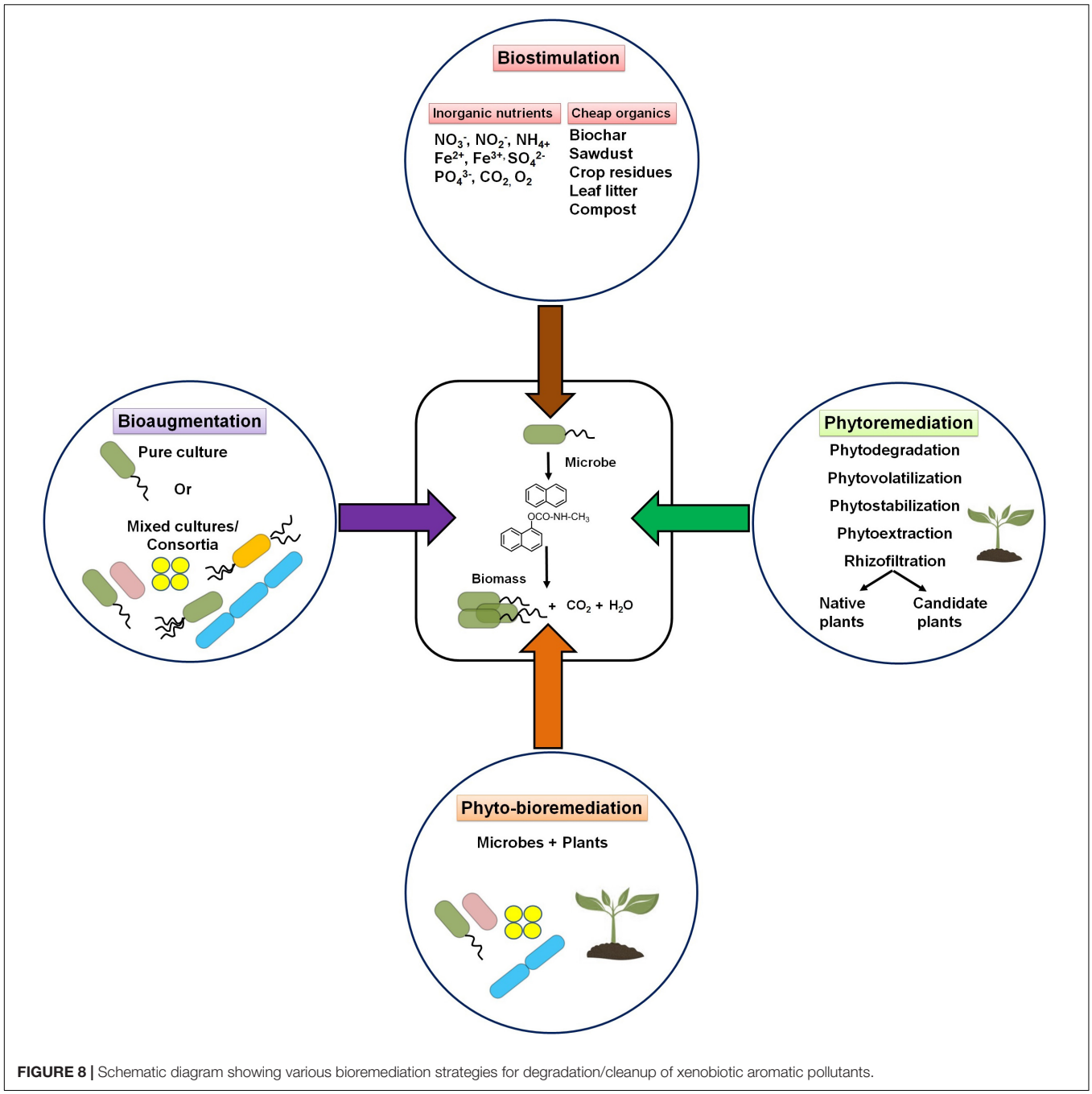


FIGURE 8 | Schematic diagram showing various bioremediation strategies for degradation/cleanup of xenobiotic aromatic pollutants.

surface/membrane fluidity, compartmentalization of pathway, and biosurfactant production are advantageous for the enhanced metabolism of PAHs. Furthermore, organisms capable of degrading a broad range of PAHs with genotypic and phenotypic stability of degradation trait, i.e., chromosomal origin and microbes with preferential utilization of such pollutants even in the presence of a simple carbon source like carbohydrates or organic acids, are of immense importance for efficient bioremediation. The use of next-generation and high-throughput molecular and computational tools is most valuable for understanding system biology of suitable xenobiotic degrading microbes. Since natural bioremediation is a slow and complex process, a system biology-based platform will be of much value for metabolic engineering (patchwork assembly, gene shuffling, and genome editing) of microbes for the reconstruction of new pathways and robust microbial chassis. The use of BESs incorporating metabolically robust microbial hosts as microbial fuel cells and microbial electrocatalytic cells might be an alternative approach for the treatment of a wider varieties of pollutants. The combined implication of omics—genomics, metagenomics, metagenome-assisted genome assemblies, transcriptomics, proteomics, metabolomics, and

phenomics—would further aid in understanding the complex behavior of microbes, fine-tuning of selected biochemical routes, its networks, and whole-cell biocatalysis. In addition, more advanced computational tools are required to fully exploit the omics-derived data for better functional assignments and understanding metabolic fluxes and their interactions. In recent days, adaptive laboratory evolution has been frequently used to transform the desired properties in environmental bacteria and for the fine-tuning of recombinant microorganisms. Exposing microorganisms with desirable target chemical pollutant(s) for prolonged duration enabled the isolation of potent degrading bacteria. Screening and identification of potent plant growth-promoting and pollutant-degrading microbes would be of added value for increasing crop yield/productivity in agroecosystems and the biodegradation of xenobiotics like pesticides simultaneously. The development of integrated platforms which might provide all the information related to bioremediation research, including data, analytical methods, and pipelines, is the current need of the hour. In the context of the use of genetically engineered microbes for pollutant biodegradation, public environmental concerns and regulatory constraints have delimited the field tests of microbes and also have affected the quality and progress of biotechnological research. So, better knowledge dissemination, popularization of achievements, and mass awareness on the beneficial use of engineered microbes must be a priority. Together with this, cross-border coordination through scientists/working groups around the world in different laboratories for data sharing and database maintenance would further help in rationalizing bioremediation plans. The use of cybernetics, artificial intelligence, nano-bioremediation (nanoscale zero-valent metals/nano-organics), agricultural/agronomic management practices, etc. will enable further value addition and cost reduction of sustainable on-site bioremediation strategies in the near future.

