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A review on unit operations, challenges, opportunities, and strategies to improve algal based biodiesel and biorefinery

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Globally, the demand for energy is increasing with an emphasis on green fuels for a sustainable future. As the urge for alternative fuels is accelerating, microalgae have emerged as a promising source that can not only produce high lipid but many other platform chemicals. Moreover, it is a better alternative in comparison to conventional feedstock due to yearlong easy and mass cultivation, carbon fixation, and value-added products extraction. To date, numerous studies have been done to elucidate these organisms for large-scale fuel production. However, enhancing the lipid synthesis rate and reducing the production cost still remain a major bottleneck for its economic viability. Therefore, this study compiles information on algae-based biodiesel production with an emphasis on its unit operations from strain selection to biofuel production. Additionally, strategies to enhance lipid accumulation by incorporating genetic, and metabolic engineering and the use of leftover biomass for harnessing bio-products have been discussed. Besides, implementing a biorefinery for extracting oil followed by utilizing leftover biomass to generate value-added products such as nanoparticles, biofertilizers, biochar, and biopharmaceuticals has also been discussed.

KEYWORDS

biodiesel, lipid productivity, biorefinery, genetic engineering, algae, carbon dioxide sequestration

Introduction

Over the decade, limitation in availability of fossil fuels and increasing population has resulted in enhanced energy demands. Furthermore, continuous exploitation of non-renewable energy sources has embarked the accumulation of carbon dioxide (CO₂) in the atmosphere from 295 parts per million (ppm) to more than 400 ppm in 2018 leading to global warming and climate change (Tang et al., 2020) (Rosa et al., 2019). According to some studies, it is estimated the current rate of fossil fuel consumption including natural gas, coal, and crude oil will lead to their exhaustion in approximately 50 years. Therefore, the green energy demand is set to increase from 43 billion L/day in 2020 to 55.96 billion L/

day by 2045 (Organization of the Petroleum Exporting Countries - OPEC, 2020). Additionally, reports published by International Energy Agency (IEA) states that the biofuels market was valued at nearly 110 billion USD in 2021 and will increase to 2012 billion USD by 2030 (IEA, 2021). Therefore, current studies are focused on generating alternatives to fabricate green energy. Numerous sources are required which are not only renewable but also environment friendly to develop sustainable carbon neutral fuels. sequestered from CO₂ the atmosphere gets converted into biomass, and upon chemical conversion, into fuel. Therefore, green fuels do not contribute to any further addition or reduction of CO₂ into the atmosphere and lead to carbon neutrality (Kumar et al., 2010). According to several scientific investigations, algae can capture 10–50 times more amount of CO₂ leading to high growth rate (Mondal et al., 2017).

The major cost of fuel is dependent on the biomass which can either be crops like sugarcane or plant seeds but they possess competition with food crops and restrict product yield (first generation); lignocellulosic biomass, kitchen waste, crop residue but the high cost of processing due to lignin and expensive technologies for lipid extraction makes them unsuccessful (second generation); microalgae depict rapid growth and enhanced oil yield but it is still expensive for large scale production (third generation); genetically modified algae with advanced extraction techniques have recently been deployed for large scale production of biofuel but they also imply high initial investment and extensive research (fourth generation) (Yaashikaa et al., 2022). Amongst them, microalgae, chlorophyll harboring photosynthetic microorganism is the best candidate used for green fuel production (Gonçalves et al., 2013). They have the ability to enhance oil yield and marginalize dependence on fossil fuels. Also, they are capable to reduce greenhouse gases (GHG) emissions by 50–70% in comparison to conventional fuels (Liu et al., 2013). The major steps incorporated in the production of microalgae-derived biodiesel are cultivation, harvesting, extraction of oil, and purification followed by biodiesel synthesis from individual cells (Tang et al., 2020). Also, generate carbohydrates, proteins, vitamins, lipids, and pigments depending on species and external growth factors. The downstream processing accounts for 50% of the biodiesel production cost. Hence, novel methodologies are needed to reduce the fuel production cost.

Currently, hybrid models and by-product extractions have been employed with algae to make fuel economically viable. For instance, growing algae in a waste water treatment plant can not only reduce the cultivation cost, but also remove pollutants from it, recover nutrients, and generate high-value commodities (Chandrasekhar et al., 2022). More recently, algal industry has given attention to a diverse variety of algae-based products apart from lipids including carotenoids, proteins, nucleic acids etc., for the production of nutraceutical, cosmeceutical, bio-stimulants, bio-fertilizers,

and other environment remediation commodities like biochar. Therefore, implementation of a biorefinery will elucidate the production of such high-value chemicals/products for different industrial interventions (Gondi et al., 2022). While, novel pre-treatment methodologies such as cell wall weakening before cell disintegration *via* nanoparticles may enhance the biogas as well as biofuel yield (Gondi et al., 2022) (Yukesh Kannah et al., 2021). Therefore, valorization of algal biomass for green fuel and using the leftover to extract other industrially important products can compensate the exorbitant fuel prices in the future.

The present article aims to provide an overview on the recent information on unit operations in up and downstream processing for algae-based biofuel as well as high-value chemical/product extraction. Additionally, a detailed study on the life cycle assessment (LCA) framework along with emerging techniques including genetic, metabolomic engineering coupled with omics approach for improving lipid yield and biomass productivity has been discussed. Finally, likelihood of developing an algal biorefinery for high-value products fabrication such as carbohydrates, lipids, carotenoids, amino acids, antioxidants, fatty acids, vitamins etc., for different industrial interventions has also been conferred. Moreover, present challenges and future research directions for integrated algal biorefinery setups are outlined.

Overview to algal biomass-based biodiesel and biochemical production: Unit operations in up and down stream processes

Unit operations in up-stream processes

Algal biodiversity

Microalgae including Cyanophyceae (blue-green algae), Bacillariophyceae (diatoms), Chlorophyceae (green algae), and Chrysophyceae (golden algae) are the most economical and efficient for harboring lipid for biodiesel production, CO₂ fixation and wastewater treatment (Kumar et al., 2010). Numerous bioprocess industries rely on these microorganisms' metabolism to convert carbohydrates to other value-added products such as vitamins, amino acids and organic acids such as acetic acid. However, the cost of carbohydrate feedstock plays a significant role in the total cost of production in such industries. While photosynthetic organisms such as microalgae provide a production alternative and can entirely eliminate the cost associated with carbohydrate feedstock. They employ inexpensive and readily available sunlight as an energy source. Cyanobacteria are known to convert 3–9% of solar energy captured into biomass whereas terrestrial plants can convert only 0.25–3% of the captured solar energy into biomass (Ducat et al., 2011).

Screening and selection of microalgal strain

According to the scientific studies the strain of interest shall have the following features (Chu, 2017).

- Robust with enhanced growth rate and high lipid content.
- Capable to withstand shear stress generated during mixing and tolerate microorganism interference.
- Flexible to adapt according to diverse physiochemical parameters encountered during culturing.
- Genetically manipulable for obtaining high growth rate, lipid productivity, and other related bioproducts.

In order to obtain the above characteristics in the algal strain several techniques have been described for their isolation and screening including gravimetric separation, single-cell isolation by serial dilution, micromanipulation, and atomized cell spray (Mutanda et al., 2011). These conventional methods for strain selection are not only complex but also time-consuming, and labor-intensive (Kwak et al., 2016; Lim et al., 2017). In order to simplify the selection process, Kim et al. reported the advancements in the field of microfluidic systems to study microalgal biotechnology (Kim et al., 2017). Likewise, Challagulla et al. reviewed the applications of various technologies for the analysis of lipid content and biomass productivity of the microalgal cell. These technologies include raman spectroscopy, near-infrared spectroscopy, fluorescent lipid-soluble dye-based detection, nuclear magnetic resonance spectroscopy, and fourier transform techniques (Challagulla et al., 2016). Moreover, flow cytometry, microfluidics, and spectroscopy can either be used alone or in combination with other technologies for screening high lipid and biomass yielding microalgal strains (Shokravi et al., 2020). While there are several strategies for rapid selection of microalgal strains such as bioprospecting is the most prominent approach for screening high biomass producing strains. Also, high throughput techniques including vibrational spectroscopy or flow cytometry which are highly accurate and rapid for characterizing metabolites (Shokravi et al., 2020).

Identification of high lipid yielding strains

Solvent extraction and gravimetric methods are used for identifying high lipid yielding strains. These procedures are not only accurate but also provide reproducible results. However, they are time-consuming, labor intensive, and require a large amount (10–15 mg wet weight) of biomass (Morschett et al., 2016). On the contrary, chromatographic methods procure high reproducibility, require less time, and provide a single report with both qualitative and quantitative estimates of fatty acids. Such methods entail cell disruption to release cellular contents and derivatives of released fatty acids but they comprise highly expensive instrumentation (Dołowy and Pyka, 2015). Another technique is Nile red staining, an *in situ* colorimetric detection technique for lipid estimation. Though it

is simple, rapid, and highly efficient, still the accuracy of results can be influenced by several factors. Although, these methods are quick, cheap, and simple in nature but cannot detect fatty acid chains having less than 12 carbon atoms (Morschett et al., 2016). Time-domain nuclear magnetic resonance (TD-NMR) an *in situ* technique can be used for estimation as it is highly rapid, simple, and economical but the accuracy of this method depends on cellular lipid content. Therefore, it requires additional analytical equipment (Romel Malapascua, 2012). More recently, inverted fluorescence microscopy has been used for determining lipid content and growth for several microalgal species (Hathwaik and Cushman, 2017). Also, fluorescence-assisted cell sorting (FACS) has been employed for the detection of high lipid yielding cells in *Chlorococcum littorale*. Similarly, a microfluidic cytometer has been applied to quantify lipid accumulation and photosynthetic efficiency in *Phaeodactylum tricorutum*. While fourier-transform infrared spectroscopy (FTIR) has been used for examining both the lipid yield and growth in the case of *Dunaliella salina* (Romel Malapascua, 2012). Similarly, for *Chlamydomonas reinhardtii* mutants, droplet microfluidics-based screening has been applied for analyzing growth and lipid content (Hathwaik and Cushman, 2017).

