



# Strategic Design of Synthetic Consortium with Embedded Wastewater Treatment Potential: Deciphering the Competence of Isolates from Diverse Microbiome

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Microorganisms play a vital role in efficient biological treatment. Supplementation of external microorganisms with high degradation rates can enhance the process efficiency significantly. Potential strains were isolated from long term wastewater treating reactors and identified using phylogenetic analysis of 16S rRNA gene fragments with the nearest neighbors extracted during BLAST search. Later the study was designed in two phases which revealed interesting findings. Phase I evaluates the potential of isolated strains viz., *Pseudomonas otitidis*, *Bacillus firmus*, *Bacillus subtilis*, and *Bacillus circulans* for their individual ability in terms of COD and nutrients removal. *B. circulans* showed highest carbon (COD) removal (70%; 0.56 kg COD<sub>R</sub>/m<sup>3</sup>-day), while maximum nutrients removal (nitrate, 81%; phosphate, 90%) was observed with *B. subtilis*. *B. firmus* showed maximum volatile fatty acid (VFA) production. Based on Phase I results, four synthetic consortia were designed in phase II with diverse combination of isolates and evaluated for their remediation efficiencies. Consortium 4 (*P. otitidis*, *B. subtilis*, and *B. firmus*) illustrated higher treatment potential [COD, 86%; SDR (cum): 0.64 kg COD<sub>R</sub>/m<sup>3</sup>-day; Nitrate, 87%; Phosphate, 97%]. The exploitation of such consortia can overcome the inefficiencies pre-existing with the biological wastewater treatment plants by acting as prospective candidates for bio-augmenting the native microflora. This communication illustrated development of the efficient consortia using lab isolated strains to improve the performance of wastewater treatment.

**Keywords:** phylogenetic analysis, carbon load, nutrients, bacterial interactions, bioelectrochemical analysis

## INTRODUCTION

Biological treatment process assumes importance and relevance due to its sustainable nature which makes this technology as one of the most important biotechnological application. Biological treatment processes have their own design parameters and economic relevance but in these processes there is a common parameter which is its “microbes” or the “microbial consortia.” Microbes as the central drivers have regulatory influence on treatment functioning and efficiency. Wastewater systems may provide diverse conditions for the microbial growth and metabolism. It stimulates development of indigenous microbial populations, toward utilization of the nutrients and specific substrates in the waste as their sole carbon and energy sources thereby degrading

the contaminants. There are many strategies applied toward the enhancement of the wastewater treatment efficiency (Venkata Mohan et al., 2001, 2007). Understanding the relationship among microbial communities, individual microbe's role, treatment rate and efficiency is one of the most important criteria in wastewater treatment.

Modern molecular techniques and environmental genomics, explored many microbial key players in wastewater treatment for nutrient removal, sludge bulking and foaming and provided many exhilarating insights into the functions, diversity and niche differentiations of these microorganisms (Daims et al., 2006). Studies have been done to understand the microbial dynamics in wastewater treatment systems (Muszyński et al., 2005; Shi et al., 2015). Several bacterial species have been identified that have the ability for wastewater degradation. Majorly, *Bacillus* spp. have been identified as substrate degraders. The genus *Bacillus* belongs to the phylum Firmicutes and members of this genus are spore-forming aerobic or facultatively anaerobic bacteria (Gander et al., 2000). Bacteria belonging to this genus can easily degrade proteins, starch and lipids. Choi et al. (2002), reported that an advanced wastewater treatment system in Korea operated with *Bacillus* strains pre-dominating was able to remove nitrogen and phosphorus as well as organic matter efficiently. Iriye and Takatsuka (1999) reported that the wastewater system operated in Japan with *Bacillus* spp. dominance showed suitable removal rates for waste organic matter combined with high de-nitrification and de-phosphorization activities and less offensive odor. Other than *Bacillus* spp., *Pseudomonas* is also considered as a potential species prevailing in wastewater microbiome. *Pseudomonas* spp. can easily break down carbon and can reduce biological oxygen demand (BOD) levels far more quickly. Since such properties of the bacterial cultures are needed for wastewater treatment, methods have been developed to enhance their growth in the wastewater treatment systems (Iriye and Takatsuka, 1999; Reddy et al., 2012). These microorganisms may be indigenous to contaminated wastewater or they are isolated from various wastewaters and brought to the contaminated area. The success rate of any individual wastewater treatment systems depends on the most abundant microbial culture or the assemblage of the different microbial communities in the system. There are various bacteria which perform treatment efficiently and effectively; but each one is meant for specific function. These competent isolated and studied strains can be added into the treatment system which is known as bioaugmentation. Broadly, it is the general practice of adding specialized microbial strains to a native community for enhancing the ability of the microbial community to degrade certain compounds or to respond to process fluctuations resulting in improved treatment. The characteristics of the microbial community in the wastewater treatment plant can be easily improved through manipulating the already present microbial community by including specific microbes individually or in effective consortia (Raghavulu et al., 2011). Mixed cultures have an apparent advantages as the microbial consortia can communally perform the treatment efficiently which cannot be performed individually. Researchers started developing consortia for increasing the efficiency of wastewater treatment (Venkata Mohan et al., 2009), removal of contaminants like phenol (Azaizeh et al., 2015), nitrogen removal (Chen et al.,

2015), to treat tobacco wastewater (Wang et al., 2009) etc. Treatment of different wastewaters differs as it dependent of the different contaminants they contain. Under such stipulation, microbial consortia with different organisms having different specificities can perform the treatment more efficiently which is intricate for individual strains or species. Efficient and effective wastewater treatment can be anticipated when the co-metabolic activities within a microbial consortia complements each other. Hence, the present study was undertaken to explore the wastewater treatment potential of isolated strains and design of synthetic consortia based on carbon and nutrient removal. The study also depicts comprehensive evaluation of both isolates and designed consortia with respect to substrate degradation efficiency, nutrients removal potential, enzymatic analysis, and bioelectrochemical behavior to comprehend the relative suitability of microbiome to the complex wastewater system.

## MATERIALS AND METHODS

### Biocatalysts

Facultative aerobic cultures from the long term operated bioreactors used for wastewater treatment were used for isolation of strains by direct plating and enrichment techniques using nutrient broth (pH 7; 24h under aerobic conditions). One milliliter of the culture suspended in sterile deionized water (9 ml) was serially diluted from  $10^{-1}$  to  $10^{-8}$  times with saline water. Diluted sample (0.1 ml) was spread on the nutrient agar plates using spread plating technique and was incubated at 37°C for 24 to 48 h. Pure colonies were obtained after repeated streaking.

### DNA Extraction and PCR Amplification

Genomic DNA from the isolated pure colony was extracted and purified using phenol–chloroform method. The concentration of DNA obtained was measured at a wavelength of 260 nm using UV/Vis-spectrophotometer (Thermo Scientific, USA). The variable V3 region of 16S rDNA was amplified by PCR using primers 8F (5'-AGAGTTTGATCCTGGCTCAG- 3') and 1542R (5'-AAGGAGGTGATCCAGCCGCA- 3'). All PCR amplifications were conducted in 50 µl containing 2 µl of total DNA containing 100 ng/ µl concentrations, 200 µM each of the four deoxynucleotide triphosphates, 15 mM MgCl<sub>2</sub>, 0.1 µM of individual primers and 1 unit of Taq polymerase. An automated thermal cycler (Eppendorf) was used for PCR amplification with program for an initial denaturation at 96°C for 5 min, 35 cycles of denaturation (40 s at 94°C), annealing (50 s at 52.6°C) and extension (1 min at 72°C) and a final extension at 72°C for 8 min. The PCR amplified product (5 µl) was subjected to agarose gel electrophoresis to confirm product recovery and to estimate product concentration. The amplified PCR product was stored at 4°C until further use.

### Sequencing, Characterization, and Phylogenetic Analysis

Amplified PCR product after purification (Fermentas) was sent to MWG Biotech for sequencing. 16S rRNA sequence (1500 bp) was aligned with those of the reference micro

organisms existing in the same region of the closest relative strains available in the GenBank database, using the BLASTN facility and was also tested for possible chimera formation with the chimera check program Decipher (online: <http://decipher.cee.wisc.edu/FindChimerasOutputs.html>). These sequences were further aligned with the closest matches found in the GenBank database with the CLUSTALW function of Molecular Evolutionary Genetics Analysis package (MEGA). Neighbor-joining phylogenetic tree was constructed with the MEGA version. 6.0. A bootstrap analysis with 100 replicates was carried out to check the robustness of the tree. Bootstrap re-sampling analysis for the replicates was performed to estimate the confidence of tree topologies. Strains were named as *Bacillus circulans* strain SLBEEC1, *Bacillus firmus* strain IICTSVMHH, *Bacillus subtilis* strain IICTSVMH13 and *Pseudomonas otitidis* SVMIICTP2 based on the BLASTN facility that showed 99–100% sequence similarity with the nearest phylogenetic neighbors. The nucleotide sequence identified in this study was deposited in the GenBank database under accession numbers for *P. otitidis*, *B. firmus*, *B. subtilis*, and *B. circulans* as HE612874, FR849707, FR849706, and KJ501091 respectively.

The isolated strains viz., *P. otitidis*, *B. firmus*, *B. subtilis*, and *B. circulans* were used as parent biocatalyst for the experiments. The stock cultures were maintained on nutrient agar slants overlaid with 20% (v/v) glycerol and kept at  $-20^{\circ}\text{C}$ . Prior to inoculation, the organisms were enriched with designed synthetic wastewater (DSW) under aerobic microenvironments (120 rpm;  $28^{\circ}\text{C}$ ) to maximize the nutrient removing capacity of the biocatalyst.