AUTHOR CONTRIBUTIONS

BM and PP conceptualized, organized, wrote, and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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Strategies for Enhancing *in vitro* Degradation of Linuron by *Variovorax* sp. Strain SRS 16 Under the Guidance of Metabolic Modeling

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Phenyl urea herbicides are being extensively used for weed control in both agricultural and non-agricultural applications. Linuron is one of the key herbicides in this family and is in wide use. Like other phenyl urea herbicides, it is known to have toxic effects as a result of its persistence in the environment. The natural removal of linuron from the environment is mainly carried through microbial biodegradation. Some microorganisms have been reported to mineralize linuron completely and utilize it as a carbon and nitrogen source. *Variovorax* sp. strain SRS 16 is one of the known efficient degraders with a recently sequenced genome. The genomic data provide an opportunity to use a genome-scale model for improving biodegradation. The aim of our study is the construction of a genome-scale metabolic model following automatic and manual protocols and its application for improving its metabolic potential through iterative simulations. Applying flux balance analysis (FBA), growth and degradation performances of SRS 16 in different media considering the influence of selected supplements (potential carbon and nitrogen sources) were simulated. Outcomes are predictions for the suitable media modification, allowing faster degradation of linuron by SRS 16. Seven metabolites were selected for *in vitro* validation of the predictions through laboratory experiments confirming the degradation-promoting effect of specific amino acids (glutamine and asparagine) on linuron degradation and SRS 16 growth. Overall, simulations are shown to be efficient in predicting the degradation potential of SRS 16 in the presence of specific supplements. The generated information contributes to the understanding of the biochemistry of linuron degradation and can be further utilized for the development of new cleanup solutions without any genetic manipulation.

Keywords: phenyl urea herbicide, linuron, 3,4-dichloroaniline, biodegradation, genome-scale metabolic model, *Variovorax* sp. strain SRS 16

INTRODUCTION

Phenyl urea herbicides are among the most widely used herbicides for weed control in several crops (mostly cereals) through their pre- or post-emergence applications. These substances interrupt electron transfer in photosystem II, leading to the formation of reactive oxygen species and resulting in cell damage (Liu, 2010). The increased rate of application of xenobiotics such as the phenyl urea herbicides in recent years enhanced their burden to the environment due to their persistence in the surroundings (Hasanuzzaman et al., 2020). These compounds are consistently found to have negative effects on the ecosystem, including hazards to human health (de Souza et al., 2020; Garcês et al., 2020). Linuron has been one of the most widely applied phenyl urea herbicides in agriculture practice, also reported as an environmental pollutant (Dejonghe et al., 2003; Horemans et al., 2016). Remediating the environment from the accumulated toxic substances is the focus of a growing number of researches efforts. The degradation or the removal of such pollutants is reported to be conducted through physical, (photo-)chemical, and chemical processes (Katsumata et al., 2011; Reddy and Kim, 2015; Kovács et al., 2016; Hao et al., 2019; Bhat et al., 2020). Microorganisms play a major role in the biological removal of linuron. So far, different strategies have been approached for enhancing biodegradation and bioremediation (Raman and Chandra, 2009; Pimviriyakul et al., 2020). By promoting the growth of soil microbial degraders, soil amendments are being used in order to accelerate the removal rate of pollutants (Bao et al., 2020). However, the selection of the amendments is mostly based on trial and error.

Biodegradation of linuron is generally initiated by amidase hydrolases, which leads to the formation of a more toxic intermediate, 3,4-dichloroaniline (DCA). DCA further degrades to metabolites that can be consumed in the central metabolism of the microorganisms. However, partial degradation of linuron produces chloroanilines that are more toxic than linuron itself. Bacterial genera such as *Arthrobacter*, *Bacillus*, *Comamonas*, *Pseudomonas*, *Sphingobium*, and *Variovorax* are able to degrade/transform linuron in various environments either in isolation or as part of a consortium (Turnbull et al., 2001; Dejonghe et al., 2003; Lerner et al., 2020; Zhang et al., 2020). *Variovorax* SRS 16 is widely studied for its ability to utilize linuron as a sole carbon and nitrogen source. *Variovorax* SRS 16 also possesses a modified chlorocatechol ortho-cleavage pathway, allowing the utilization of linuron as a substrate for growth. The presence of the amidase (specific linuron hydrolase) and gene clusters which are responsible for the degradation of linuron and dichloroaniline in SRS 16 has been described in detail with the support of proteomic studies (Bers et al., 2011). Based on the relatively extensive phenotypic information, together with the publication of its genome sequence (Sørensen et al., 2005; Bers et al., 2011), *Variovorax* SRS 16 is recognized as a good model for exploring biodegradation of phenyl urea herbicides.

Processing genomic information into a metabolic model is increasingly used as a route for generating a predictive tool to elucidate and manipulate cellular biochemical activity. Genome-scale metabolic modeling has been proven as an efficient approach to decode the genomic and functional information for a specific phenotype by investigating the gene–protein interactions on a cellular level (Thiele and Palsson, 2010; Gu et al., 2019). To construct metabolic models, some preliminary information on physiological requirements (mainly of growth) of the organism is needed for better curation and to ensure the appropriate functioning of the model (Covert et al., 2001). This approach generally follows some defined steps such as collecting basic information related to the conditions required for growth of cells together with description of the metabolism of the organism based on its genome sequence. Conversion of this information into a mathematical framework, as a model, is the next step. Further, the behavior of the model can be predicted under specific growth conditions along with biomass generation and exchange (uptake or release) of relevant compounds (metabolites). In the final step, experiments are carried out to validate the predictions (Devoid et al., 2013). Genome-scale modeling is considered advantageous as it allows screening of multiple conditions in a short time and lowering of cost by providing a limited set of solutions that can be further tested. Solutions that enhance a desired or improved behavior of the organism can be predicted based on media supplementations without any requirement of genetic modification (García-Jiménez et al., 2018).

The importance of these models as a tool for rapid biodegradation and for the development of effective bioremediation strategies is recognized (Scheibe et al., 2009) and demonstrated in a few studies. For example, a metabolic model of *Ralstonia eutropha* H16, a microorganism with multiple potential applications in environmental biotechnology, was used to analyze its metabolic characteristics (Park et al., 2011). The genome-scale model of *Pseudomonas veronii* 1YdBTEX2, a degrader of monoaromatic compounds, was used together with 'omics data (transcriptomics and exometabolomics) to characterize active metabolic processes at different growth stages. The matching of predictions with bacterial response indicates the importance of modeling to enhance the success rate of bio-inoculants in complex systems (Hadadi et al., 2020). Such studies help to predict the changes in cellular processes under a specific given condition (e.g., xenobiotic biodegradation). In addition, such integrative "omics modeling" approaches have also been used for optimization of predefined processes (Calmels et al., 2019). Xu et al. (2019) used models constructed for indigenous species whose abundance in soil was affected by exposure to the herbicide atrazine. The community modeling revealed interspecies metabolic interactions that support enhanced growth and degradation. The simulations pointed at a contribution of non-degrader species to the *in situ* process achieved thorough trophic exchanges with the degrader (Faust, 2019). The effect of specific metabolites on the degradation rate of atrazine by *Paenarthrobacter aurescens* TC1 was predicted by a genome-scale model and was further validated *in vitro* (Ofaim et al., 2020). The predictive biology facilitated the identification of optimal conditions for degradation that can promote the development of bioaugmentation or biostimulation approaches for better controlling the degradation processes. One bottleneck of bioaugmentation is that in many cases, the exogenous degrading microorganisms fail to establish in

a new environment and do not produce enough biomass to eliminate contaminants *in situ*. Stimulating the growth of native degraders through adding specific compounds to the contaminated site is a practical alternative to bioaugmentation. The present study aims to elucidate the effect of nutritional supplements on linuron degradation by *Variovorax* SRS 16 (as a model for native degraders) and estimate their potential usage as biostimulants in order to harness the full potential of indigenous degraders. SRS 16 was selected due to the availability of a fully sequenced genome and extensive biochemical knowledge of its full pathway in linuron degradation – a pollutant that belongs to a large contaminant group of the phenyl urea herbicides. A genome-scale metabolic model of SRS 16 was constructed following automatic and manual protocols and was applied to explore the stimulating effect of different supplements through iterative simulations Compounds pointed by simulations as strong enhancers of degradation were further validated through wet laboratory experiments.