Microalgae breeding strategies

In comparison to terrestrial plants, microalgae entail several advantages including a short life cycle from hours to days as compared to seasonal cycles seen in plants, are unicellular organisms that can rapidly breed in a confined space thus reducing the setup/land cost, and can do both sexual as well as asexual reproduction and boost the genetic diversity of the species. A variety of species can be screened on the basis of desired phenotypes *via* flow cytometry and other high throughput techniques. According to recent studies, transgenic microalgae are gaining more interest because of efficient phenotype construction (Hallmann, 2007). Novel strains can be developed by targeting specific genes through forward or reverse genetics. The former approach has much to offer by screening the results of random knockout events. Currently, there is a lack of transformation technologies that can be applied to biodiesel-producing algae. However, alternative strategies such as isolation and breeding of highly potent non-genetically modified organism (GMO) strains can be employed. One such method is the screening of libraries produced through UV or chemical mutagen treatment. This method also bypasses the regulatory procedure for GMOs for outdoor systems (Larkum et al., 2012).

Microalgal cultivation

Traditionally, open ponds and land-based photobioreactors (PBRs) have been used for algal cultivation. However, in recent times, the focus has been shifted to ocean-based floating microalgal culture systems due to the availability of large areas and wave energy for continuous mixing. In addition to

this, seawater provides nutrients and impregnates thermoregulation (Park et al., 2018). However, there is still a need for the development of novel and advanced technologies for the harvesting of microalgae biomass, and lipids from the cells. So, to enhance lipid productivity, two-stage microalgal cultivation setups have been proposed 1) Efficient growth stage: either in culture medium or in wastewater effluent medium, and; 2) High lipid accumulation inducing stage: salt stress stage (Hang et al., 2020).

To date several attempts have been made to reduce the production cost of biodiesel, some of them are as follows: (Gong and Jiang, 2011):

- Sequester CO₂ from thermal power plants to enhance biomass productivity
- Use nutrient-enriched wastewater for culturing microalgae
- Design and develop outdoor photobioreactors for large-scale algae cultivation
- Utilize by-products obtained after oil extraction effectively
- Incorporate genetic and metabolic engineering for the optimization of biodiesel production

Common strategies utilized for the cultivation of microalgae are: (Pavithra et al., 2020).

- 1) Single stage–Cultivation of microalgae using continuous, semi-continuous, or fed-batch system with lower lipid content due to nutrient deprivation in continuous or fed-batch setup.
- 2) Two-stage–Cultivation of microalgae in nutrient-rich medium followed by subjecting to stressful conditions.

Single-stage cultivation of microalgae

Open pond cultivation system

The operation mode of a cultivation setup not only improves biomass but also lipid yield. Such cultivation setups comprise of artificial ponds, open water bodies, or containers. Besides, on the basis of architecture, they can be shallow raceways, and circular unstirred ponds, out of which, raceway ponds are the most widely implemented. Besides, raceway ponds provide continuous tender agitation, minimal energy requirements, and low cost. Usually, they are cemented/concrete construct divided by baffles arranged in a series to support gentle mixing of nutrients as well as algal biomass. A depth of 1.5 ft promotes efficient sunlight penetration and optimum CO₂ sequestration (Chandra et al., 2020). However, they need an extremely large area and are capable of getting contaminated by microorganisms and birds. Moreover, the productivity quotient of microalgae cultivated in an open pond system is directly proportional to the location of the system, layout, inoculated species, and the prevailing weather conditions. For ponds with water levels higher than 200 mm, the light intensity for ponds, except for the thin upper layer, is below the photo-compensation point and thus, limiting the algal

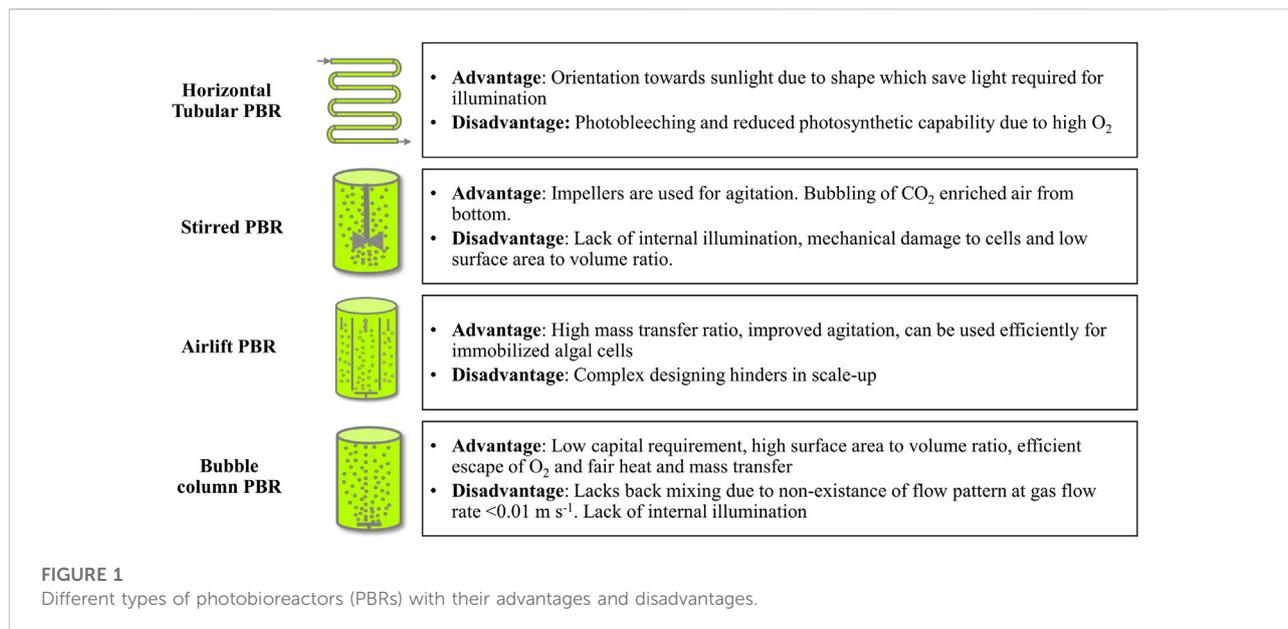
growth (Doucha and Lívanský, 2006). For such cases, thorough mixing can improve light penetration as well as cultivation efficiency. Additionally, such setups are majorly used for synthesizing, lipids, carotenoids (beta-carotene), proteins, carbohydrates and, other industrially important products. Though they are low-cost systems, but variable temperature, high water and weather dependence, and low biomass concentration limits their use for large-scale production.

Photobioreactor system

PBRs are a closed artificial setup which can incorporate both mechanical as well as non-mechanical mixing strategies. Usually, they comprise of a sparger having specialized nozzles to release CO₂ bubbles for non-mechanical agitation. While in some cases impellers can be installed for mechanical mixing in the PBR sets. These systems offer enhanced growth rates, higher cell density, and biomass compared to open pond cultivation systems (Huang et al., 2017). Additionally, CO₂ sequestering PBRs have a high biomass transfer rate due to increased resistance to mass transfer from a gaseous phase to algal cells *via* the lipid phase. Moreover, such setups are composed of glass, polyvinyl chloride (PVC), polyethylene (PE), polycarbonate (PC), plexiglass, or fibre glass and provide appropriate growth conditions with desired amount of carbon, water, light, nutrients, temperature, and pH. Amongst the different bioreactor types, airlift reactors are the most suitable for sequestering flue CO₂. Moreover, they provide uniform mixing, high gas transfer rate, low hydrodynamic stress, and an easy control system (Mondal et al., 2017). The selection of suitable PBR depends on the desired strain, space availability, and the preferred product's nature. Specific advantages of using PBRs are 1) concentration monitoring and control system, 2) minimal or no chances of contamination, 3) no media evaporation, 4) high concentration of microalgal cells, and 5) provides several critical cultural conditions simultaneously such as pH, temperature, CO₂, mixing and others (Pavithra et al., 2020). However, high capital and operational cost owing to complex configuration, an artificial illumination system, cooling, and agitation system, deoxygenation procedures, and other complex operating conditions are some of the limitations that prevent commercial implementation of these setups (Peng et al., 2020). Additionally, biofouling, overheating, and cellular damage due to shear stress are also some drawbacks. The PBRs are further classified into horizontal tubular, stirred tank, airlift, and bubble column PBR. Some advantages and disadvantages of these systems are discussed in Figure 1.

Operational parameters for efficient PBR Working

An effective PBR shall not only allow easy light penetration but also provide equal distribution of it. Besides it shall promote uniform mixing and mass transfer under favorable pH and temperature conditions. Also, cost is an important criterion for a long-term investment. So, it should be cost effective. Some of the aspects are discussed as follows.