## Synthetic Consortia

The consortia was developed by aseptically transferring the 12 h grown culture of each individual strains studied in phase I, in different combinations [*P. otitidis*+ *B. circulans* (1); *P. otitidis* + *B. circulans*+ *B. subtilis* (2); *P. otitidis*+ *B. subtilis* + *B. circulans* + *B. firmus* (3); *P. otitidis*+ *B. subtilis* + *B. firmus* (4)] in 500 ml Erlenmeyer flasks containing 250 ml of DSW at similar operating conditions for single cultures. Individual strains were inoculated with 2% (v/v) aliquots of 12 hour grown culture, respectively, to maintain the same cell count in the pure culture and in the consortium. All the cultures were added in equal proportion in order to maintain the same initial OD 600.

## Experimental Methodology

Prior to inoculation, the biocatalysts were re-suspended in sterilized (15 min;  $121^{\circ}\text{C}$ ) designed synthetic wastewater for 12 h at room temperature. Designed synthetic wastewater (DSW; glucose  $3\text{ g l}^{-1}$ ;  $\text{NH}_4\text{Cl}$   $0.5\text{ g l}^{-1}$ ,  $\text{KH}_2\text{PO}_4$   $0.25\text{ g l}^{-1}$ ,  $\text{K}_2\text{HPO}_4$   $0.25\text{ g l}^{-1}$ ,  $\text{MgCl}_2$   $0.3\text{ g l}^{-1}$ ,  $\text{CoCl}_2$   $1\text{ l}^{-1}$ ,  $\text{ZnCl}_2$   $11.5\text{ mg l}^{-1}$ ,  $\text{CuCl}_2$   $10.5\text{ mg l}^{-1}$ ,  $\text{CaCl}_2$   $5\text{ mg l}^{-1}$ ,  $\text{MnCl}_2$   $15\text{ mg l}^{-1}$ ,  $\text{NiSO}_4$   $0.16\text{ g l}^{-1}$ ,  $\text{FeCl}_3$   $0.03\text{ g l}^{-1}$ ) was used to evaluate the biocatalytic activity. The resulting pellets of *P. otitidis*, *B. subtilis*, *B. firmus*, and *B. circulans* were inoculated individually into each of the four 500 ml flasks, respectively and were operated for six cycles each with a retention period of 48 h. All the four systems were operated under similar conditions and feed replacement was done with sterilized DSW at an organic load of  $3\text{ g COD l}^{-1}$ . Prior to loading and autoclaving, pH of the DSW was adjusted to 7 using 2 N ortho phosphoric acid or 1 M NaOH.

After performing the individual strains performance experiment consortia were made using different strains as per discussed in Section 2.2. and studied in the similar condition for individual strain studies for two cycles and average of two was reported in the study, thus sample collection was done more frequently. All the experimental operations were carried out in fed-batch mode. Before every feeding event, the inoculum was allowed to settle down (30 min; settling) and the exhausted feed was decanted (15 min; decanting). The inoculum settled at the bottom was used for subsequent operations. Feeding, decanting and recirculation operations were performed and the operation was properly carried out to ensure that sterile microenvironment is maintained. All the experiments were performed at ambient temperatures ( $29 \pm 2^{\circ}\text{C}$ ).

## Analysis

Chemical oxygen demand (COD-closed refluxing titrimetric method), volatile fatty acids (VFA), nitrate, phosphate and pH were estimated by the standard methods (APHA, 1998). Optical density (OD) of the bacterial cultures was measured at 600 nm using spectrophotometer (Genequant 1300). Cyclic voltammetry (CV) was employed to evaluate the electron discharge properties of the biocatalyst, using a potentiostat–galvanostat system (PGSTAT12, Ecochemie). CV was operated and voltammograms were recorded against Ag/AgCl (3.5 M) by applying a potential ramp (+0.5 to  $-0.5\text{ mV}$ ) at a scan rate of  $20\text{ mV/s}$  employing platinum wire and carbon rod as counter and working electrodes respectively.

## RESULTS AND DISCUSSION

### Phylogenetic Architecture Analysis of Isolates

The isolated strains based on 16S rDNA method were identified as *P. otitidis*, *B. firmus*, *B. subtilis*, and *B. circulans* and submitted to the GenBank database under NCBI Accession Numbers HE612874, FR849707, FR849706, and KJ501091, respectively (Table 1). The phylogenetic tree was constructed based on the partial sequences of 16S rDNA gene of four isolates with other related bacterial strains as shown in Figure 1 along with bootstrap values. *B. circulans* exhibited 92% of bootstrap values with *B. circulans* strain NBRC 13626 (NR\_112632.1). *P. otitidis* exhibited 82% of bootstrap values with *P. otitidis* MCC10330 (NR\_043289.1). *B. subtilis* exhibited similarity with *B. subtilis* subsp. *spizizenii* TU-B-10 (CP002905.1) while *B. firmus* exhibited 97% of bootstrap values with *B. firmus* NBRC 15306T (NR\_112635.1). In the phylogenetic tree clear clusters were observed with the bootstrap value of 99–100%. *B. firmus*, *B. circulans*, *B. subtilis* are Gram positive bacteria while *P. otitidis* is Gram negative bacteria, which can easily be seen in the tree because of their different clusters. Majorly, genus *Bacillus* is a group of proteolytic, cellulolytic, lipolytic, aerobic or facultative anaerobic bacteria and it is widely used in wastewater treatment (Groudev, 1987). *B. circulans* is a typical chemo-organoheterotrophic bacterium, which can use mono-, di- and polysaccharides and polyhydroxylic alcohols as a source of carbon, energy and electrons (Groudev, 1987). *B. subtilis* possesses multiple enzyme systems producing amylase, lipases,

**TABLE 1 | List of strains isolated identified with phylogenetic analysis with accession numbers, classification, GenBank closest match, identity similarities and their properties.**

S.No	Phylogenetic architecture analysis				
	Accession No./Strain name	Bacteria class	Genbank closed match	Identity similarity (%)	Characteristics
1	HE612874/SVMIICTP2	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales Pseudomonadaceae; Pseudomonas	<i>Pseudomonas otitidis</i> strain MCC10330 (NR_043289.1)	99	Bioplastics production, and dye degradation
2.	FR849707/IICTSVMHH	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	<i>Bacillus firmus</i> NBRC 15306T (NR_112635.1)	100	Proteolytic activity, Produce acid from D-glucose, arabinose, D-xylose, lactose, sucrose etc
3.	FR849706/IICTSVMH13	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> TU-B-10 (CP002905.1)	99	Degrades pectin and polysaccharides, produce amylase, lipases, and proteases enzymes
4.	KJ501091/SLBEEC1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	<i>Bacillus circulans</i> strain NBRC 13626 (NR_112632.1)	100	Use mono-, di- and polysaccharides and polyhydroxylic alcohols as source of carbon, energy and electrons

and proteases, which are useful in breakdown of complex molecules and fats in wastewater. *B. firmus* is capable of producing xylanases (Min-Jen et al., 2002) at alkaline conditions. It widely used of dye removal from textile wastewater (Ogugbue et al., 2012). *P. otitidis* was studied for its bioplastics production and dye degradation (Wu et al., 2009; Reddy et al., 2012). *Pseudomonas* sps. is also exploited for pyridine degradation (Venkata Mohan et al., 2003). Hence, these isolated strains mark themselves as the potential candidates for wastewater treatment.