MATERIALS AND METHODS

Constraint-Based Reconstruction

As a first step, a draft model of *Variovorax* sp. SRS 16 was reconstructed by following a bottom-up approach as elaborated by Ofaim et al. (2020). Briefly, the whole-genome sequence of SRS 16 was retrieved from the genome database of NCBI and annotated using RAST (Overbeek et al., 2014). An initial metabolic reconstruction was obtained by analyzing the annotated genome sequence through Model SEED (Faria et al., 2018). The metabolic reconstruction has three main components: a complete list of reactions and their associated genes, information of interactions between the gene and protein associated with the metabolic reaction, and the components involved in biomass generation (Devoid et al., 2013). The list of reactions contains the biomass (which includes all the biomass constituents and their fractional status), cytosolic, transport, and exchange reactions. The model was improved by adding potentially missing reactions through an automated gap-filling process (Henry et al., 2010). The improved draft model was exported to Systems Biology Markup Language (SBML) format from Model SEED.

The reactions in the original model were complemented by reactions described for *Variovorax* SRS 16 in additional genomic annotation databases including BiGG (Norsigian et al., 2020), KEGG (Kanehisa et al., 2016), UniProt (The UniProt Consortium, 2019), IMG (Chen et al., 2019), and MetaCyc (Karp, 2002). Reactions were included according to EC accessions. Reaction stoichiometries were verified for all reactions, and non-balanced reactions were fixed. Directionality and stoichiometry of reactions were determined according to the KEGG scheme. Specific reactions involved in linuron degradation were added based on a literature survey (**Figure 1**). The reconstruction was curated to verify that it allows the generation of all biomass components under physiologically feasible minimal conditions – minimal salt solution and linuron (**Supplementary File 1**). The *Variovorax* sp. SRS 16 model in SBML format is provided in **Supplementary File 2**.

Simulations of Growth and Degradation of Linuron Using Flux Balance Analysis

Simulations were carried out using flux balance analysis (FBA), allowing us to depict cellular processes based on cellular reconstructions (Rana et al., 2020). Briefly, FBA follows the law

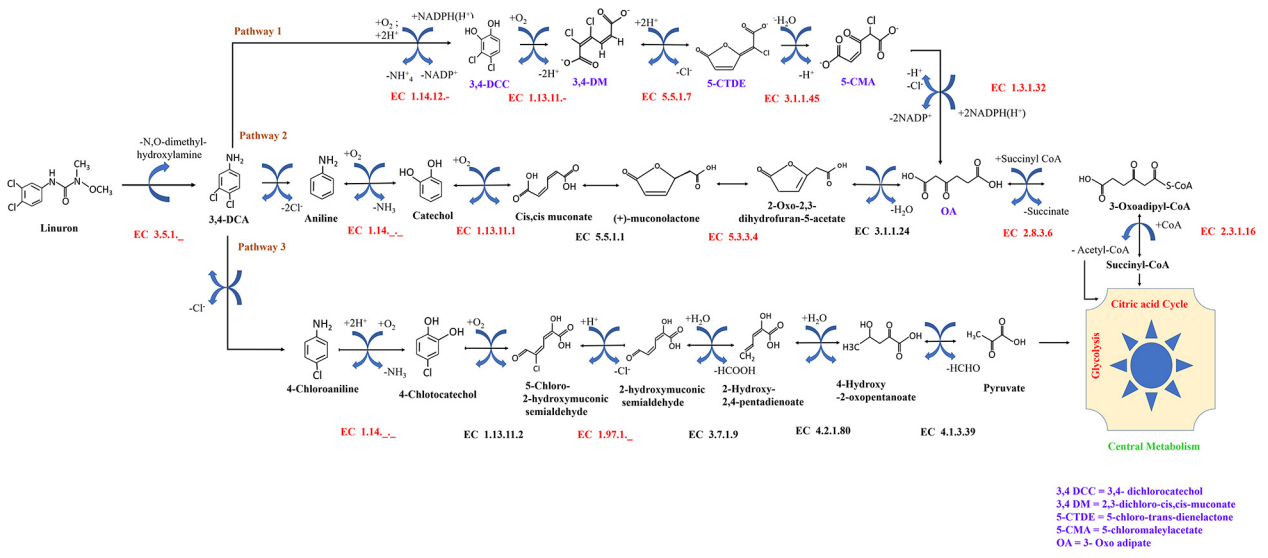

FIGURE 1 | Possible fate of linuron and 3,4-DCA in SRS 16. Reactions in black were included in the automatic reconstruction. Reactions in red were manually added to the model. Pathway 1 (modified ortho-cleavage pathway) was retrieved from BioCyc based on direct evidence for *Variovorax* SRS 16 strain (https://biocyc.org/META/NEW-IMAGE?type=PATHWAY&object=PWY-7496). Pathways 2 and 3 (ortho- and meta-cleavage pathways, respectively) were completed by following the generic KEGG pathways.

of mass conservation and consider the metabolic framework as a static stoichiometric matrix (metabolites × reactions). FBA describes the predicted flux distribution through linear optimization by targeting specific cellular objectives (mainly growth). In the present study, the growth of the model was chosen as the objective function through the maximization of the biomass reaction. Flux variability analysis (FVA) was carried out to account for the possible flow of fluxes involved in secretion and uptake of all metabolites (Mahadevan and Schilling, 2003). The simulations were applied to predict biomass generation and linuron degradation over a time period under a range of defined conditions – *in silico* growth media. All simulations were carried out under definitions that follow experimentally verified viable conditions in minimal media with linuron (**Supplementary File 3**) and 120 exchange metabolites (one at a time) representing an alternative carbon/nitrogen source or other supplements (**Supplementary File 4**).

Dynamic modeling was used for the prediction of the profile of consuming metabolites typical to the biomass increase and linuron degradation across time. To this end, we simulated the behavior of our metabolic model across time. The simulation process follows (Xu et al., 2019; Ofaim et al., 2020) and is illustrated in **Supplementary File 5**. Briefly, the model works under the following assumptions: (1) a finite start amount of media components is available; (2) a maximal amount of uptake a single cell can acquire from the media in a given time point is defined (the lower bound of the exchange reaction value); (3) new substrate concentrations in each time point are determined by the predicted substrate concentration for the previous step augmented with any additional substrates provided or consumed in the current iteration. The maximum uptake was set to a ratio of up to 1 unit of each metabolite available in the media; (4) after each time tick, the biomass amount was updated according to the flux amount of the biomass reaction in the model at this time tick. As the biomass production rate serves as a proxy for the size of the population in the simulated environment and substrate uptake/secretion is mainly affected by population size, the model was used to evaluate the actual substrate uptake and growth rate given the supplied media across time.