Light

Intensity of light and UV-Visible spectrum (400–700 nm) are some of the critical factors responsible for improved biomass yield. Usually, distribution of light is non-uniform inside a PBR due to scattering and absorption by individual cells (Quan et al., 2004). The radiation attenuation depends on cell concentration, PBR geometry, distance of light, and visible range. Also, according to studies intensity of light decreases with distance. So, light can penetrate only several millimetres when cell density is more than 10 g/L in a PBR. Additionally, growth decrease as the reactor depth increases. In some cases, investigators revealed that if light is above the critical value so the growth will be inhibited *via* photoinhibition (Richmond, 2004). On the contrary if it is below the necessary level then it will lead to photolimitation and stunted growth. Therefore, based on cell growth, PBR's can be divided into 3 zones namely: strong illuminating zone, light energy fulfils the cell demand; weak illumination zone, light energy just meets the cell demand; and dark zone, cells are unable to grow due to limiting light (Bitog et al., 2011). Hence, for enhanced biomass productivity thickness of a PBR should be minimum.

Mixing

Efficient mixing not only provides uniform distribution of nutrients, gas but also avoids stagnation of cells in dark zones, prevent fouling, and maintain the cultures (Anjos et al., 2013). Additionally, mixing exposes light equally to all the cells and endorse efficient mass transfer. But, excess of mixing might cause cell damage due to shear force. Also, it is recommended to keep the mixing velocity below 1 m/s to prevent micro eddies formation that can damage cells. A velocity of 20–50 cm/s is recommended for good mixing (Lehr & Posten, 2009). Mixing in

PBR is induced *via* agitator, impeller, static mixer, or a CO₂ enriched sparger.

Mass transfer

Algae growth can be distributed into three processes including photosynthesis, photorespiration, and dark respiration (Wang et al., 2012). Most of the identified algal species undertake the C3 pathway or Calvin Cycle for CO₂ fixation. An enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) catalyzes the formation of two molecules of the 3-carbon compound from the combination of CO₂ and a 5-carbon containing compound. While, Photosynthesis is carried out in the chloroplast as a result of biophysical and biochemical reactions happening at thylakoid and stroma respectively. Biophysical reactions lead to photon absorption *via* chlorophyll and other accessory photosynthetic pigments. Therefore, water is oxidized and release oxygen. Free electrons from water molecules result in the formation of ATP and NADPH. The energy produced by the splitting of water is used for CO₂ fixation in the dark reaction. Whereas, biochemical reactions lead to the formation of sugar and other organic molecules for cellular functions and metabolism (Raven and Beardall, 2003). Therefore, photosynthesis elucidates light and CO₂ to make ATP and O₂ as a by-product. However, increased amount of dissolved O₂ may cause photoinhibition which is toxic and inhibit algae growth. So, closed PBR's are equipped with a degasser to prevent such condition and mediate smooth mass transfer.

Temperature

Microalgae are capable of growing only under specific temperature range. Exorbitant temperatures lead to growth inhibition followed by cell death. It is seen that Open pond cultivation maintain an approximate temperature of 40°C due to

evaporation (Anto et al., 2020). While closed PBR are well equipped with thermal jackets and cooling water units which prevents desired temperature deviations. However, in tropical regions where the maximum temperature reaches upto 47°C there is a constant need of heat exchangers, IR-radiation, and light dilution is used to enhance biomass productivity. This additional cost can be compensated *via* solar photovoltaic panel setup (SPV) or other bioactive compounds extraction after biofuel production (Stephens et al., 2010) (Gondi et al., 2022).

pH

Optimal pH for algae growth is in the range of 7–9. It greatly affects the CO₂/carbonates solubility, and functioning of intracellular enzymes. Acidic media may enhance nutrition uptake by the cells, produce metabolites and increase metal toxicity. While a basic media interferes with the cell division. Any deviation from the standard pH range will reduce the photosynthetic efficacy. Generally, freshwater algae including cyanobacteria can tolerate altered pH, but marine algae are inflexible to any such deviations and need an optimum value around 8.2. (Lackner, 2003; Kumar et al., 2010).

Carbon dioxide sequestration

Microalgal biodiesel is found to be a carbon-neutral renewable liquid fuel as half of the dry weight of the algal biomass is carbon which is sequestered from the atmosphere. Therefore, cultivation setups are continuously supplied with CO₂ for enhanced biomass production. For instance, approximately 183 tons of CO₂ can produce 100 tons of microalgal biomass (Chisti, 2008). Some sources of CO₂ supply include atmospheric CO₂ (approximately 0.0387% (v/v) in the air), industrial emissions (flue or flaring gases which typically contain approximately 15% (v/v) CO₂ in the exhaust emission), and chemically fixed soluble carbonates (e.g., sodium bicarbonate and sodium carbonate). However, for large-scale setups, industrial emissions have been found to be adequately fulfilling the CO₂ demands (Lackner, 2003; Kumar et al., 2010). Methods for supply and its efficacy vary with the nature of the cultivation system that is either open surface ponds or closed photobioreactors with diverse configurations. So, a closed setup is fortified with membrane transfer, gas injection, and gas exchange which offers fair CO₂ supply, gas transfer, and uniform mixing of nutrients in the cultivation systems (Yamaguchi, 1996; Carvalho et al., 2006). According to the studies extracellular enzyme, carbonic anhydrase (CA)—a zinc metalloenzyme facilitates atmospheric CO₂ uptake by microalgal cells. They are responsible for catalyzing the conversion of CO₂ to bicarbonates. These bicarbonates can easily be taken up by the microalgal cells *via* specialized cell transporters. In addition to this, depending upon the microalgal species, captured CO₂ can be stored in the cells as lipids, proteins, or carbohydrates. (Kumar et al., 2010; Mondal et al., 2017).

Two stage cultivation of microalgae

Microalgae cultivation setup defines fuel quality and quantity extracted from the feedstock (Pavithra et al., 2020). Under ideal cultivation conditions, the algal strain proliferates with a synchronous increase in the lipid content. However, the culture condition required for cellular growth differs from the one required for lipid production. Thus, the culture condition favors biomass production with a lower lipid content (Alishah Aratboni et al., 2019). In order to enhance the lipid content of the microalgal cells, other approaches must be employed, such as inducing nitrogen or phosphate limitation and increased salinity during growth. However, these conditions may reduce the cell growth rate. Therefore, a balance is required so that lipid content and cell growth occur (Qiu et al., 2017). A two-stage cultivation setup provides this balance for microalgae growth. Initially, a nutrient-rich medium is used to maximize biomass production in heterotrophic conditions, followed by inducing stress in the phototrophic mode for enhanced lipid productivity. The most commonly used two-stage strategies are based on: starvation, inducer addition, metabolic switch, and irradiation (Nagappan et al., 2019) (Yap et al., 2021).