## Evaluation of Isolates for Treatment Efficiency

### Biomass Growth

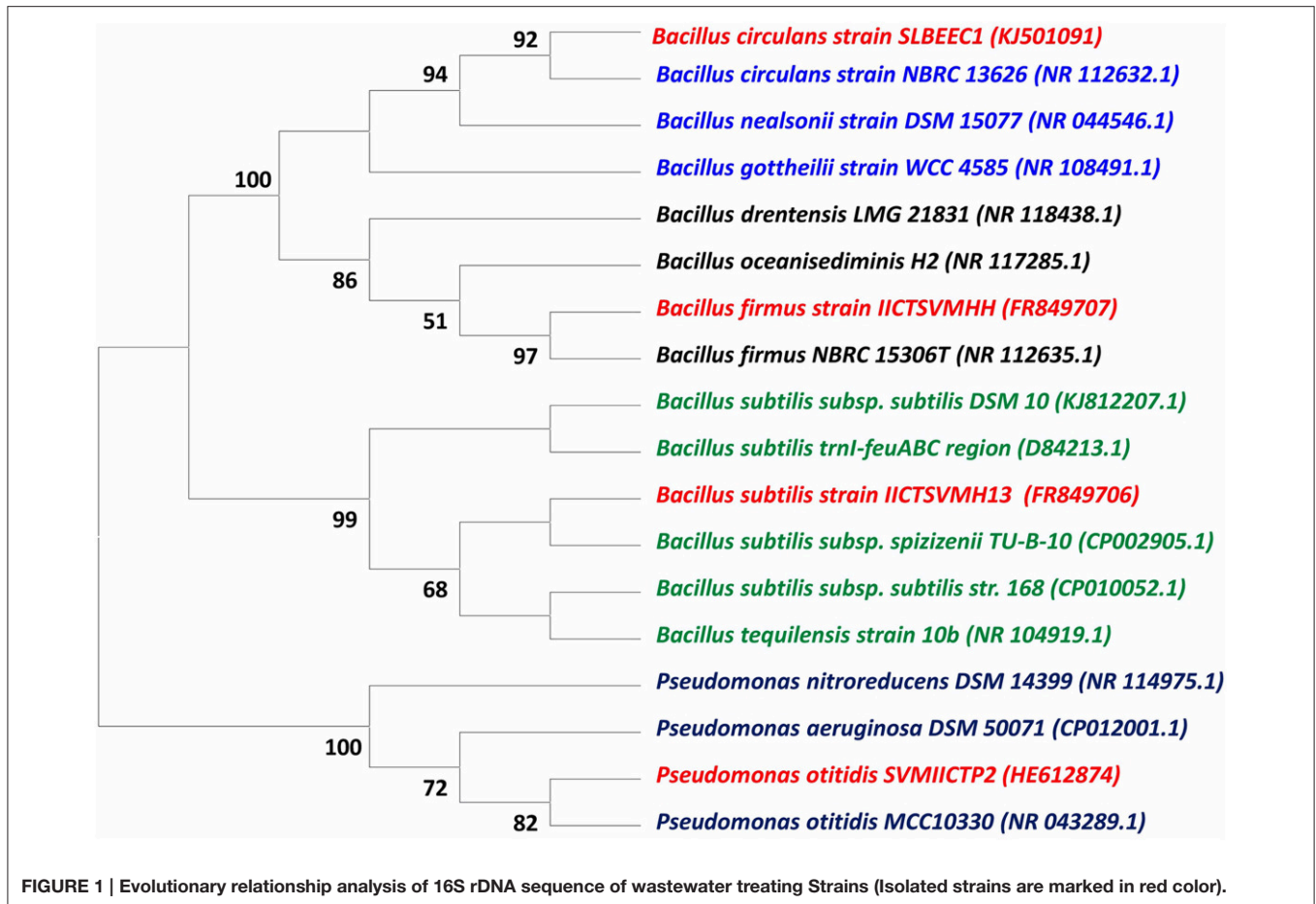
The pattern of growth of the cultures was observed in the form of growth curves (OD 600; **Figure 2A**). For all isolates, cells were growing well for the wastewater composition. *B. subtilis* showed maximum growth of about 0.4 OD at the end of exponential phase followed by *B. circulans* (0.37 OD) and *P. otitidis* of around 0.35 OD in the third cycle. *B. firmus* showed a long lag phase compared to all other strains. Interestingly, floc formation in *B. firmus* operated system was observed after 12 h, which may be attributed to production of polysaccharides by this culture. Formation of polysaccharides by *B. firmus* was already being reported (Salehizadeh and Shojaosadati, 2002). Microbial activities in the system allow the mineralization of pollutants into carbon dioxide and water, and microbial transformation is measured as an important route for complete degradation of pollutants in the wastewater. Hence, efficiency of a treatment

system is dependent on the growth of the culture. If a viable culture is inoculated into a solution which contains a suitable carbon nitrogen source, other micro and macro-nutrients necessary for growth, and physiological temperature and pH are maintained, it will grow as seen in *B. subtilis*. However, if the conditions are adverse the growth can be withheld, while some cultures can withstand the adverse conditions. Similarly in the wastewater systems, if the conditions are not suitable then growth of bacteria cannot be observed. If the subjected inoculum can endure the adverse conditions prevailing in the system and grows well, the treatment efficiency will automatically increase. The rate of biomass synthesis is proportional to biomass present in the system.

## Wastewater Treatment

### Carbon load removal

COD removal is considered as a critical parameter to assess the wastewater treatment efficiency. A gradual depletion in substrate (COD concentration) was observed with operation time among all strains. COD removal efficiency (**Figures 3A,B**), cumulative (**Figure 3C**) and non-cumulative substrate degradation rate (SDR) (**Figure 4**) were calculated to track the decrement in the carbon load. Observation revealed that COD (%) and SDR (%) varied depending on the inherent capabilities of strains. Among the isolates, higher average COD removal efficiency (COD<sub>R</sub>) was observed for *B. circulans* (COD<sub>R</sub>: 70%) followed by *B. subtilis* (COD<sub>R</sub>: 60%) and least with *B. firmus* (COD<sub>R</sub>: 44%). *B. circulans* showed highest SDR (cumulative) with 0.56 kg COD<sub>R</sub>/m<sup>3</sup>-day

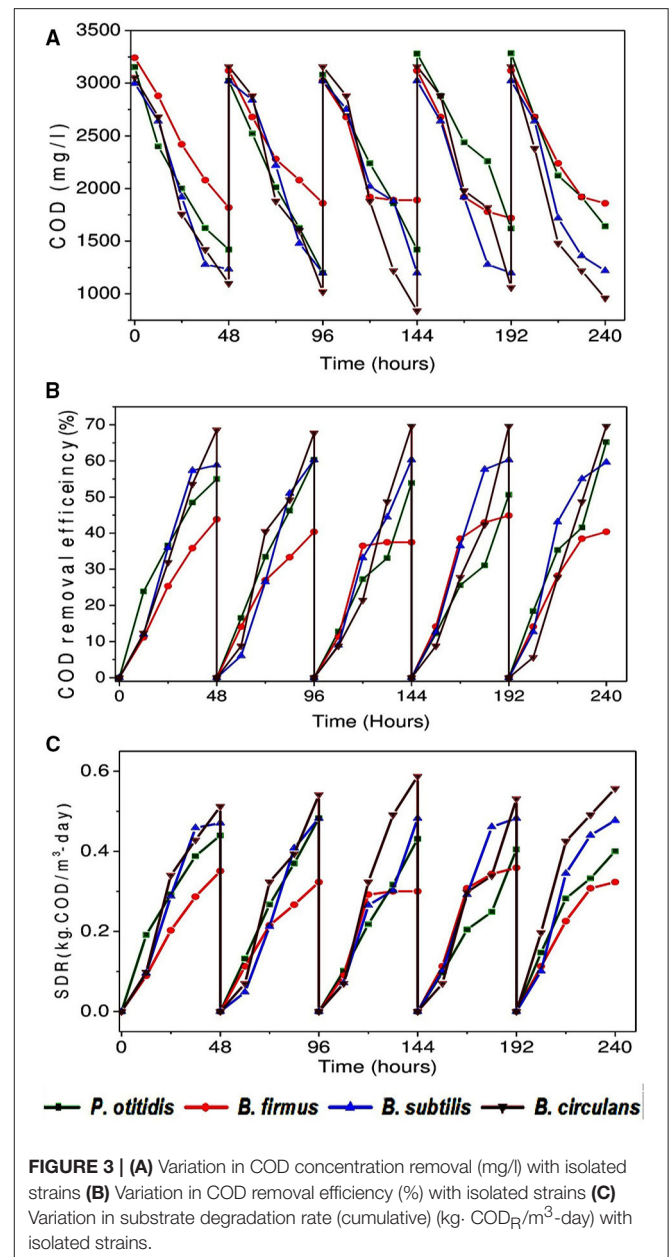
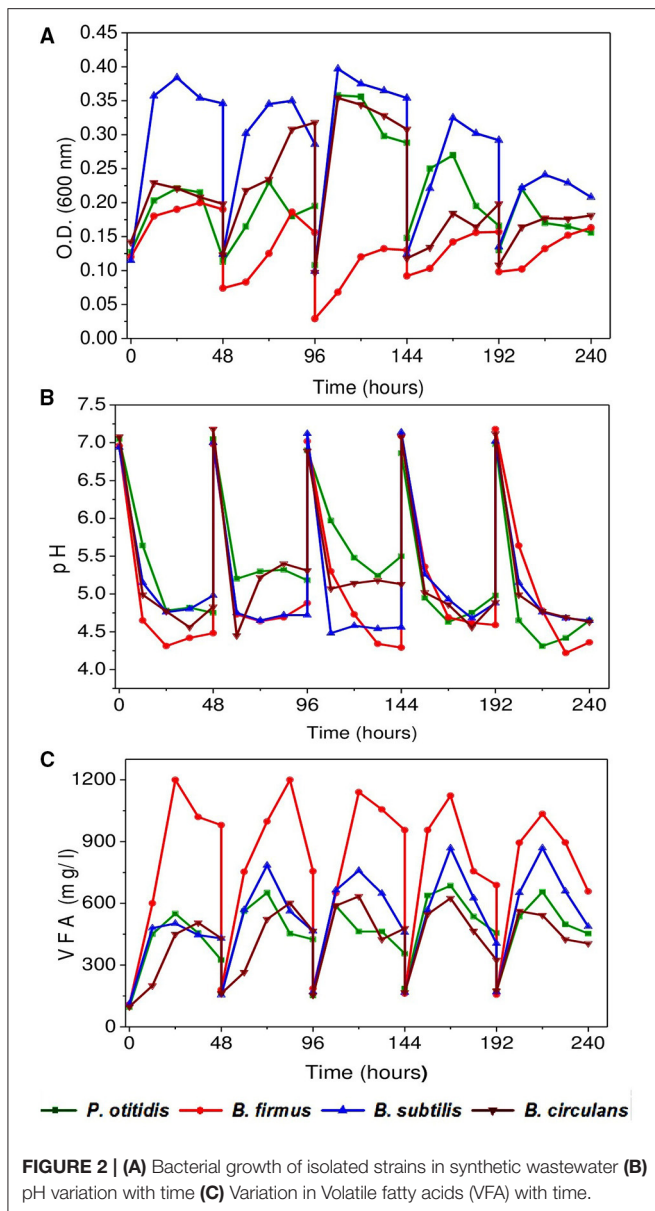


followed by *B. subtilis* (0.48 kg COD<sub>R</sub>/m<sup>3</sup>-day), and *B. firmus* (0.32 kg COD<sub>R</sub>/m<sup>3</sup>-day). The SDR (non-cumulative; SDR<sub>T</sub>) of all the strains increased till the 24<sup>th</sup> hour and later decreased with time. Growth of all isolates directly supported the SDR<sub>T</sub> while, in stationary phase there was some decrement in SDR<sub>T</sub> (Figure 5). Lowest SDR<sub>T</sub> at 48<sup>th</sup> hour was observed in *B. firmus* and highest rates in *B. circulans*. *B. circulans* can produce metabolites which have specific functions such as acidolysis, alkalysis and complexolysis (Dragutinovi et al., 2012). Tannery wastewater treatment was reported with *B. circulans* (Renitha et al., 2014) where good COD removal was observed. *B. subtilis* was also exploited for COD removal from tannery wastewater (Yusuf et al., 2013), where COD removal efficiency of 87.6% was observed compared to *P. fragii* (85.2%).