Simulations were carried out until a state where time cycles did not lead to an increase in biomass was reached. Initial concentration values for all metabolites were set to a fixed amount of 50 units (represented as the initial lower bounds, LB, of the exchange reactions). The upper bound of the exchange reactions was set to 1,000 units to allow the secretion of all the exchange metabolites. Reversible non-exchange reactions' lower bound was −1,000 units, and for non-exchange non-reversible reactions, it is 0 units; the upper bound for non-exchange reactions was 1,000 units. At each growth cycle, the generation of biomass is updated on the basis of flux flow in the biomass reaction. The algorithm assumes that media components are available to all growing cells with equal probability. The growth rate and the flux of substrate (consumed or secreted) associated with the model in the given media conditions are calculated at every time point, where, in each cycle, we try to maximize the biomass of each member of the community and then we fixate the biomass and minimize the uptake of metabolites of each member while maintaining the max biomass found. The cycles of predictions were carried out until the saturation point appeared in the biomass production (no growth recorded reflecting the exhaustion of given resources). Starting with one bacterial cell, the flux balance model was used to predict the uptake of carbon and nitrogen sources (including linuron) across time.

All model simulations were done on an Intel i7 quad-core server with 32 GB of memory, running Linux. The development programming language of our simulators was JAVA, and our linear programming software was IBM CPLEX.

In vitro Experiments of *Variovorax* sp. SRS 16 Strain: Growth and Linuron Degradation

Variovorax sp. strain SRS 16 (NCBI: txid282217, kindly provided by Dirk Springael, KU Leuven) was revived from stock cultures stored in glycerol at −80°C. The purity and authenticity of the strain were checked by 16S rRNA gene sequencing. The minimal medium (**Supplementary File 3**) described by Sørensen and Aamand (2003) was prepared, and bacterial cells were grown on agar plates at 25°C. For the quantitative analysis, 250 ml flasks were used to prepare the minimal medium (50 ml of the medium per flask) and autoclaved. The effects of the seven selected substrates – representing strong, moderate, and weak enhancers of linuron degradation – on growth and linuron degradation were tested. The supplements (filter sterilized) were added to a final concentration of 0.12 mM in media (equivalent to 30 ppm of linuron). The linuron concentration was selected on the basis of a previous study. MS, MS + C, and MS + N consist of only minimal medium, minimal media added with only carbon (glucose), and minimal media added with only nitrogen (ammonium salt), respectively. MS was treated as a negative control. The autoclaved minimal medium was supplemented with the substrates (separately) and linuron (30 ppm) followed by bacterial inoculation to a final OD (at 600 nm) of 0.05–0.1. The mother culture was raised in MSCN (succinic acid and ammonium salt) medium with linuron, by inoculating fresh agar plate-grown bacterial cells followed by incubation at 25°C (120 rpm) for 24 h.

All the inoculated flasks were incubated at 25°C (120 rpm) for 7 days. Bacterial growth and linuron degradation were monitored at definite intervals (zeroth day, third day, fifth day, and seventh day) from the day of inoculation. For bacterial growth, 200 μl was taken, and OD was measured at 600 nm by using Infinite® 200 PRO (Tecan Trading AG, Switzerland). Linuron degradation was measured through HPLC with standard procedures. Briefly, 1 ml of sample was taken and centrifuged at 10,000 × *g* for 5 min. The supernatant was filtered through a 0.22 μm PTFE syringe filter and transferred to the HPLC vials for the detection of residual linuron. Linuron and DCA were analyzed by using Agilent 1100 HPLC (Waldbronn, Germany). Detection of linuron was done at 240 nm using the external calibration method (sensitivity 0.5 mg L^{-1}). The mobile phase of 70% methanol at a flow rate of 1 ml min^{-1} with a reverse-phased (Phenomenex, Torrance, CA) of

250 mm length × 4.6 mm inner diameter with particle size 5 μm was used for separation.

Linuron biodegradation and biomass buildup experiments were carried out in biological triplicates. The effect of supplements was statistically analyzed by performing repeated-measures ANOVA at $p < 0.05$ in SPSS v19.

RESULTS

Reconstruction of Genome-Scale Metabolic Network for *Variovorax* sp. Strain SRS 16

The genome of *Variovorax* sp. strain SRS 16 is about 7.7 Mb in size, including its chromosome (5.7 Mb) and four plasmids with an approximate size of <1 Mb each (Öztürk et al., 2020). The complete sequence was retrieved from NCBI and annotated for reconstruction using the Model SEED pipeline. This initial reconstruction contained a list of 2,150 gene–protein–reaction associations that were classified as exchange, transport, and cytosolic as well as a list of all relevant metabolites and a biomass reaction. Based on the taxonomic classification, the biomass reaction was defined as Gram-negative bacteria in the Model SEED reconstruction pipeline. The composition of the biomass reaction summarizes the fractional contribution of generalized microbial biomass precursors (e.g., amino acids and lipids) to the synthesis of a new cell and is similar to the previously published genome-scale reconstruction of *Escherichia coli* strain K-12 (Monk et al., 2017). Initial simulations were carried out for debugging and removing futile or erroneously energy-generating loops. To this end, all external fluxes were blocked (upper and lower bounds set to zero). Next, a minimal medium was used, verifying that growth requires the supply of both carbon and nitrogen sources. After establishing no growth under infeasible conditions, we tested growth (biomass production) under experimentally verified conditions (succinic acid and ammonium salt as a carbon and nitrogen source, respectively) as described by Sørensen et al. (2009). Fine tuning of growth simulations to correctly represent the bacteria's biology was done by manual curation. This included manual gap filling (addition of spontaneous and literature-supported reactions) and curation of reaction directionality.

The reconstructed metabolic network presented here covers 19.2% of the open reading frames (ORFs) present in the genome (**Table 1**). The coverage is lower than gold standard models of other Gram-negative models such as iAF1260 for *Escherichia* (Feist et al., 2010) (29%), iPC1209 for *Pectobacterium* (Wang et al., 2015), and iPAO1 for *Pseudomonas* (25.8%) (Zhu et al., 2018). It is, however, similar to coverage obtained for the genome-scale reconstruction of the closely related genus *Rhodoferax ferrireducens* (from the same family *Comamonadaceae*) – 15.6% (Risso et al., 2009). Total number of reactions is similar to gold standard reconstruction including the updated model of *E. coli* str. K-12 (Orth et al., 2011). A detailed description of the network including the reactions, metabolites, genes, and compartments that comprise the network is provided in **Table 1**.

TABLE 1 | Functional details of the *Variovorax* sp. SRS 16 model.

Serial no.	Category	Total number
1	Metabolites	2,185
2	Exchange reactions	127
3	Reversible reactions	1,432
4	Transport reactions	277
5	Biochemical reactions	1,746
6	Total reactions	2,150
7	Genes associated with a reaction*	1,425 (total 7,411) (Öztürk et al., 2020)

**19.2% coverage of genome.*

The model is also available as an SBML file (Hucka et al., 2003) in **Supplementary File 2**. The SBML file can be used with tools such as MATLAB or other SBML-compliant software. The minimal media used for simulations are available in **Supplementary File 1**. The set of metabolites, reactions, and exchanges are provided in **Supplementary Files 1, 4**.