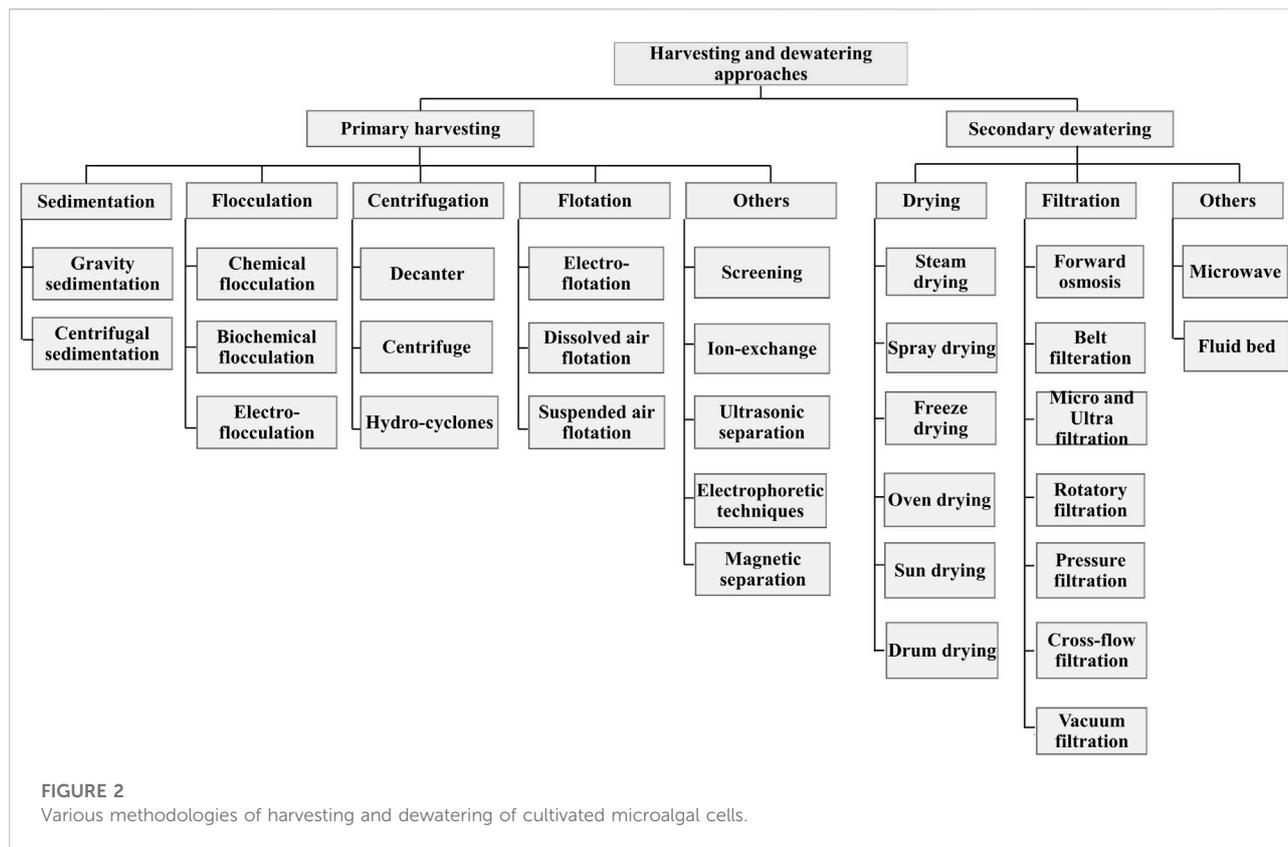
Microalgal culturing system

There are two major culturing systems 1) Suspended cell cultivation and 2) Immobilized cell activation system. The suspended cell cultivation system is highly energy intensive and time-consuming. Circular and raceway ponds are preferred for commercial algae cultivation. The commercial ponds should be shallow, with a depth ranging between 0.2 and 0.5 m so that light can penetrate to the bottom of the pond. Microalgae absorb and utilize CO₂ as a carbon source from the atmosphere. A suspended cell cultivation system has an effortless operation, low construction, operational and production cost, and functions under high CO₂ concentration. The significant disadvantages of this cultivation method include a lack of light and temperature control system, and the entire system is prone to contamination. The second method, the immobilized cell activation system, is an alternative cell cultivation method. Biofilm containing microalgal cells are attached to their specific natural surface through effective adsorption, immobilization, semipermeable membrane capture, polymer traps, and covalent joint. Some widely used immobilization methods include cell adhesion, solid surface biofilm formation, and polymer matrix cell entrapment employing polymers such as alginate and carrageenan (Vasilieva et al., 2016; Lin-Lan et al., 2018).

Unit operations in down-stream processes

Harvesting and dewatering

Microalgal biomass harvesting contributes about 20–30% of the total production cost (Molina Grima et al., 2003). A detailed



discussion on the advantages and disadvantages associated with different unit operations adopted in algal biofuels has been presented elsewhere (Kumar et al., 2022a). Since the harvested biomass is highly dilute and contains more than 99.6% of the water on a weight basis, dewatering is required for further downstream processing (Mata et al., 2012). Both harvesting and dewatering are bottlenecks for microalgae-based biofuels as they are energy intensive and incorporate the high cost of production (Peng et al., 2020). Furthermore, these methodologies are categorized as primary harvesting and secondary dewatering, as described in Figure 2.

After the synthesis and accumulation of lipids in microalgal cells, biomass is harvested, followed by lipid extraction and purification before transesterification reaction for biodiesel production. Numerous methods can be sequentially applied in the downstream process or directly to the bioreactors. Additionally, bulk harvesting is performed to separate algal biomass from the bulk suspension, accompanied by thickening protocols further to concentrate the slurry (Das, 2016). Harvesting algae cells are expensive due to their small size, which possesses terminal velocity and hinders mechanical separation. Also, these small-sized cells impart volume reduction and make the water removal process expensive. Algal cells are usually negatively charged, which keeps them in a dispersed state. Therefore, thermal drying and centrifugation can be

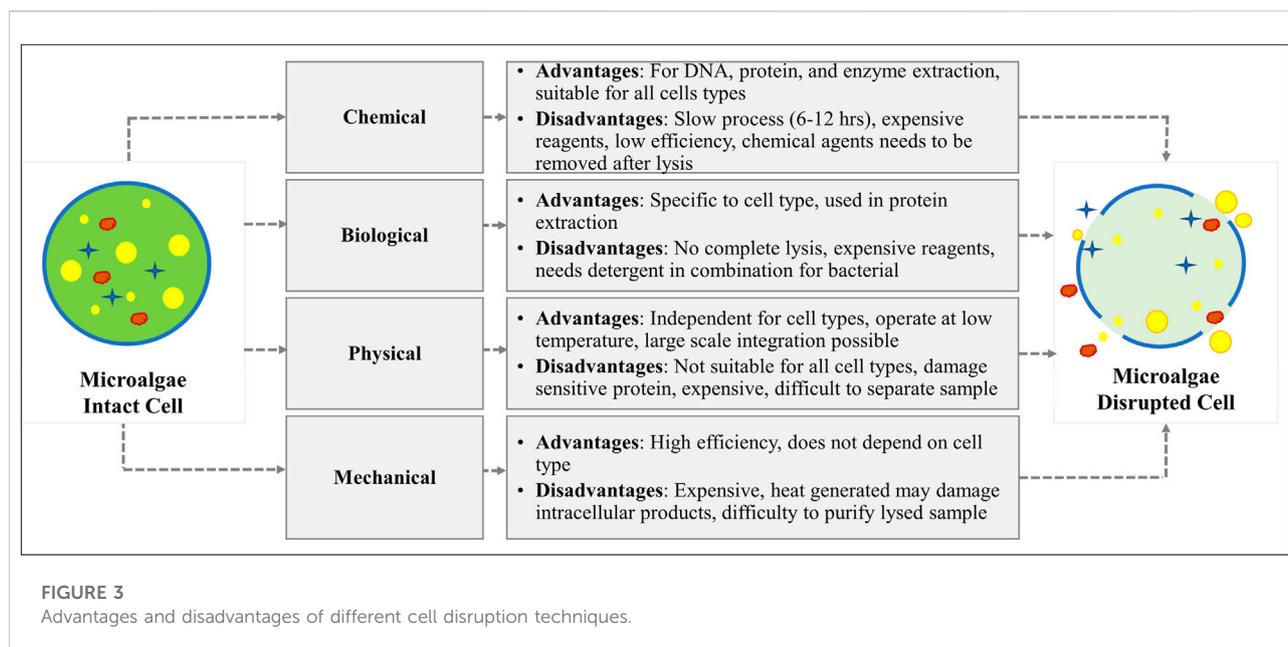
incorporated to remove water. However, thermal drying is expensive, and centrifugation demands high energy making separation expensive. For economical separation, flocculation, sedimentation, and flotation can be performed before centrifugation or filtration (Jeevanandam and Danquah, 2020).

Cell disruption

It is essential to release the lipid content produced inside the microalgal cell. Many frequently physical, chemical, biological, and mechanical cell disruption methods are incorporated for algal cells as described in Figure 3. Though algae cell walls are sometimes complex, varied chemically, and robust structurally so more energy intensive techniques are required to extract intercellular compounds which are referred as high-value products. Numerous techniques have been studied to break algal cell wall from a variety of species including the use of surfactants, ultrasound, microwave, sonication, bead milling, enzymatic lysis, high-pressure homogenization, and steam treatments (Alhattab et al., 2019). Some disruption techniques with advantages and disadvantages have been discussed below in Table 1.

Microalgal lipid extraction methods

Amongst all the extraction techniques available for the recovery of microalgal oil, an appropriate solvent should be selected to achieve enhanced yield and minimize the



production cost. A comparative analysis of lipid extractions methods is depicted in Figure 4.

Organic solvent extraction

It is a widely applied method for microalgal oil extraction. It involves organic solvent selection on the basis of toxicity, recyclability, and cost. For instance, some common solvents are -hexane, benzene, acetone, cyclohexane, and chloroform. According to some recent studies, the use of Bligh and Dyer's method which amalgamates a mixture of chloroform, methanol, and water to extract microalgal oil is the most efficient process for lipid extraction. Some studies have reported the use of the Folch method for lipid extraction which makes use of a 2:1 ratio of chloroform-methanol by volume. Currently, the Matyash method of lipid extraction is used which is comparatively more rigorous and is a modification to both of the methods. It employs methyl-tert-butyl ether (MTBE) as a solvent for lipid extraction (Kumar et al., 2015). However, their toxicity is a major concern that can be eradicated by employing cell disruption methods preceding the extraction step (Sati et al., 2019). To minimize the toxicity of chloroform, several other solvents such as ethanol, MTBE, hexane, and acetic acid esters can be employed. Recent studies have also suggested the employment of 2-ethoxyethanol (2-EE) for the enhanced extraction of lipids as compared to the traditionally used solvents (Kumar et al., 2015).

Supercritical fluid extraction

Supercritical fluids are compounds that act both as liquid or gas when exposed to varying temperatures and suppress above

their critical values. These fluids are an alternative to organic solvents and are used for the extraction of lipids, essential oils, and other functional groups from plants and microalgae (Mata et al., 2012). Based on improved solvation powers of fluids such as CO₂, water, ethane, methanol, and butane above their critical points these fluids have mass transfer properties similar to gases and solvating properties and diffusion coefficient comparable to that of liquids. Most commonly used is supercritical CO₂ (scCO₂) for oil extraction due to low critical temperature (31.1 °C) and pressure (72.9 atm). scCO₂ is a green way of oil extraction and it also permits complete characterization of extracted lipid and the resultant biofuel. However, owing to low polarity it is relatively inefficient in the extraction of polar compounds. In order to enhance the extraction capability, modifiers or co-solvents are used. Modifiers are highly polar in nature and when added in small amounts can enhance the solvation properties of scCO₂ (Mata et al., 2012). This technique offers several advantages over organic solvent extraction such as no toxicity levels, does not lead to oxidative degradation of extracts, and enabling easy separation of the bioproducts (Li et al., 2014).