### Nitrate removal

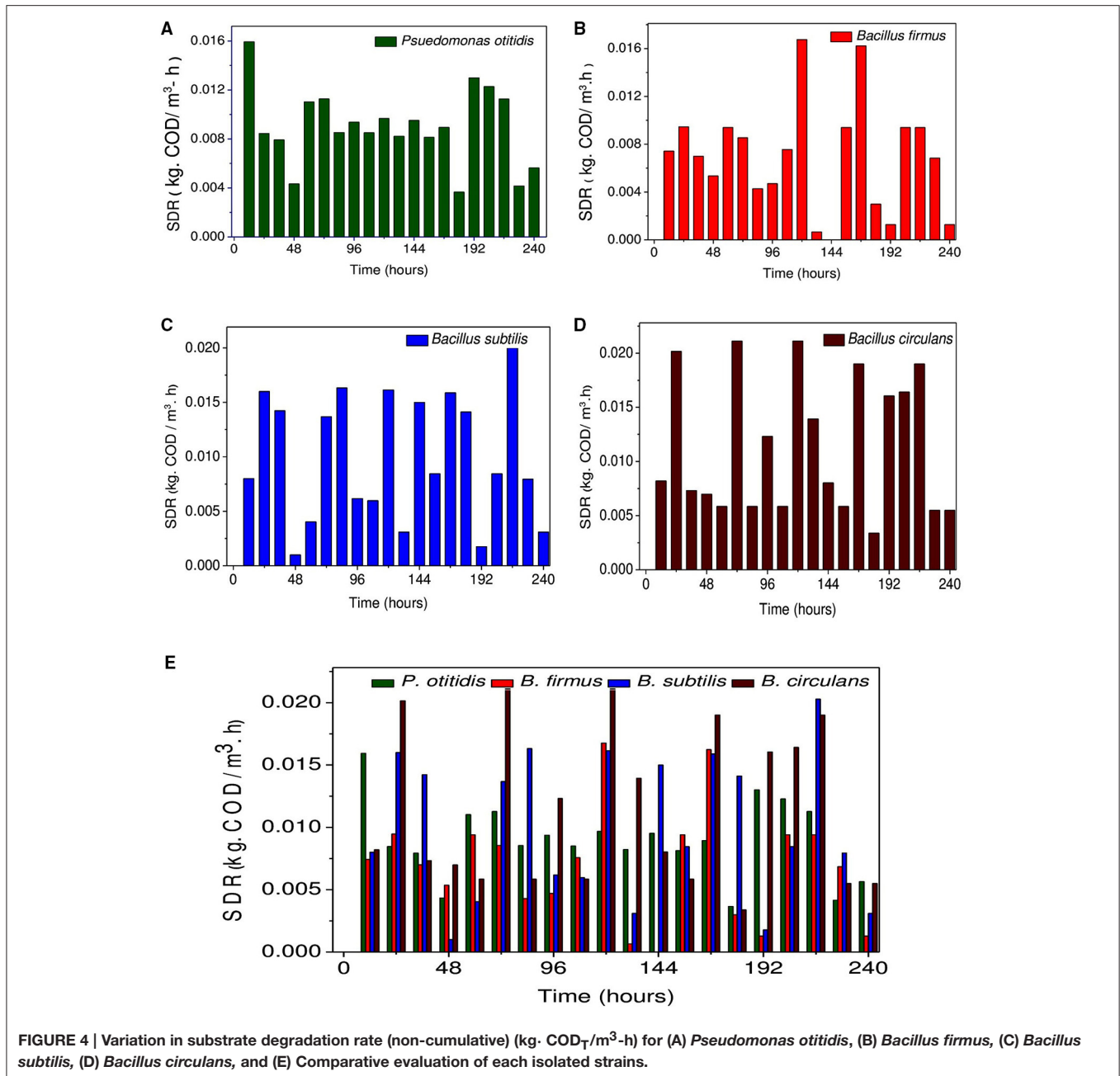
Nitrogen-containing compounds when present in environment can cause serious problems, hence its removal becomes necessary from wastewater. Therefore in this study nitrate removal was considered as an important parameter. All isolates documented good nitrate removal but was found to depend on the individual strain (Figures 5A,B). Operation with *B. subtilis* showed highest removal efficiency of nitrate accounting for nearly 80%. Subsequently in the system operated with *P. otitidis* showed

higher nitrate removal efficiency (56%) than *B. firmus* (46%). Least nitrate removal was found in the system operated with *B. circulans* (41%). Biological treatment includes both anaerobic and aerobic treatment operations where nitrate is metabolized by microorganisms in three different ways, i.e., assimilation to ammonia under aerobic conditions, dissimilation to nitrogen or to ammonia under micro aerobic or anaerobic conditions. Thus, aerobic nitrate-respiring bacteria may be particularly important in environments with fluctuating or minimal oxygen availability as mostly studies were done with anaerobic nitrate reduction. These bacteria may be obligate aerobe, anaerobe or facultative aerobes or anaerobes. In wastewater, microbes with the complete denitrification pathway can catalyze the dissimilatory reduction of nitrate to nitrogen. The aerobic metabolism of *Bacillus* and *Pseudomonas* sps. have revealed many fascinating features of assimilatory nitrate reduction to ammonia via nitrite. Few microbes, can support anaerobic growth or dissipate excess reducing power using dissimilatory reduction of nitrate to ammonia via nitrite like *B. subtilis* viz. a facultative aerobe can utilize nitrate or nitrite as sole nitrogen source, and nitrate and nitrite are also utilized as electron acceptors for anaerobic respiration under anaerobic conditions. Karunai Selvi and Ravindran (2012) observed that *B. subtilis* can effectively utilize nitrate as nitrogen source under



aerobic conditions. In a nitrogen cycle, nitrate are converted to nitrite by assimilatory or dissimilatory nitrate reductases. Nitrate reduction takes place by the action of a membrane-bound nitrate reductase, which has an active site in the cytoplasm and is prevalent amongst nitrate-respiring bacteria, including nitrate-denitrifying and ammonifying species (Carter et al., 1995). Nitrate reductases are also reported in periplasmic compartment in various organisms like *T. pantotropha*, *P. stutzeri* and many more (Carter et al., 1995). Reduction of nitrate in the periplasm is insensitive to the oxygen inhibition of nitrate transport across the cytoplasmic membrane that stops the reduction by the membrane-bound enzyme (Carter et al., 1995). *B. subtilis* has two reductases the nitrate and nitrite reductases and both with two different physiological functions. Nitrogen limitation subjects these enzymes to catalyze the reduction of nitrate to ammonia via nitrite for the anabolic integration of nitrogen

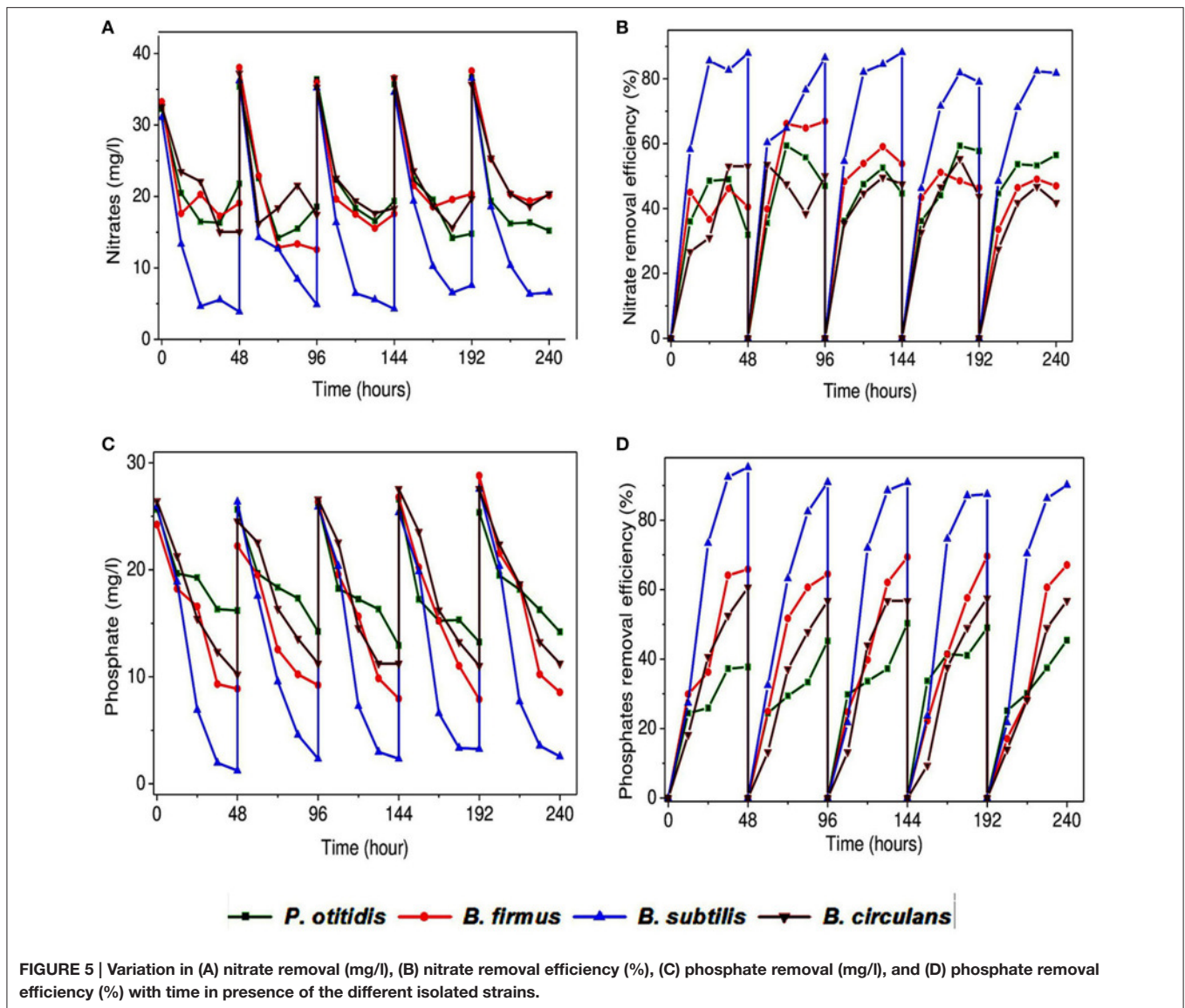
into biomolecules and under anaerobic respiration they use nitrate and nitrite as terminal electron acceptors (Nakano et al., 1998). These two distinct nitrate reductases, encoded by *nasBC* and *narGHI*, functions in catabolic and anabolic nitrogen metabolism, respectively. *B. subtilis* can exploit nitrate or nitrite as sole nitrogen source, and nitrate and nitrite are also utilized as electron acceptors for anaerobic respiration. Nakano and co-workers described the aerobic assimilatory function by NADH-dependent reductase (*NasDE*) in *B. subtilis* (Nakano et al., 1998). Kim and co-workers used dominant isolates viz. *B. cereus*, *B. subtilis* and *B. licheniform* in flask experiment for nitrogen removal under aerobic growth conditions. They supported that the *Bacillus* strains were able