Adapting and Curating iRZ1425 for Modeling Linuron Degradation

Model reactions involved in linuron degradation and their complete link to the core metabolism were added manually based on the detailed reports of the relevant pathways in the specific strain as well as in other bacterial species, forming three possible pathways (**Figure 1**). In Pathway 1, degradation occurs via modified ortho-cleavage, where in the first step, there is a ring cleavage in DCA; then, chloro intermediates transform into maleylacetate and finally enter the citric acid cycle. The pathway was described by Bers et al. (2011) based on the study of linuron degradation in *Variovorax* SRS 16 and is supported in part by molecular evidence. Notably, none of the reactions in the modified ortho-cleavage pathway was identified by automatic reconstruction. Alternatively, we also included two hypothetical pathways (not specifically detected for SRS 16), Pathways 2 and 3 (**Figure 1**), for the degradation of the herbicide hydrolysis product (Arora, 2015; Hussain et al., 2015). Pathway 2 follows the removal of successive dehalogenation and the formation of aniline from DCA. The product aniline enters the ortho-cleavage pathway through catechol to the core metabolism. Pathway 3 begins with the dehalogenation of DCA with the formation of 4-chloroaniline. 4-Chloroaniline is further converted to catechol derivatives, which leads to the meta-cleavage pathway. Some of the enzymes involved in these pathways were detected as part of the automatic reconstruction, and missing reactions were manually added (**Figure 1**). In the present study, simulations were carried out considering all three pathways, with Pathway 1 only and with Pathways 2 and 3 together; the same results were obtained when considering all the pathways together and with Pathway 1 only. The *Variovorax* sp. SRS 16 strain is documented to grow on linuron alone as it utilizes linuron as a sole carbon and nitrogen source in minimal media (Sørensen and Aamand, 2003). Sørensen et al. (2009) reported a strong enhancing effect of nitrogen (ammonium sulfate) on the degradation rate but not of carbon. These experimental

observations were successfully captured by model simulations, indicating a stronger impact of nitrogen supplement (ammonium sulfate) over a carbon supplement (glucose) as enhancers of degradation. Simulation outcomes are consistent while using different pathway alternatives (**Figure 2**). In accordance with the documented mineralization of linuron (Sørensen et al., 2009), simulations indicate that linuron is converted into central-metabolism metabolites and biomass, where none of the degradation by-products (**Figure 1**) accumulate in the medium. The predicted production of biomass is fully correlated with linuron degradation (**Supplementary File 6**), providing additional evidence of full degradation.

Simulation-Based Predictions for Potential Enhancers of Linuron Degradation

The SRS 16 linuron degradation behavior under the influence of specific carbon (succinic acid) and nitrogen (ammonium salt) sources was extensively studied and reported (Sørensen et al., 2009). Here, we used simulations to further explore the effect of media supplements that can serve as potential degradation enhancers. We simulated growth in 120 different media combinations, each supplementing the linuron-containing minimal mineral media with a single exchange metabolite. The list of metabolites, following omission of 20 toxic and other non-relevant substances (considering future application in soil) such as diuron and dipeptides, is provided in **Supplementary File 4**. The effect of 28 exchange supplements selected across the full scale of degradation efficiency is shown in **Figure 3**. Selected metabolites were chosen to represent the biochemical diversity of carbon (simple sugars, organic acids, and biopolymers) and nitrogen (mainly amino acids, amine, and ammonium) sources that can act as future biostimulants in terms of regulation, costs, and accessibility. Simulations for all compounds were carried out considering both the SRS 16-specific pathway (Pathway 1, **Supplementary File 7**) and generic degradation pathways (Pathways 2 and 3, **Figure 3**). Different supplements were predicted to have variable impact on the growth of *Variovorax* SRS 16 and the degradation rate of linuron. In a reference medium containing linuron only (MS), 40% of the linuron was degraded at the 11th simulation round. The predictions stratify a group of enhancers with variable degrees of linuron degradation in the 11th iteration (>40–100%), in comparison to non-enhancers (40% linuron degradation, as in MS). All the carbon sources and several amino acids (arginine, lysine, methionine, cysteine, and tryptophan) were classified as non-enhancers vs. nitrogen sources that are predicted to expedite degradation at various degrees, with some variations depending on the simulation pathways used. Overall, considering the different pathway options, the rate of degradation broadly follows the same trend where most amino acids act as moderate enhancers, where on the 11th round, 50–75% of linuron is degraded. Fast enhancers include glutamine and asparagine (∼100% degraded in the final, 14th, round in comparison to 60% in MS). Though groups of enhancers vs. non-enhancers are similar when considering both simulation pathways (**Figure 3** and **Supplementary File 7**), differences are observed in internal ranking as presented in **Figure 4**. The major difference found is that in Pathway 1, methionine and cysteine influence the

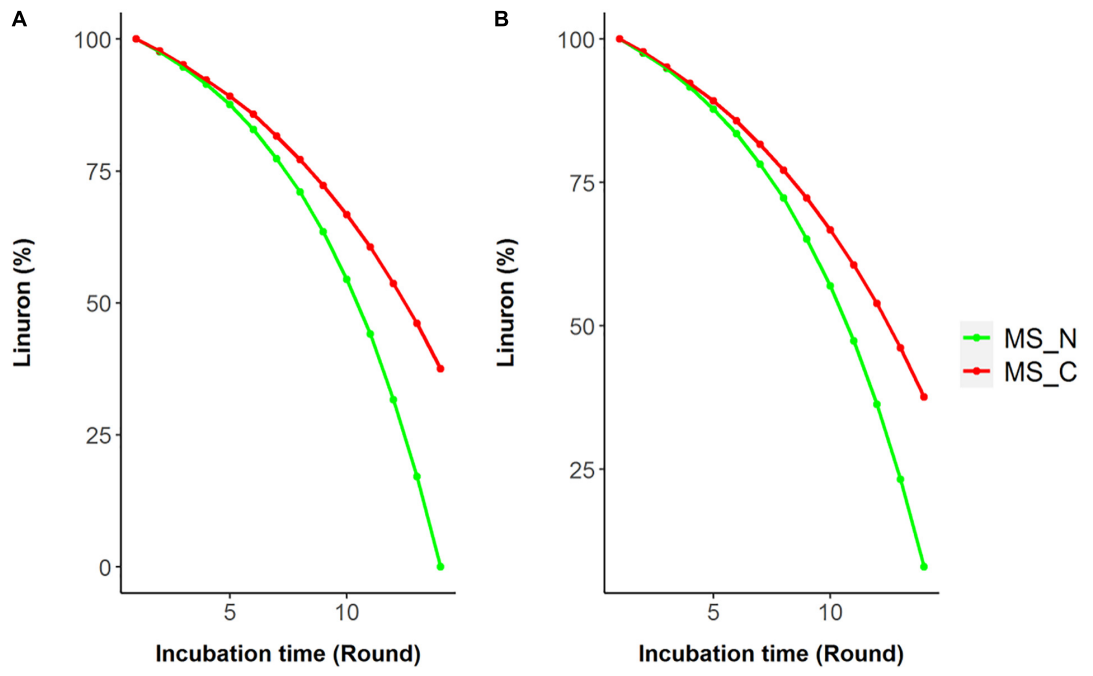

FIGURE 2 | Simulated linuron degradation in the modified media SRS 16 model considering Pathway 1 **(A)** and Pathways 2 and 3 **(B)**. Simulations were carried out in minimal mineral media (MS) with linuron supplemented by nitrogen (ammonium sulfate) or carbon (succinate) sources (MS_N and MS_C, respectively).