Ionic liquid-mediated extraction

Ionic liquids (ILs) are non-volatile, thermally stable, and have high solvation capabilities. They are a promising alternative for toxic and volatile organic solvents. ILs are also referred to as "green" solvents (Gholami et al., 2020). ILs are salts of large asymmetric cations coupled with smaller organic or inorganic anions. ILs can be maintained in a liquid state at temperatures ranging from 0 to 140°C. ILs are the non-aqueous solutions of desirable salts. IL-methanol system has

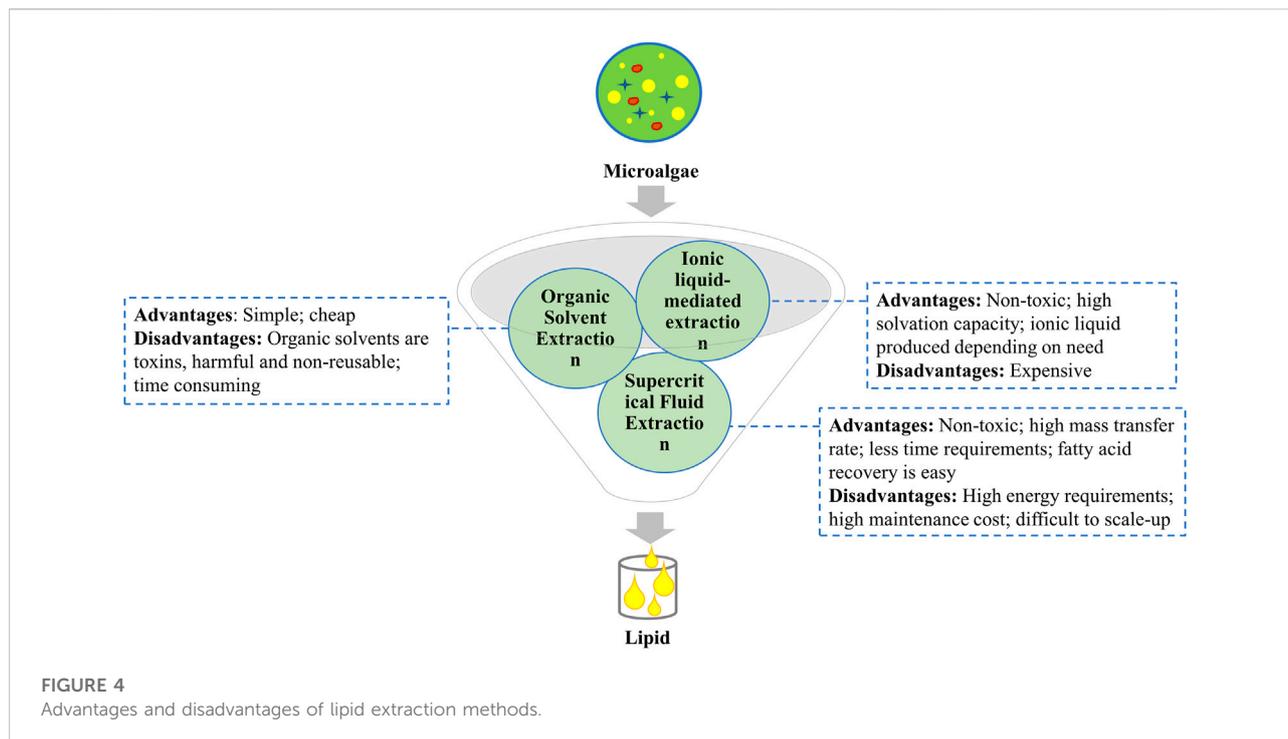
TABLE 1 Advantages and disadvantages of different cell disruption techniques.

Cell disruption type	Cell disruption technique	Mode of action	Advantages	Disadvantages	References
Chemical	Alkali and detergent lysis	Cell wall degradation <i>via</i> different surface chemical reactions	<ul style="list-style-type: none"> - Easy and simple - Suitable for all cell types 	<ul style="list-style-type: none"> - Expensive - Environment pollution - Chemical contamination - Slow process 	Islam et al. (2017)
Biological	Enzymatic	Enzymes hydrolyse the chemical bonds by binding to specific molecules in the cell wall	<ul style="list-style-type: none"> - Specific to different cell types - No harmful chemical - No harsh physical condition 	<ul style="list-style-type: none"> - Does not do complete lysis - Expensive reagents 	Venkata Subhash et al. (2017)
Physical	Ultrasonication	Acoustic streaming, liquid shear stress, cavitation effect	<ul style="list-style-type: none"> - Less extraction time - Eco-friendly - Enhanced penetration of solvents 	<ul style="list-style-type: none"> - Require energy - Difficult to scale up - High operational cost 	Liu et al. (2013)
	Microwave	Temperature, energy increase	<ul style="list-style-type: none"> - Less solvent used - Less extraction time - Simple, rapid and can be scaled-up 	<ul style="list-style-type: none"> - High temperature cause lipid oxidation - High operational cost - Easy scale up 	McMillan et al., (2013)
Mechanical	Osmotic Shock	Osmotic pressure applied for cell lysis	<ul style="list-style-type: none"> - For sensitive product extraction 	<ul style="list-style-type: none"> - Long treatment time - Cannot scale up 	Karim et al. (2019)
	Oil Press	Mechanical pressing and shear force	<ul style="list-style-type: none"> - Simple process - No requirement of solvent 	<ul style="list-style-type: none"> - Slow/time consuming - Higher quantity of sample needed - Not fit of high moisture content samples 	Karim et al. (2019)
	High Speed Homogenization	Turbulent force with hydrodynamic cavitation	<ul style="list-style-type: none"> - Simple method - Efficient extraction - Less contact time - Solvent free 	<ul style="list-style-type: none"> - Energy intensive - High heat dissipation 	Karim et al. (2019)
	Bead Milling	Mechanical pressure and shear force	<ul style="list-style-type: none"> - No solvent required - Suitable for samples with high moisture content 	<ul style="list-style-type: none"> - May not extract majority of lipid - Low efficiency for rigid cells - May cause lipid degradation - Additional process to remove undesirable products and beads 	Suarez Garcia et al. (2019)
	Steam Explosion	Sudden pressure drop	<ul style="list-style-type: none"> - Do not release any hazardous waste 	<ul style="list-style-type: none"> - Species specific 	Lorente et al. (2018)
	Pulse Electric Field	Permeabilization of cell membrane leading to pore formation	<ul style="list-style-type: none"> - No chemical used - No cell debris - Quick technique - Enhanced yield 	<ul style="list-style-type: none"> - depend on media composition 	Vorobiev et al. (2020)

been used as an alternative to imidazolium-based ILs as they are cheap and less toxic to aquatic habitat and thus, prove to be an environment-friendly method of oil extraction from microalgae (Sati et al., 2019). ILs make it possible to use varied combinations of anion and cation which in turn allows for deciding the polarity of the solvent, conductivity, hydrophobicity, and solubility (Kumar et al., 2015).

From algal lipid to biodiesel

Microalgae store excess photosynthetic products as triacylglycerol, a storage lipid. Many microalgal species can be artificially induced to accumulate an increased amount of storage lipids under nutrient deficient conditions. These storage lipids are chemically similar to vegetable oils and can be extracted and converted to fatty acid methyl ester (FAME) for their use as



biodiesel. Biodiesel has several advantages such as they are renewable, and biodegradable leading to the emission of fewer particulate matter into the environment upon its combustion (Dunahay et al., 1995). Extraction of lipid from biomass requires specific solvents having a high affinity for the microalgal lipids. Most commonly employed extraction methods for microalgal lipids involves the use of organic solvents, supercritical fluids, and ionic liquids (Amaro et al., 2011; Mercer and Armenta, 2011; Taher et al., 2011; Kim et al., 2012). Although these extraction techniques can be applied directly to the dewatered biomass, the efficiency is very less because the cell wall of microalgae prohibits these solvents from reaching the cytoplasm where these lipids are stored (Cravotto et al., 2008; Chen et al., 2009). Thus, in order to increase efficiency, lipid extraction is preceded by cell wall disruption methods (Gonçalves et al., 2013). Additionally, conversion of microalgal lipids to biodiesel is also done chemically by the following methods: 1) micro emulsification of oils; 2) direct use or blending of recovered oil; 3) transesterification/ alcoholysis; 4) thermochemical methods such as thermal cracking, pyrolysis, thermochemical liquefaction, gasification and; 5) biological conversion employing anaerobic digestion, fermentation, and photobiological oxygen production. Amongst all the known conversion methods, transesterification of microalgal oils is the most popularly employed methodology for oil separation (Suali and Sarbatly, 2012; Nandhini et al., 2022).

Transesterification

The most widely used multi-step process which converts triglycerides to low molecular weight alcohols in the presence of a

catalyst (homogenous acid or alkali) is termed transesterification. This reaction is carried out in batch mode, stirred reactors. Initially, triglycerides are converted to diglycerides, monoglycerides, and then to glycerol. Such conversion reactions are reversible and each conversion step yields approximately 1 mol fatty acid ester (Quader and Ahmed, 2017). The process constitutes high conversion rates, easy process control, mild reaction temperature, and pressure. In addition to this molar ratio of alcohol, amount of free fatty acids (FFA), type of catalyst, reaction conditions and water content are the factors that affect biomass yield during the reaction (Chozhavendhan et al., 2020). However, prolonged reaction time, complex separation stage after reaction, and batch operation mode limits the use of this method extensively.

Moreover, this reaction is susceptible to the purity of feedstock (water content should be greater than 0.5% w/w and FFA content should be greater than 1% w/w). In order to obtain a pure product, some pre-treatment steps are incorporated with this method. This not only avoids low yield but also saponification. Transesterification can either be applied to the extracted microalgal oil or directly to the biomass, known as *in situ* transesterification. This can reduce the production cost by eliminating the cost involved in the extraction of microalgal oil (Chisti, 2008).