to perform simultaneously aerobic nitrification/denitrification process (Kim et al., 2005). *Pseudomonas* also showed relatively higher nitrate removal efficiency. For studying nitrogen removal Ayyasamy et al., attained assimilatory denitrification process using *Pseudomonas sp.* (RS7) in the ground water system under aerobic conditions. *Pseudomonas* species possess periplasmic nitrate reductase providing ability to respire nitrate in the presence of oxygen (Ayyasamy et al., 2007). Studies were done with *Pseudomonas*, *Aeromonas*, and *Moraxella*, which have expressed a nitrate reductase with an active site located in the periplasmic compartment (Carter et al., 1995). These interesting facts can support the nitrate removal in aerobic conditions.

### Phosphate removal

Phosphate is one of the major nutrients in wastewater. In this study, phosphate removal was observed and found to be varied with the type of strain used (Figures 5C,D). Observation showed that the maximum phosphate removal till the end of the cycle was observed in the system operated with *B. subtilis* (91%) followed by *B. firmus* (58%), and *B. circulans* (54%). *P. otitidis* showed least phosphate removal (47%). Phosphate removal here becomes an important factor as phosphorus is one of the most essential nutrients and is an important component of different cellular structures, like cell membranes and nucleic acids. However, when present in excess it can



cause eutrophication. Phosphate from wastewater bodies are biologically removed by absorption of dissolved orthophosphate, organic phosphate and polyphosphate by microorganisms. The phosphorus removal biological techniques are based by co-metabolism wherein the microorganisms involved could be able to remove solubilized phosphate in the wastewater by accumulating them intra-cellularly in the form of polyphosphates or solubilizing the phosphate. Phosphate removal varies with different conditions. Along with the carbon source, pH also plays a crucial role in phosphate removal as acidic pH favors acid phosphatase which helps in the removal of phosphate. Increase in biomass concentrations of the *B. subtilis* showed a greater phosphate uptake capacity. Torriani-Gorini (1987) observed that the genes and proteins of microbial cells involved in the hydrolysis of organic phosphate with oxygen as the terminal electron acceptor. Karunai Selvi and Ravindran (2012) studied *B. subtilis* for its phosphate solubilisation activity in

different carbon and nitrogen sources. They observed *B. subtilis* showed maximum phosphate solubilisation when glucose and ammonium sulfate were used as carbon and nitrogen source, respectively. The bacterial species like *Pseudomonas* are well studied for polyphosphate accumulations. It uses the stored carbon reserves to produce energy for growth and to replenish their stores polyphosphate resulting the net removal of phosphate (Krishnaswamy et al., 2009). The phosphate is taken up by cells for growth and to reform polyphosphate under aerobic condition. Studies also revealed that presence of both carbon and phosphate under aerobic conditions at same times can have negative effects on phosphorus uptake. When carbon sources are present it will be utilized for PHA formation, which is property of *P. otitidis* and only when carbon sources gets exhausted the phosphorus uptakes occurs (Mino et al., 1998). This may be the reason why less phosphate removal was observed with *P. otitidis*.



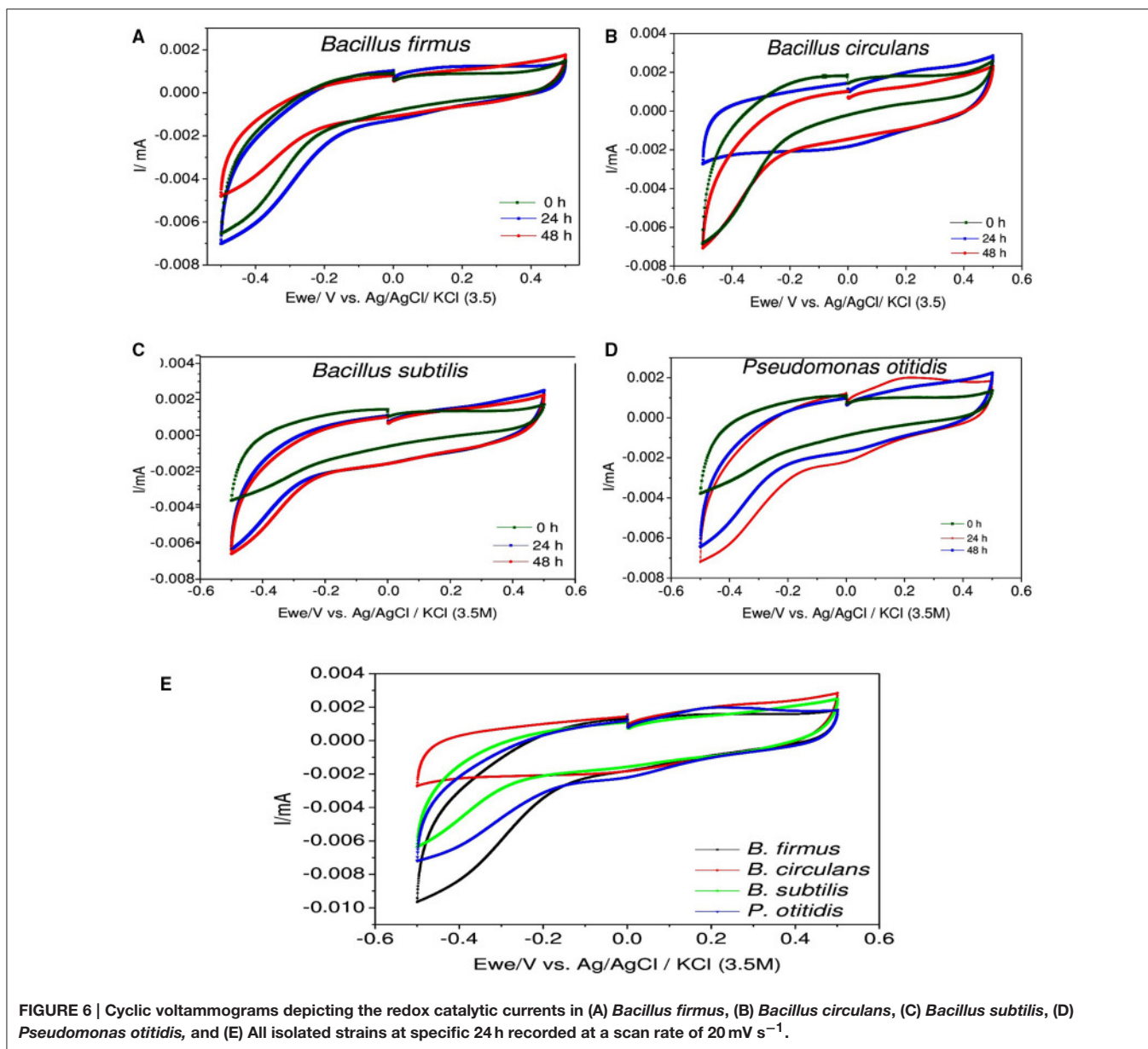


FIGURE 6 | Cyclic voltammograms depicting the redox catalytic currents in (A) *Bacillus firmus*, (B) *Bacillus circulans*, (C) *Bacillus subtilis*, (D) *Pseudomonas otitidis*, and (E) All isolated strains at specific 24 h recorded at a scan rate of  $20 \text{ mV s}^{-1}$ .