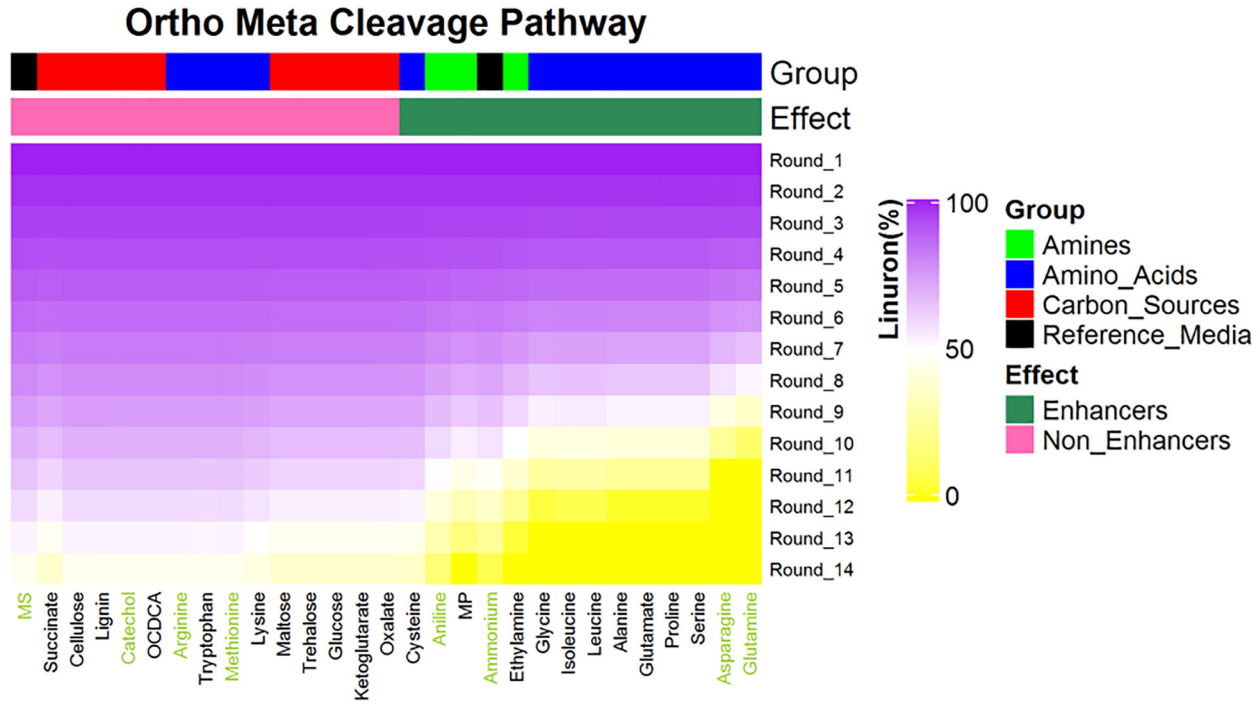

FIGURE 3 | Predictions for linuron degradation by SRS 16 in minimal media supplemented with linuron and 28 compounds (selected carbon and nitrogen sources). Compounds are ranked according the linuron amount in the ninth simulation rounds. All media contain linuron. Reference medium: MS = minimal solution + linuron + no supplement, MP = 2-methyl propanamine. A total of seven metabolites (green font) were selected for experimental validation. Simulations assuming Pathways 2 and 3; simulations assuming alternative pathways are provided in **Supplementary File 7**.

degradation rate positively, whereas in Pathways 2 and 3, they are categorized as non-enhancers.

In vitro Validation of Potential Enhancers of Linuron Degradation by *Variovorax* sp. SRS 16

Seven metabolites representing a range of predicted degradation enhancement potential and biochemical characteristics (**Figure 3**) were selected for validation. These metabolites include glutamine and asparagine as strong enhancers; aniline as a moderate enhancer; arginine, oxalic acid, and catechol as weak enhancers (**Figure 3**); and methionine whose enhancement potential depends on the choice of simulation pathway (non-enhancer in Pathway 1 and strong enhancer in Pathways 2 and 3, **Figure 4**). The simulations (growth and degradation) for the seven selected substrates assuming degradation Pathways 2 and 3 are shown in **Figure 5**. Simulations considering Pathways 1 and degradation Pathways 1–3 are shown in **Supplementary File 8**. Overall, the laboratory experiments supported predictions in the majority of the cases. Growth experiments (**Figure 5B**) are fully consistent with predictions, dividing supplement as non-enhancers vs. enhancers. In agreement with predictions, a slow (negligible) growth rate was found in MS and in MS supplemented by arginine, catechol, aniline, and methionine. A significantly higher growth rate was recorded in the presence of glutamine and asparagine ($p < 0.05$). Degradation results (**Figure 5D**) are overall consistent with growth results (**Figure 5B**), with significant stratification of enhancers (glutamine and asparagine) vs non-enhancers ($p < 0.05$). Aniline and methionine had a non-significantly stronger enhancement effect in comparison to MS and were classified into the non-enhancers group ($p < 0.05$ repeated-measures ANOVA), consistent with simulations in Pathways 2 and 3.

The results provide an insight that some supplements (associated with nitrogen metabolism) have a strong enhancing effect that helps in the conversion of linuron into potential cellular building blocks.

DISCUSSION

Linuron is considered a stable phenyl urea herbicide. Under field conditions, the half-life of linuron varies from 30 to 150 days in different soil types, with an estimated average half-life of 60 days (Swarcewicz et al., 2013). DCA, its primary degradation intermediate, is reported to be more toxic. Therefore, complete mineralization of this compound is desired. *Variovorax* SRS 16, a member of a genus reported as an efficient degrader of phenyl urea herbicide, is widely known for its ability to mineralize linuron (Sørensen et al., 2009). Stimulating the growth of this degrader through adding compounds to a contaminated site is a potential strategy for enhancing its activity while