Algal cell components recovery

The multiple product recovery majorly depends on the fractioning ability of several components and maintenance of

the quality and yield of the recovered products. The most crucial step is selecting an appropriate extraction technique and cell wall disruption to release the desired product. Additionally, optimal parameters and gentle extraction of different components manifest multiple product recovery (Gifuni et al., 2019). A large-scale microalgal culturing system currently produces extremely dilute cultures (0.08–3.6 g/L), and weather conditions and light penetration hamper their productivity. In order to minimize energy consumption in harvesting and extraction, a minimum of ~100 g/L cell concentration is required (Gifuni et al., 2019). Microalgae possess an oil yield in the range of 58,700–136,900 L/ha. With 15–77% of oil on a dry basis, making them the best candidate for biodiesel production. The exact percentage of oil content is dependent on the algal species. The average cost of algal biodiesel is USD 0.48 to 2.8 per L, while the average price of conventional biodiesel produced from vegetable oil or palm oil is USD 0.52 per L. The significant expense of algae-based biodiesel depends on cultivation and harvesting techniques. In the current scenario, crude oil prices have exceeded USD 80–100 per 100 L. Therefore, microalgal biodiesel proves to be an economical and sustainable alternative.

Algal lipid synthesis

To obtain microalgae-based fuel, species selection followed by elucidation of lipid metabolism and the connected pathway is crucial. In addition, comprehensive knowledge of carbon flux throughout cellular processes is required. Lipid catabolism plays an integral role in the metabolic processing of lipids throughout cell growth and during nutrition deficiency and is involved in lipid accumulation and homeostasis (Trentacoste et al., 2013). Analysis of various lipid classes, fatty acids, and membrane stability in transgenic strains suggested the role of lipase/phospholipase enzyme in maintaining lipid homeostasis and in membrane lipid turnover. Lipid catabolism provides acyl group for membrane recognition as a change in environmental conditions leads to the synthesis of polar lipids during dark cycles and remobilizes cell membrane when conditions get better. It is hypothesized that the knockdown of the lipase enzyme can lead to high lipid accumulation (Trentacoste et al., 2013). Understanding the microalgal lipid biosynthesis pathway paves the way to accumulate lipids in the microalgal cells, followed by the production of biodiesel and other bio-based products. In addition, genetic engineering, the addition of stimulators or repressors, or changing culture conditions may improve lipid storage inside the cells (Tang et al., 2020).

Genetic engineering approaches

Numerous enzymes inducing biochemical reactions are responsible for synthesizing Triacylglycerol (TAG) and fatty acid (FA) in microalgae cells. It has been reported that over-

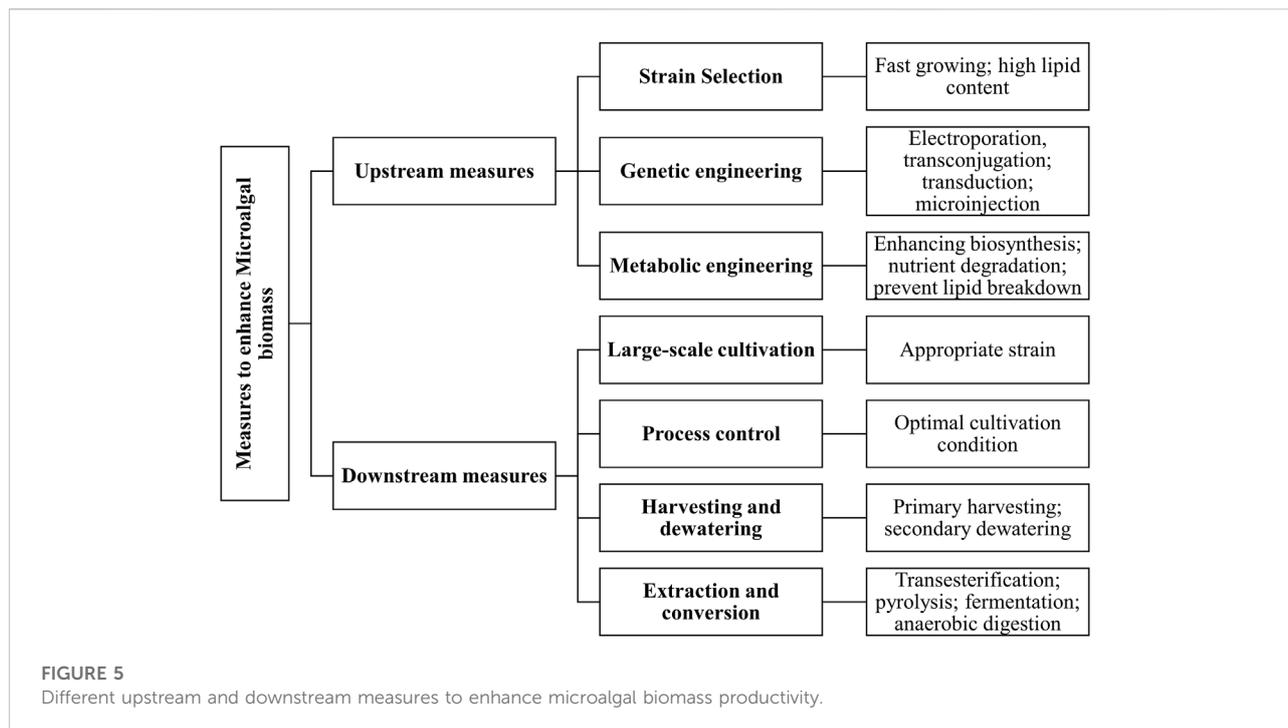
expression of these enzymes would enhance lipid accumulation (Bajguz and Piotrowska-Niczyporuk, 2013). One of the most exploited enzymes is Acetyl-CoA carboxylase (ACCCase), a multi-subunit enzyme found in the chloroplast of algae. According to some studies, overexpression of ACCCase alone does not improve lipid accumulation (Ng et al., 2017). However, over-expression of ACCCase subunit (accD) in association with malic enzyme (ME) increased lipid productivity in *Dunaliella salina* (Salama et al., 2014). Furthermore, overexpression of ME enhanced 2.5 times the lipid production in *Phaeodactylum tricorutum* without causing any negative impact on biomass productivity. Suppression of the competitive pathways such as lipid and carbohydrate catabolism are an alternative strategy to enhance oil yield (Abomohra et al., 2020). Carbohydrate metabolism is an essential microalgal pathway for storing carbon and producing starch. Therefore, the knockdown of starch metabolism may alter the carbon pathway toward lipid synthesis.

Lipid accumulation strategies

TAG accumulation can remarkably be enhanced by introducing environmental stress such as nitrogen scarcity. Limiting iron, sulfur, zinc, phosphorus, and salt may also trigger lipid and starch synthesis in microalgae. Major steps involved in lipid biosynthesis are: 1) fatty acid production in plastids; 2) glycerol-lipid formation in the endoplasmic reticulum (ER); 3) packaging of glycerol-lipids into oil bodies. Fatty acid synthesis is catalyzed through ACCCase, yielding malonyl-coenzyme A from acetyl-coenzyme A and bicarbonate (Maity et al., 2014). ACCCase through its two catalytic centers biotin carboxylase (BC) and carboxyl transferase (CT), catalyzes irreversible carboxylation of acetyl-CoA to produce malonyl-CoA, which is essential for fatty acid biosynthesis (Cronan and Waldrop, 2002; Fan et al., 2014).

Nitrogen starvation

Microalgal biomass is initially produced under nitrogen-rich conditions, while TAG accumulation is induced by creating nitrogen deficiency (Peng et al., 2020). Nitrogen limitation reduces metabolism and leads to excess photon dissipation as heat or fluorescence. The percentage reduction in photosynthetic capacity under stressful situations is species-specific and influences the amount of available energy for the synthesis of TAG. Also, it causes a reduction in photosynthetic proteins, pigments, and centers for photosystem reaction in order to minimize overexcitation-associated damage (Chisti, 2008; Kumar et al., 2010). For instance, in *Neochloris oleoabundans* only 8.6% of the generated electrons were used for TAG biosynthesis and the rest of the energy got dissipated in photosystems or utilized in catabolic processes (Trentacoste et al., 2013). Thus, it is highly recommended to choose a species that retains the high photosynthetic capability and



TAG productivity under nitrogen depletion (Klok et al., 2014). TAG content can further be improved by employing techniques such as selective cell sorting, targeted engineering, and random mutagenesis (Remmers et al., 2018). It has been studied that the efficiency of bulk lipid production is less due to low solar-lipid conversion. Several attempts have been made to increase solar-lipid conversion efficiencies such as rational strain selection, improvement of selected strain, and process condition optimization (Yamaguchi, 1996; Carvalho et al., 2006; Mondal et al., 2017).