## Redox Condition

Volatile fatty acids (VFA) and pH are the active intrinsic expressers of acid and base reactions of the system. Inlet pH of all the reactors were adjusted to 7 and a consistent drop in pH was observed in all the systems throughout the operation (Figure 2B). Maximum pH drop was observed with *B. firmus* corresponding to maximum VFA generation followed by *B. subtilis* and *P. otitidis* (Figure 2C). The reduction in pH may be due to the production of various organic acids in the culture medium which was observed in *B. firmus*. Higher VFA generation was observed with in *B. firmus* (1200 mg/l) followed by *B. subtilis* (800 mg/l) and *P. otitidis* (500 mg/l). Least VFA production was observed with *B. circulans* (400 mg/l). Phosphate utilizing microorganisms produced various organic acids and consequently a fall in pH of the medium (Kundu and Gaur, 1984; Satar and Gaur, 1984).

## Bioelectrochemical Analysis

For understanding the electrochemical activity of individual bacterial strain during the process, CV analysis was carried out (Figure 6). CV helps to elucidate the appropriate information of the nature and the rate of metabolic activities by evaluating bioelectrochemical activity of bacteria in whole cell form. It gives detailed electrochemical reaction occurring on the electrode surface and measures redox activities of the components which are involved in solution as well as components bound to the bacteria (Naresh Kumar et al., 2014). In this study, CV revealed observations related to the electron discharge (ED) pattern as a function of bacterial strain used. In biological metabolism protons and electrons are generated and utilized continuously in the bacterial cells. Voltammograms visualized significant variation in the bioelectrochemical behavior with respect to particular organism. Voltammetric signatures of

**TABLE 2 | Synthetic consortia composition designed based on the individual isolated strains study.**

S. No.	Consortia Designed based on individual strain studies		
	Consortia	Constituent strains	Basis of Consortia
1.	Consortium 1	<i>Pseudomonas otitidis</i> + <i>Bacillus circulans</i>	Interspecies, high COD removal
2.	Consortium 2	<i>Pseudomonas otitidis</i> + <i>Bacillus circulans</i> + <i>Bacillus subtilis</i>	Interspecies, high COD and nutrient removal
3.	Consortium 3	<i>Pseudomonas otitidis</i> + <i>Bacillus subtilis</i> + <i>Bacillus circulans</i> + <i>Bacillus firmus</i>	Interspecies, high COD and nutrient removal, VFA generation
4.	Consortium 4	<i>Pseudomonas otitidis</i> + <i>Bacillus subtilis</i> + <i>Bacillus firmus</i>	Interspecies, high nutrient removal

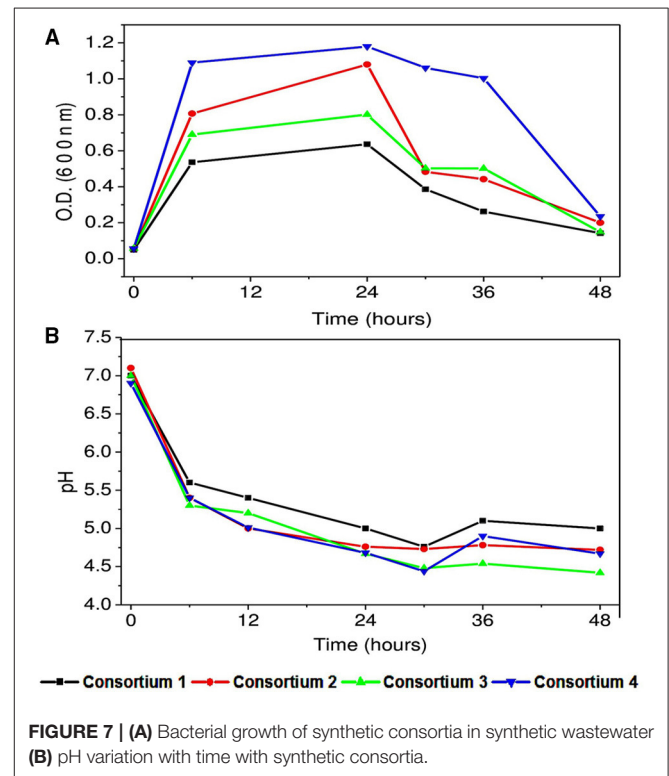
individual culture illustrated marked variation with the function of microbial cycle indicating the variation in electron transfer mechanism. In case of *B. firmus* reduction currents (RC) were significantly higher than the oxidation currents (OC) which were more or less similar (RC:  $-7.0 \mu\text{A}$  and OC:  $1.7 \mu\text{A}$ ) during all the time intervals. The RC distinctively increased during 24<sup>th</sup> hour (RC:  $-7.0 \mu\text{A}$ ) from 0<sup>th</sup> hour and slightly decreased at 48<sup>th</sup> hour. The RC currents were found to be almost equal at both 0<sup>th</sup> and 48<sup>th</sup> hour but OC increased with time and was found to be marginally higher at 48<sup>th</sup> hour than the 0<sup>th</sup> hour depicting decline phase with slight activity of the bacterial activity. Like *B. firmus*, in *B. subtilis* also higher RC was observed than the OC, which were more or less similar during all the time intervals. The RC increased with time (0<sup>th</sup> hour  $-3.8 \mu\text{A}$ ; 24<sup>th</sup> hour,  $-6.5 \mu\text{A}$ ) and marginally increased till 48<sup>th</sup> hour (RC:  $-6.7 \mu\text{A}$ ). The OC currents also increased from 0<sup>th</sup> hour and were found to be almost equal at both 24<sup>th</sup> and 48<sup>th</sup> hour which shows that *B. subtilis* got well adapted to the system which was supported by the highest growth rate. For *B. circulans*, RC decreased and OC increased from 0<sup>th</sup> hour to 24<sup>th</sup> hour, but the RC and OC were distinctively equal (RC:  $-3.0 \mu\text{A}$  and OC:  $3.0 \mu\text{A}$ ) depicting its electrogenic activity that aids in effective discharge and transfer of the redox equivalents as well as their reduction at a higher rate. For comparative evaluation, voltammograms were also compared for each strain at 24<sup>th</sup> hour (active cultures; **Figure 6E**). Maximum RC was observed for *B. firmus* (RC  $-9.0 \mu\text{A}$ ) followed by *P. otitidis* (RC:  $-7.0 \mu\text{A}$ ). *B. circulans* showed almost equal OC and RC (OC/ RC: 3/-3  $\mu\text{A}$ ). The increment in both the oxidation and reduction catalytic currents at 24<sup>th</sup> hour indicates the biochemical reactions of substrate utilization where the role of oxygen is negligible as electron acceptor. Highest COD removal by *B. circulans* can be supported by cyclic voltametry where equal oxidation reduction currents were observed.

## Performance of Designed Synthetic Consortia

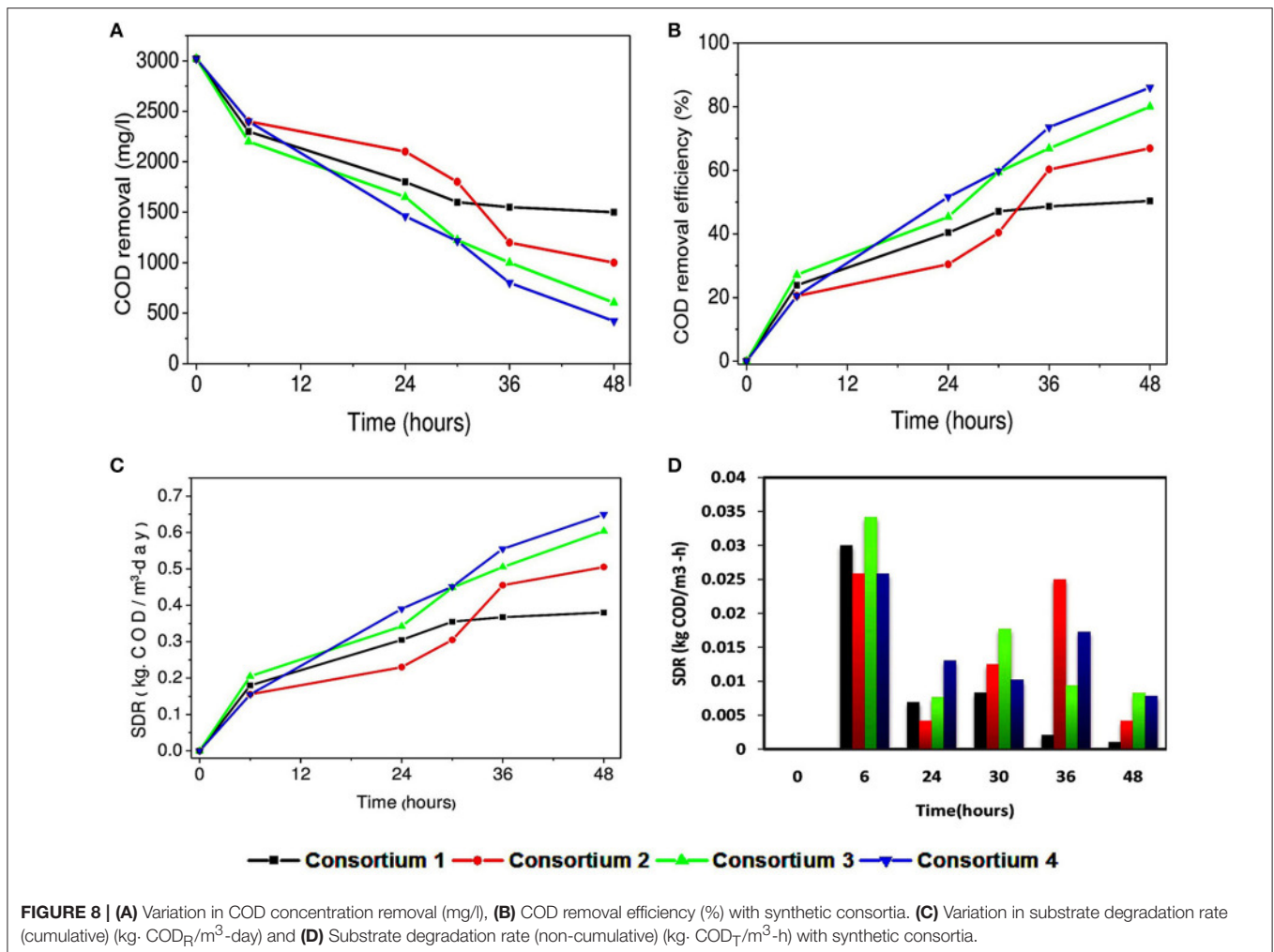
Individual strains were studied and based on their performance four different consortia were developed and studied for their effective wastewater treatment potential as depicted in **Table 2**.