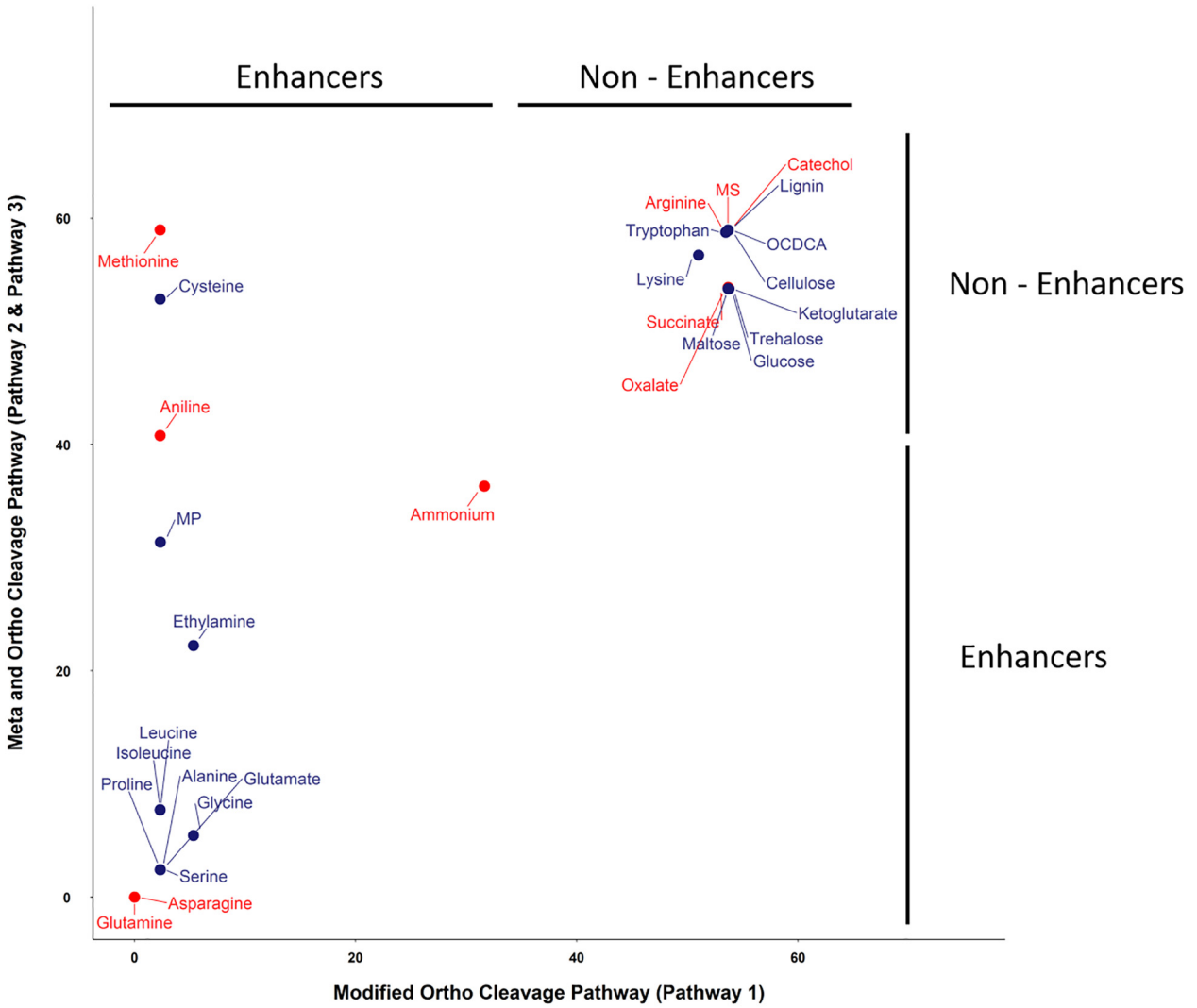

FIGURE 4 | Linuron degradation status (at round 10) under the influence of supplements with the ortho- and meta-cleavage pathway (Pathways 2 and 3) vs. the modified ortho-cleavage pathway (Pathway 1). Substrates in red were selected for further validation in the lab experiment. The x and y scales represent the amount of linuron (%).

bypassing the need of introducing exogenous microbes. Here, we describe the construction of a genome-scale metabolic model of *Variovorax* sp. strain SRS 16 aiming at finding model-based solutions for accelerating its linuron degradation activity. Construction followed automatic and manual protocols, and iterative simulations were applied for predicting potential compounds that promote the degradation of linuron. Predictions for significant degradation enhancers were confirmed by wet laboratory experiments.

Although, overall, the study demonstrates that model predictions can guide metabolic exploration toward predicting biochemical routes leading to enhanced degradation activity, several limitations of this work should be acknowledged. First, further adaptations are required for the current version of iRZ1425 to meet the requirements of gold standard models (Thiele and Palsson, 2010). Mainly, the biomass composition is an approximation that relies on automatic protocols and genomic information (Oh et al., 2007; Henry et al., 2010), similar to other recent works (Bordel et al., 2019; Naizabekov and Lee, 2020; Ofaim et al., 2020). The construction of a biomass reaction specific to the SRS 16 reaction requires layers of experimental data that are currently missing, including the precursors of general biomass constructions (for example, proteins, lipids, and species-specific components). Collection of currently lacking experimental data will be incorporated in future versions of the model to define an accurate biomass objective function using, for example, the BOFdat computational platform (Lachance et al., 2019). Second, though the current analysis demonstrates the use of model-based solutions for accelerating linuron degradation activity, the mechanism explaining the different effects of the metabolites tested remains unaddressed here. Simulations, supported by experimental evidence from the