Lipid biosynthesizing enzymes

A variety of enzymes including acetyl-CoA synthetase (ACS), Acetyl-CoA carboxylase (ACCase), acyl-ACP esterase (AAE), diacylglycerol acyltransferase (DGAT), D5-elongase (D5 Elo), D6-elongase (D6 Elo), glycerol-3-phosphate-dehydrogenase (G3PDH), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAT), phosphatidic acid phosphatase (PAP) is involved in lipid biosynthesis (Klok et al., 2014). According to some experimental pieces of evidence, overexpression of cytosolic ACCase in *Chlamydomonas reinhardtii* lead to a 2-fold increase in starch content and a 2.4-fold higher accumulation of TAG (Rengel et al., 2018). Similarly, overexpression of Acetyl-CoA carboxylase (ACCase) in *Chlamydomonas reinhardtii* increased FA synthesis by approximately 56%. It is highly possible that the homeostatic regulation as in the case of redox activation of ACCase under

stressful conditions ensures sufficient synthesis of fatty acyl groups (Shahid et al., 2020). Overexpression of AAE, led to fatty acid profile changes in *Phaeodactylum tricoratum* and *Chlamydomonas reinhardtii* whereas an increased lipid content was observed only in *P. tricoratum* (Dunahay et al., 1995; Lin et al., 2018). While, overexpression of type 2 DGAT in *P. tricoratum* leads to only 35% increase in TAG levels (Muñoz et al., 2019) and overexpression of some type 2 DGAT enzymes results in an increased TAG yield in *C. reinhardtii* (Xue et al., 2015; Sharma et al., 2021). In *Chlorella minutisma*, it has been studied that heterologous expression of yeast-derived LPAT, PAP, GPAT, DGAT, and G3PDH enzymes leads to a two-fold increase in TAG content as compared to overexpression of any one of the genes (Han et al., 2021). Heterologous expression of gene D5 Elo and D6 Elo from *Ostreococcus tauri* into *P. tricoratum* resulted in an increase of docosahexaenoic acid (DHA) (Klok et al., 2014).

Challenges to sustainable production of algal biodiesel production

Numerous biological barriers are still present for industrial-scale algal biodiesel production have been discussed. Figure 5 describes various upstream and downstream strategies to enhance microalgal biomass production.

- Organism survival: Current systems have been unable to maintain the best laboratory organisms under field conditions. Laboratory culture when exposed to environmental conditions

gets contaminated by indigenous organisms found in the environment (Gressel, 2007).

- Carbon dioxide enrichment: Carbon dioxide levels above 5% slow the growth of higher plants and animals, yet many algae and cyanobacteria grow happily when “aerated” with 100% carbon dioxide or directly with ca. 14% CO₂ flue gas from power generators. Still, the response of algae to added carbon dioxide is not as good as it could be, and there is a place for improvement of carbon assimilation (Gressel, 2007).

- Light penetration: For autotrophic cultivation of algae light is an important parameter. Its intensity decreases with the increase of water depth. Moreover, the photosynthetic efficacy of the species is highly dependent on the source of light. In open ponds, the shallow depths provide a high surface area with efficient mixing and light penetration. It can be calculated by Beer-Lambert’s law (Wang et al., 2015).

- Environmental dependence: The high yields attained in ponds is usually seasonal. Therefore, algal growth is a function of temperature. Some species do not grow well in cold temperatures while some face difficulty at high temperatures. Additionally, they require a special cooling unit (Gressel, 2007).

- Limitations: According to the literature, a major drawback of algae-based biodiesel production is the display of contrasting features such as high biomass productivity accompanied by low lipid accumulation, or high lipid accumulation with low biomass productivity. The growth rate of oleaginous algal species is often slow (Shokravi et al., 2020). It is well established that in order to produce biodiesel economically, a balance is required to be maintained between biomass productivity and lipid accumulation.

- Challenges associated with biomass production:

- 1) Daily and seasonal variations of sunlight during cultivation.
- 2) High levels of dissolved oxygen in closed PBRs inhibit photosynthesis in microalgal cells.
- 3) High oxygen concentration and sunlight intensity cause photo-oxidative damage to microalgal cells.

The maximum tolerance of dissolved oxygen for microalgal cells should not exceed 400% of air saturation in PBRs (Chisti, 2008).

Strategies to make microalgal-based biodiesel economical

The microalgal lipids can be classified into two major groups: polar lipids (phospholipids and glycolipids) which constitute about 41–92% of the total lipid content and non-polar or neutral lipids (sterols and free fatty acids) having 5–51% of lipid content (Tang et al., 2020). Genetic and metabolic engineering can (Chisti, 2008) enhance the photosynthetic efficiency and increase the biomass yield on

light, increase biomass growth rate, elevate the oil content in algal biomass, and improve temperature tolerance of algae so that there is a reduced need for cooling consequently reduces the cost of production, and provide tolerance against photoinhibition. Therefore, elucidating the genetic makeup can improve the strain yield.

Abiotic manipulations

Increased levels of polar lipids inside the cell may lead to a reduction in permeability of membranes and also lead to reduced fluidity which limits an excessive flow of sodium and chloride ions into cells. A high concentration of neutral/non-polar lipids enables microalgal cells to resist osmotic pressure by enhancing membrane rigidity. The addition of salts such as sodium bicarbonate increases the lipid content of cells by enhancing the photosynthetic capability and nitrogen utilization by microalgal cells (White et al., 2013). While, ferric, cupric, zinc and chromium salts, have been reported to increase lipid content in some of the algal species (Sharma et al., 2012). However, these salts cannot be used on a large scale due to their extreme harmful environmental impact. Sodium chloride supplementation can be used more effectively and safely for enhancing lipid accumulation in microalgal cells (Xia et al., 2013). Table 2 describes various attempts to enhance algal productivity by employing various abiotic strategies.

Biotic manipulations: GM algae

In recent times, researchers have proposed a number of strategies for increasing lipid content in microalgal cells such as genome manipulation, random mutagenesis, metabolic engineering, blocking carbohydrate metabolism pathways, and lipid degradation which competes with the biosynthesis of lipids. As a result, engineered microalgal strains have high lipid content but slow growth rates. Amongst all the devised strategies, blocking competitive pathways has been recognized as a potential alternative to increase lipid yield (Sun et al., 2019). Researchers have suggested that inhibition of enzymes responsible for converting lipids into free fatty acids may help in improving lipid accumulation. Lipid catabolism provides acyl groups that help in membrane reorganization and acknowledgment of the photosynthetic system. Some recent studies depicted that knockdown of genes associated with lipid catabolism has a lesser impact on the microalgal growth rate as compared to the strains having disrupted carbohydrate pools. Therefore, lipid catabolizing enzymes are the best candidates for improving lipid content through knockout mutations (Nguyen et al., 2020). Table 3

TABLE 2 Abiotic strategies to enhance algal productivity.

Strategies	Algae and growth conditions	Change in biomass productivity (BP)/Cell growth	Change in biochemical components	Possible reasons behind outcome	Remarks	References
Nanoemulsion (NE)-based growth media (1% silicone oil (SE) and 1% paraffin oil (PE) in BG-11)	<i>Chlorella pyrenoidosa</i> (LI: 46.5–50 $\mu\text{mol m}^{-2} \text{ s}^{-1}$; LP: 12:12 h; T: $25 \pm 1^\circ\text{C}$; shaking at 150 rpm)	3.20 g L^{-1} (PE), 2.75 g L^{-1} (SE) as compared to control 1.03 g L^{-1} in BG-11	Increase in Chlorophyll a synthesis by 76% and 53%; lipid content increased to 23.6% and 26.8% as compared to control 18.05%. Carbohydrates increased from 13.6% to 18.9% and 17.20% for SE and PE respectively.	Effective CO_2 supplementation for microalgal growth media. NE acts as CO_2 delivery agent to algal growth medium.	Biomass productivity and lipid both increased.	Nigam et al. (2021)
Phytohormones- Auxin, Indole-3-acetic acid (IAA) and Brassinosteroids (IAA induced BRs in algal cells)	<i>Chlorella vulgaris</i>	—	Increase in total chlorophylls, protein, and carbohydrate.	BRs could prevent loss of photosynthetic pigments by an activation of enzymes participating in chloro- phyll biosynthesis or an induction of their synthesis	BRs promote photosynthesis and growth by positively regulating synthesis and activation of a variety of photosynthetic enzymes	Bajguz and Piotrowska-Niczyporuk, (2013)
Phytohormones - Auxin, Indole-3-acetic acid (IAA) and Diethyl aminoethyl hexanoate (DAH)	<i>Scenedesmus obliquus</i> GU732418 (BBM media; Shaking at 150 rpm; T- and 27°C ; LI-45–50 $\mu\text{mol photon m}^{-2}/\text{s}$)	1.9 and 2.5 fold increase in growth (Cell counts) due to IAA and DAH respectively.	Increase in lipid content was reported for both IAA and DAH.	Phytohormones stimulate algal growth.	At higher concentrations, the proportion of unsaturated fatty acids increased while saturated fatty acids decreased.	Salama et al. (2014)
Phytohormones -Jasmonic acid (JA)	<i>Chlorella vulgaris</i> (Trebouxiophyceae) (F/2 medium; T- $24 \pm 2^\circ\text{C}$; 80 $\mu\text{mol m}^{-2}\text{s}^{-1}$)	Increase in cell growth	Lipid productivity enhanced.	Phytohormones stimulate algal growth.	Induced production of saturated SFA and MUFA without compensating the production of PUFA.	Jusoh et al. (2015)
Phytohormones, Cytokinin- Kinetin (K) and Zeatin (Z)	<i>Acutodesmus obliquus</i> (BG-11 media)	Biomass productivity increased by 50% (K) and 60.7% (Z).	Lipid productivity increased upto 63–65% for both K and Z.	Cytokinin, by enhancing enzymatic activity, improves the photosynthetic efficiency and carbon fixation.	Z was found to be more effective than K for increase in BP.	Renuka et al. (2017)
Phytohormones (PHs), Kinetin, Indole-3-butyric acid (IBA), Methyl jasmonate (MJ), and Antioxidants- Butylated hydroxyanisole (BH)	<i>Chlorella protothecoides</i> (Tris-acetate-phosphate (TAP) medium; LP-14:10; T- 27°C ; LI-4,800 Lux)	Significant increase in biomass productivity for each phytohormone.	Increase in lipid content and alpha-linolenic acid (ALA). An increase of 26.5% and 60.5% in ALA content by kinetin and BH respectively.	PHs (Cytokinin/ Auxin) play a stimulatory role for algal growth.	An increase in biosynthesis of saturated fatty acids and decrease in PUFA was observed.	Parsaeimehr et al. (2017)
Monoethanolamine (MEA)	<i>Chlorella fusca</i> LEB 111 (BG-11 growth media)	Increase in Biomass productivity.	Enhanced accumulation in protein and lipid content.	It enhances CO_2 biofixation.	Chemical absorption of CO_2 with MEA solutions is a promising method.	Rosa et al. (2019)
Phytohormones- IAA, and Diethyl		Increase in biomass productivity by 22%	Increase in lipid productivity by 49%	Phytohormones, under N-limitation,	Amount of saturated fatty acid (SFA) and	Giridhar Babu et al. (2017)

(Continued on following page)

TABLE 2 (Continued) Abiotic strategies to enhance algal productivity.