### Biomass Growth

All the consortia showed good biomass growth and depicted that the cultures got well adapted to the conditions implemented and have some positive association. Highest biomass at OD 600 was observed in consortium 4 constituted with *P. otitidis*, *B. subtilis*, and *B. firmus* (OD 600/1.2) followed by consortium



2 constituted with *P. otitidis* + *B. circulans* + *B. subtilis* (OD 600/1). Least biomass was observed in consortium 1 constituted with *P. otitidis* + *B. circulans*. Highest growth in consortium 4 may attribute to high COD and nutrient removal. Interestingly, consortium 4 showed maximum stationary phase till 36 h while others showed decline phase after 24<sup>th</sup> hour itself (**Figure 7**). Growth of the cultures in any environment is the indication of favorable condition for the cultures to nurture themselves. Growth of the individual microorganism is relatively easy when compared to growth of consortium having different organisms. The bacteria can form various complex associations with other organisms present in the system. These interactions may be positive, negative or may be neutral. In neutral associations, two different bacterial species occupy the same environment without affecting each other. Such association might be transitory; as conditions change in the environment or might be the change in relationship. Positive associations help the species to grow with mutual benefits which might have happened in consortium system 4. Negative association inhibits or adversely effects the growth of other organism. This might have happened in the



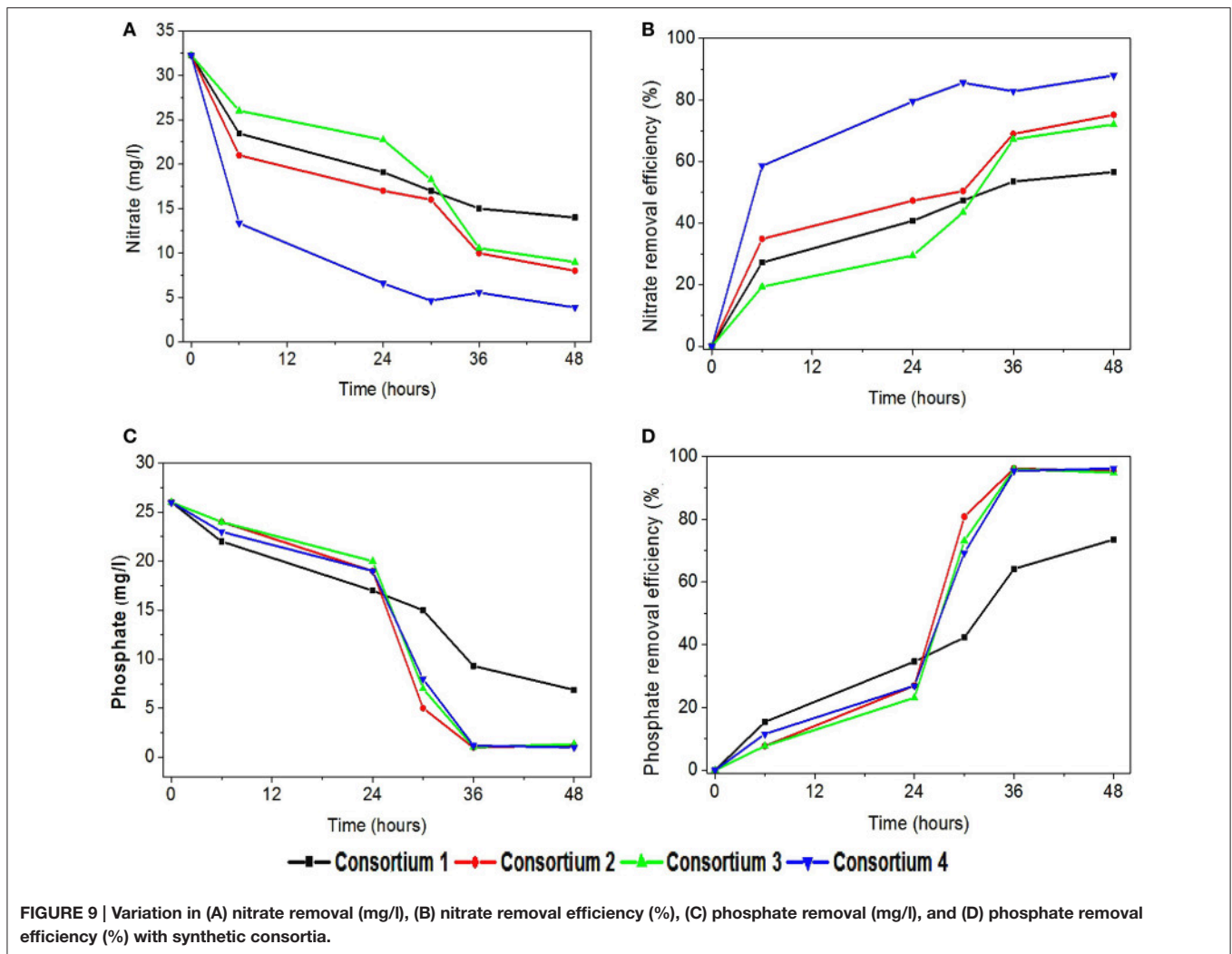
consortium 3 where although four strains were present but the growth was less when compared to consortium system 2 and 4. Mutualism helps bacteria to form close spatial associations with the others that are essential for their survival. Hence, growth is also an important parameter in consortia study. Along with growth, pH drop in consortia system was observed (Figure 7B) but the VFA production was negligible (data not shown) which may be attributed to the utilization of electrons and protons in the co-metabolism of the consortium (Annie Modestra and Venkata Mohan, 2014).

## Wastewater Treatment

### Carbon load removal

COD removal for the consortia systems interestingly was higher when compared to the individual strains (Figures 8A,B). Highest COD removal was observed in consortium 4 with COD<sub>R</sub> efficiency of 86%. This may be attributed to the availability of carbon source to the culture and the co-metabolic activity of the culture is favorable for wastewater treatment. Lowest COD removal in consortium 1 (COD<sub>R</sub>: 50%) may be attributed to the combination of the isolates with which it is constituted. This combination may not be favorable for substrate

degradation, which can be supported with cyclic voltammogram of consortium 1. Cumulative SDR of 0.64 kg COD<sub>R</sub>/m<sup>3</sup>-day was found to be maximum with consortium 4 (Figures 8C,D). Interestingly, when SDR per hour (non-cumulative) (SDR<sub>T</sub>) with consortia was calculated it showed variation from the strains. Sudden increase in SDR<sub>T</sub> was observed in all systems and thereafter a drop in SDR<sub>T</sub> at 24<sup>th</sup> hour was observed. Consortium 1 constituted with *P. otitidis* and *B. circulans* showed minimum SDR<sub>T</sub> at individual hours. At 36<sup>th</sup> hour and 48<sup>th</sup> hour SDR<sub>T</sub> for consortium 1 was 0.0025 kg COD<sub>T</sub>/m<sup>3</sup>-h and 0.001 kg COD<sub>T</sub>/m<sup>3</sup>-h, respectively which was very less when compared to other consortia. Sudden increment at 6<sup>th</sup> hour is attributed to growth of the strains in the culture. Later decrement at 24<sup>th</sup> hour may be attributed to the competition among the strains in the consortia to uptake the substrate or transformation of the substrate towards metabolite production. At 30<sup>th</sup> hour again increase in SDR% was observed which may be because of the survival of the fittest. At 36<sup>th</sup> hour slight decrement was observed for all consortia except for consortium 2. The reason for this may be either the surviving strains were trying to uptake the residual COD, or may be the strains were adapting to the depleted carbon and nutrients condition in the wastewater. The



**FIGURE 9 |** Variation in (A) nitrate removal (mg/l), (B) nitrate removal efficiency (%), (C) phosphate removal (mg/l), and (D) phosphate removal efficiency (%) with synthetic consortia.

consortia study gave different perspective when compared to the studies with individual strains. For better understanding, it was found that the consortia where *P. otitidis* and *B. subtilis* were present it showed enhanced treatment efficiency. Various studies also reported higher COD removal using *B. subtilis* in different consortium using different wastewaters (Sharma et al., 2010).