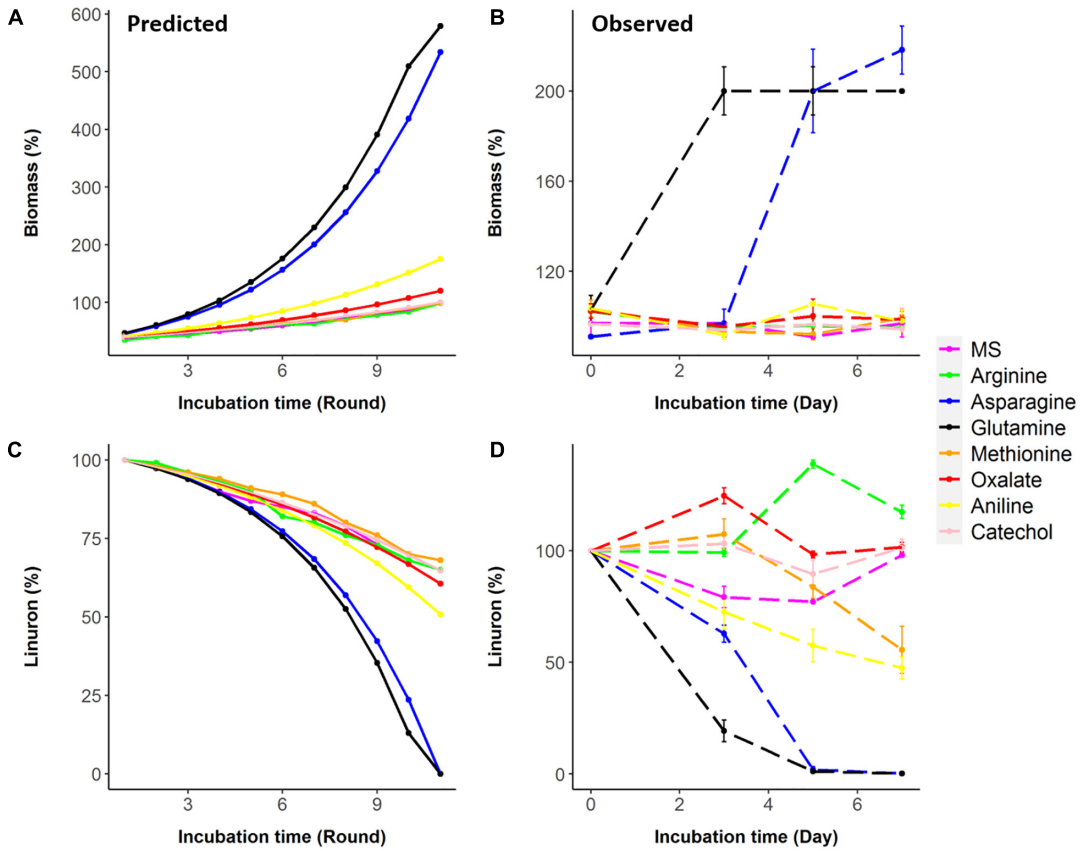

FIGURE 5 | Predictions (based on Pathways 2 and 3) vs. observations for biomass and linuron degradation by *Variovorax* SRS 16. **(A,B)** Biomass predicted and observed. **(C,D)** Linuron degradation predicted and observed.

current study as well as previous reports (Sørensen et al., 2009), clearly point to nitrogenous compounds being advantageous in comparison to carbon ones. However, nitrogen content *per se* is insufficient for predicting the stimulating effect of compounds, and degradation efficiency cannot be ranked according to N content or to the C:N ratio. For example, the efficient stimulants glutamine and asparagine have C:N ratios of 5:2 and 4:2, respectively, in comparison to 6:4 in the non-efficient supplement arginine (**Supplementary File 4**). Simulation outcomes cannot be predetermined based solely on the C and/or N content of media supplements but rather by considering the complementary stoichiometric effect of multiple chemical reaction cascades, or pathways, that construct the genome-scale metabolic network. Similarly, simulation outcomes cannot be predicted based on compound structural properties, for example, different biostimulation potential of the two peripheral aromatic compounds, aniline and catechol.

Thus, the simulation predictions reflect the complexity of cellular processes and the balance between different metabolic pathways contributing to growth. The simulations provide multiple optimal solutions for the internal fluxes involved in central metabolism and hence are not sufficient for deciphering the mechanism by which environmental (media dependent) conditions affect linuron degradation. However, the integration of simulation data with 'omics data (e.g., metabolomics, proteomics, and transcriptomics) can reduce the solution space and shed light on the fine-tuning of cellular activity, considering the need to balance between myriads of constraints. The model provides a platform for future sequential studies through the integration of metabolomic and transcriptomic data (similar to Hadadi et al., 2020), allowing the exploration of the mechanism of linuron degradation as well as other aspects of *Variovorax* sp. strain SRS 16 biochemistry. Such additional experiments are required in order to complement model predictions regarding the mechanism behind accelerating the phenomenon. For example, transcriptomics/proteomics studies targeting the effect of different media supplements (e.g., amino acids) would be beneficial to track the molecular processes considering different degradation scenarios.

Though current analysis requires additional layers on information in order to provide a description of the degradation mechanism, the analysis provides several insights that can be further explored in future studies. Model construction suggests three alternative routes for the degradation of linuron. Pathway 1 (modified ortho-cleavage pathway) relies on a detailed description of linuron degradation at strain level (Öztürk et al., 2020). *Variovorax* SRS 16 was reported to contain the gene coding for the amidase *LibA*, which hydrolyzes linuron

in *N*,*O*-dimethyl hydroxylamine and DCA. The catabolic pathway for DCA degradation into components of central metabolism is suggested to involve conversion of 4,5-dichlorocatechol to oxoadipate (modified ortho-cleavage pathway) (Bers et al., 2011). Alternative degradation pathways of DCA were reported in other bacterial genera (Dejonghe et al., 2002; Breugelmans et al., 2010). In the current study, automatic construction based on the SRS 16 genome sequence suggested the partial presence of enzymes in chloroaniline or chlorocatechol metabolism (Pathways 2 and 3), where enzymes from the modified ortho-cleavage pathway (Pathway 1) were not detected. The missing/partial degradation pathways were completed through a gap-filling procedure (**Figure 1**). Here, the possibility of the presence of the ortho-cleavage pathway and meta-cleavage pathway along with the existing modified ortho-cleavage pathway is proposed based on genome data. The presence of partial Pathways 2 and 3 indicates that SRS 16 might be using these pathways for linuron mineralization under different conditions. Simulations in the SRS 16 model with complete Pathways 2 and 3 provided similar results to those obtained with Pathway 1 for most of the supplements (such as glutamine, asparagine, and aniline), with some exceptions such as methionine.

In the present study, it is shown that reconstruction is important for the study of bacterial physiology, allowing the simulation of degradation outcomes considering multiple conditions. It is well known that the degradation process is influenced by various physicochemical and nutritional factors (Kanissery and Sims, 2011). The availability of carbon and nitrogen sources (nutrients) significantly affects the efficiency of degradation (Yassir et al., 1998; Wu et al., 2011). Here, simulations suggest the importance of specific supplements to increase the degradation rate. Simulations predicted variations in the enhancement potential of different supplements, further supported by experimental validation. As a single cell is a complex network of reactions and metabolites, supplements can have a variable effect associated with the activation of different sets of reactions under each media condition. Integration of model predictions with omics data is useful for revealing the metabolic shifts in the microbial cell (Alam et al., 2010). To date, several strategies have been adopted in order to accelerate the biodegradation of herbicides in the environment, ranging from optimizing media to constructing transgenics (Azab et al., 2018; Li et al., 2020). Genome-scale modeling approaches along with omics technology have established their importance in biotechnology and medicine (Zhang and Hua, 2016). They are continuously appreciated for their role in the degradation of pollutants (Faust, 2019; Xu et al., 2019; Cardozo et al., 2020) and in other environmental problems related to agricultural soils (Mazzola and Freilich, 2017). Recently, Ofaim et al. (2020) demonstrated the use of genome-scale modeling for optimizing atrazine degradation by *P. aurescens* TC1. The current study demonstrated that genome-scale modeling allows the optimization of microbial activity, leading to herbicide biodegradation. The integration of predictive biology helped to screen the effect of the higher number of supplements on the degradation by SRS 16. Such supplement-derived amendment might reflect exchanges in the indigenous community where the degrader can be supported by non-degrader species (Xu et al., 2019) and accelerate the degradation rate.

In this way, computational biology can reduce cost, time, and effort (mainly related to gene manipulation) and save resources to identify the optimal solutions for a specific phenomenon. The association of genome-scale metabolic models with the omics approach can help to unravel the hidden facts of metabolism (Massaiu et al., 2019). Thus, there is a strong possibility of improvement in degradation processes through the contribution of modeling as an environmental biotechnology approach.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

KD, RZ, ZR, and SF designed the study and drafted the manuscript. RZ constructed the metabolic model. KD participated in model construction, led the design and analysis of computational and experimental results, and wrote the manuscript. SM participated in computational data analysis. NK and DB performed the wet laboratory experiments. BÖ, RA, and HE analyzed and reviewed the results. All authors carried out writing and improvement of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.602464/full#supplementary-material

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Conflict of Interest: BÖ was employed by the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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