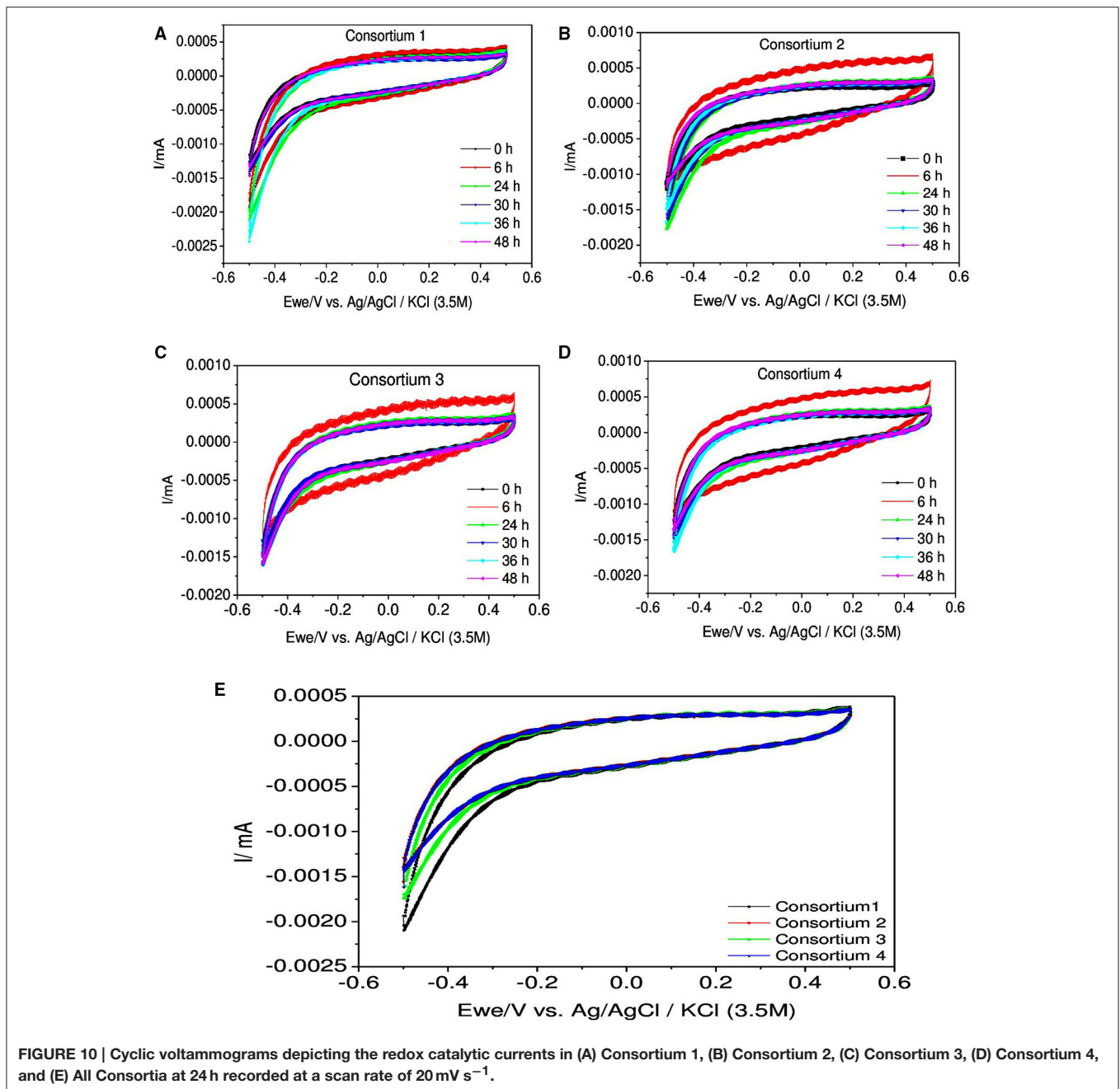
### Nitrate removal

In the consortia operated systems, nitrate removal was also observed to be higher compared to the individual strains (Figures 9A,B). Highest nitrate removal was observed in system 4 (88%). It is interesting to find the consortia having *P. otitidis* and *B. subtilis* (consortia 2, 3, and 4) showed higher nutrient removal (75–86%). This can be supported with the previous study where *Pseudomonas* sps and *B. subtilis* consortia reported higher nitrate removal (Rajakumar et al., 2008). Literature reveals that *B. subtilis* is considered as the one of the best candidate for nitrate removal with *Pseudomonas* sps. Nitrogen removal where microbial community cycled through a series of aerobic, anoxic, and anaerobic stages enables complete removal of nitrogen (N) and phosphorus (P) from wastewater (Rittmann, 2006). This removal involves

aerobic chemolithoautotrophic ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), chemolithoautotrophic bacteria, polyphosphate-accumulating organisms (PAO), and the glycogen-accumulating organisms (GAO) (Daims et al., 2006). Out of these communities very few microorganism are efficient enough to carry out mechanism, among which *B. subtilis* is one of the major organism. Many of these organisms were isolated and studied for its individual efficiencies for wastewater treatment (Hamza et al., 2009; Sharma et al., 2010).

### Phosphate removal

In consortia operated systems phosphate removal was also observed higher when compared to the individual strains operated systems (Figures 9C,D). Apart from consortia 1 (73%) other consortia showed high phosphate removal (95–97%) supporting the fact that the presence of *B. subtilis* and *P. otitidis* helps in higher phosphate solubilisation. Mino et al. (1998) reported that the enhanced biological phosphorus removal capacity also relies on the ability of some organisms that can convert volatile fatty acids to polyhydroxyalkanoate (PHA) under aerobic conditions. Presence of *P. otitidis* might have lead to lower volatile fatty acids concentrations in all the systems, as its



ability for PHA synthesis is well exploited through augmentation (Reddy et al., 2012). The presence of organic acids releases protons which involves in biological ammonium assimilation that enhances the utilization of phosphate.

### Bioelectrochemical Analysis

For better understanding CV analysis for the consortia was carried out regularly at different time intervals (Figure 10). Analysis revealed that comparatively redox currents were less compared to individual strains but interestingly RC were high in all the systems. In case of consortium 1, RC was significantly higher than the OC which were more or less similar during all the time intervals. The RC was found to increase with time till

36 h ( $-2.6 \mu\text{A}$ ) but sudden drop was observed at 48 h ( $-1.6 \mu\text{A}$ ) which was almost similar to 0<sup>th</sup> hour depicting a decline in the activity of the culture. In consortium 2, RC was found to have increased till 24 h (RC:  $-1.8 \mu\text{A}$ ) then dropped from 30 h till 48 h. Interestingly, at 6<sup>th</sup> hour high RC ( $-1.2 \mu\text{A}$ ) and OC ( $0.6 \text{ mA}$ ) were observed. In consortium 3, interestingly redox currents were found to be relatively similar with RC ( $-1.6 \mu\text{A}$ ) except OC at 6<sup>th</sup> hour ( $0.6 \mu\text{A}$ ) as it was higher compared to others. In consortium 4, the RC increased till 36<sup>th</sup> hour (RC:  $-1.7 \mu\text{A}$ ) and later decrement was observed at 48<sup>th</sup> hour. Interestingly, consortium containing *B. subtilis* showed higher OC specifically at 6<sup>th</sup> hour than the consortium lacking it. In this study, the increment in both the oxidation and reduction catalytic currents

**TABLE 3 | Comparative evaluation of synthetic consortia with the single strains for their wastewater treatment potential.**

S. No.	Wastewater Treatment efficiency				
	Strains/Consortium	COD (%)	Nitrate (%)	Phosphate (%)	CV Analysis ( $\mu\text{A}$ )
1.	<i>Pseudomonas otitidis</i> SVMIICTP2	50	56	45	OC/RC: 1/-7
2.	<i>Bacillus firmus</i> IICTSVMHH	40	46	67	OC/RC: 1/-19
3.	<i>Bacillus subtilis</i> IICTSVMH13	59	81	90	OC/RC: 2/-6.5
4.	<i>Bacillus circulans</i> SLBEEC1	68	41	56	OC/RC: 3/-3
5.	Consortium 1	50	56	73	OC/RC: 0.28/-2.1
6.	Consortium 2	66	75	95	OC/RC: 0.25/-1.75
7.	Consortium 3	80	72	94	OC/RC: 0.24/-1.5
8.	Consortium 4	86	87	96	OC/RC: 0.29/-1.4

in aerobic systems indicated the initial biochemical reactions of substrate utilization, where the oxygen is playing negligible role as electron acceptor. Broadly, drop in redox current were observed in later hours which indicates that the metabolically released protons and electrons were consumed toward substrate degradation instead of discharging toward counter and working electrodes.

The comparative evaluation of both the strains and consortia studies revealed that the consortia were effective than the individual strains, suggesting that the potential strains can be used for synthesizing a defined consortium for the effective wastewater treatment (Table 3). When consortia were designed with multiple strains, the performance varied from the individual strains (either positively or negatively) due to the competition/synergy among the strains within the microenvironment. When *B. circulans* and *B. subtilis* along with *P. otitidis* were present in the same microenvironment, the treatment efficiency was low but supplementation of *B. firmus* has lead to the increment in the wastewater treatment efficiency. Interestingly, *B. firmus* and *B. subtilis* along with *P. otitidis* showed higher wastewater treatment.

## CONCLUSIONS

In this study four different strains viz., *P. otitidis*, *B. firmus*, *B. subtilis*, and *B. circulans* were isolated, identified and further selected for the wastewater treatment. Highest efficiency of carbon removal was observed with *B. circulans* while nutrients removal was observed for *B. subtilis*. Four consortia with these isolated and studied strains were designed using different combinations. Synthetic consortium constituted with *P. otitidis*,

*B. circulans* and *B. firmus* showed highest treatment efficiency viz. is 86%, 87%, and 96% in terms of carbon, nitrate and phosphate removal, respectively. Bio-electrochemical analysis helped in understanding the release and utilization of electrons and protons toward substrate degradation. Functioning of nitrate reductase in *Bacillus* and *Pseudomonas* might have lead to nitrate reduction under aerobic conditions. Lower catalytic currents in consortia studies than the individual strains depicted utilization of the electrons and protons toward enhancing the wastewater treatment. Presence of interspecies bacterial strains and their synergetic relationship enhanced the wastewater treatment efficiency.

## AUTHOR CONTRIBUTIONS

Both the authors have Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; Drafting the work or revising it critically for important intellectual content; final approval of the version to be published; and Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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**Conflict of Interest Statement:** The 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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