Strategies	Algae and growth conditions	Change in biomass productivity (BP)/Cell growth	Change in biochemical components	Possible reasons behind outcome	Remarks	References
aminoethyl hexonate (DA-6) under Nitrogen limitation	<i>Chlorella sorokiniana</i> (BG-11 growth media)	and 43% for IAA and DA-6 respectively as compared to control.	and 84% for IAA and DA-6 respectively as compared to control.	cause concurrent increase in biomass and metabolite productivity.	monounsaturated fatty acid (MUFA) increased.	
Two stage cultivation; NaCl based lipid induction	<i>Scenedesmus obtusus</i> XJ-15 (BG-11 growth media)	Increase in biomass by 52% as compared to the first stage.	Increase in lipid content by 21.6% as compared to the first stage. Final lipid content- 47.7%.	IAA and DA-6 probably induced the activity of antioxidants enzymes, which protect cells from damages caused by abiotic stresses.	Introduction of NaCl enhanced accumulation of polar and neutral lipid or TAGs to counter salinity stress caused by it.	Xia et al. (2013) At increased polar lipid accumulation, the cell membrane permeability and fluidity decrease. It prohibits an excessive flux of NaCl ions into cells.
NaCl based lipid induction	<i>Monoraphidium dybowskii</i> LB50	Biomass decreased slightly.	Total lipid and neutral lipid contents increased to 41.7% and 17.48% respectively.	NaCl promote the conversion of Glycolipid into neutral lipids and phospholipids.	NaCl increase the neutral lipid accumulation in algal cells.	Yang et al. (2014)
NaCl-based lipid induction	<i>Tetraselmis</i> sp. KCTC12432BP in Saline seawater	At optimum concentration, BP increases by 89 and 152% in closed and open cultures as compared to regular salinity medium.	369% increase in lipid productivity in case of close culture cultivation setup.	Carbon fixation rate enhanced.	At higher salinity, contaminations from other agents were reported to be significantly reduced.	Park et al. (2018)
Phytohormones under N-limitation; Auxin -IAA, gibberellin-gibberellic acid (GA) and Cytokinin-K	<i>Chlorella sorokiniana</i>	An increase of 45.55%, 35.94% and 37.37% in BP on supplementation with IAA, GA, and K respectively.	An increase of 56.02%, 37.45% and 53.50% in LP on supplementation with IAA, GA, and K respectively.	IAA and K caused increased cell division and cell elongation resulting into enhanced growth and biomass productivity. N-stress caused enhanced lipid accumulation.	Upregulation of rbcL and accD gene expression was observed for each IAA, GA, and K.	Guldhe et al. (2019)
Synergistic effect of NaCl and CaCl ₂ stresses	<i>Chlamydomonas reinhardtii</i> (C growth medium)	Algal growth rate decreased.	Lipid content increased upto 73.4% as compared to control.	High salinity causes osmotic stress, ion (salt) stress, and oxidative stress leading to enhanced lipid accumulation. Ca ²⁺ seems to positively affect lipid synthetic pathways.	Upregulation of GPDH, LPAAT, and DAGAT genes was observed.	Hang et al. (2020)
Salinity stress	<i>Dunaliella salina</i> KSA-HS022	Decrease in BP	Enhancement in lipid accumulation.	Oxidative stress results in growth inhibition. High salinity stress positively affected the lipid biosynthesis pathways.	Fatty acid profile varies with salinity of the medium.	Abomohra et al. (2020)
Nitrogen starvation	<i>Chlorella zofingiensis</i> (BG-11: LI- 150 l mol m ⁻² s ⁻¹ ; T- 25 ± 1°C in an airlift reactor)	Decrease in BP (On 10th day of cultivation, BP for N-Starved 0.7 g/L while for N-replete- 3.1 g/L)	Increase in synthesis rate of starch (~4-fold) and lipid (~7-fold).	—	Algal cells tend to lower the degree of unsaturation of fatty acids in response to N-starvation.	Zhu et al. (2014)
N/P ratio		Increase in BP.				Choi and Lee, (2015)

(Continued on following page)

TABLE 2 (Continued) Abiotic strategies to enhance algal productivity.

Strategies	Algae and growth conditions	Change in biomass productivity (BP)/Cell growth	Change in biochemical components	Possible reasons behind outcome	Remarks	References
	<i>Chlorella vulgaris</i> (Jaworski's medium; LP-16:8; T: 23 ± 1°C)		Increase in lipid accumulation.	Increasing the N/P ratios up to 10 continuously increases the biomass production.	Biomass production is highly dependent on the N/P ratios	

describes various examples of genetic and metabolic engineering that have been carried out to enhance algal lipid productivity.

Metabolic engineering

Metabolic engineering involves the addition or deletion of genes in order to optimize the genetic and regulatory processes of an organism for altering metabolic properties in a predetermined way such as to increase the production of certain metabolites in an organism (Sun et al., 2019). DGAT enzyme involved in TAGs biosynthesis is one of the most crucial enzymes in the pathway as it catalyzes the last step of the lipid synthesis pathway which is the esterification of diacylglycerol yielding triacylglycerol. Overexpression of DGAT can lead to high lipid productivity in GM microalgal cells. Alternative strategies for enhancing microalgal lipid accumulation focus on blocking metabolic pathways competing with lipid production such as starch synthesis, oxaloacetate, or phospholipid biosynthesis pathways. Till now more than 40 microalgal species have been genetically manipulated expressing different stability and efficiencies (Díaz-Santos, 2019).

Enzymes such as glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAT), and diacylglycerol acyltransferase (DGAT) of the Kennedy pathway are responsible for catalyzing key steps in the formation of TAGs in algal cells. Overexpression of these key enzymes in strains may increase TAG contents in the cells (Muñoz et al., 2019). Overexpression of malic enzyme (ME) also leads an enhanced lipid production in the microalgal cells as ME catalyzes the irreversible oxidative decarboxylation of malate to yield pyruvate, NADH, and CO₂. NADH is required as reducing power for fatty acid biosynthesis. Morphological changes have also been observed in transgenic algae with shorter and thicker cells containing large oil bodies (Xue et al., 2015).

Genetic engineering

Despite all the ideal properties of microalgae, the use of genetic engineering and synthetic biology for genome modification is still a challenge. Genome manipulation helps in improving microalgal strains followed by enhanced biomass

yield, productivity, and efficiency of microalgal-based industries. Recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9), an advanced genome manipulation technique is gaining importance in manipulating microalgal cells for improved atmospheric CO₂ fixation which eventually would lead to efficient biofuel production. This approach is highly efficient, economical, and environment friendly too (Godbole et al., 2021). The recent methods of genome editing and advancements in metabolic engineering and synthetic biology enable us to combat issues associated with traditional genetic engineering approaches such as instability of transgenes, ineffective expression, and delivery system for effective delivery of genetic material exogenous in nature and selection and screening of recombinants (Sun et al., 2019).

The current strategies employed to improve lipid biosynthesis or to modify the fatty acid profile of microalgal cells includes: 1) fatty acid beta-oxidation elimination, 2) increment in reducing power supply, 3) use of plant-based thioesterase for optimizing fatty acid chain length and 4) thioesterase overexpression to minimize feedback inhibition due to increased levels of acyl-ACP. Genetic engineering tools such as Zinc finger nucleases, CRISPR/Cas9, and chloroplast transfection should be preferred for overexpressing and blocking the genes of interest (Shahid et al., 2020).

Genetic engineering so far has been applied to the following aspects to increase lipid content in microalgal cells:

- 1) Overexpressing enzymes responsible for lipid biosynthesis
- 2) Blocking or inhibiting competitive pathways such as inhibiting beta-oxidation, inhibiting starch synthesis, suppressing the activity of phosphoenolpyruvate carboxylase, and repression of phospholipid biosynthesis.
- 3) Constitutive expression of lipid biosynthesis transcription regulators in microalgal cells
- 4) Metabolic engineering to enhance lipid productivity (Morales et al., 2021).

Algae and cyanobacteria for third-generation biodiesel need transgenic manipulation to deal with “weeds”, light

TABLE 3 Genetic engineering strategies to enhance algae productivity.

Strategies	Algae	Change in biomass productivity (BP)/Cell growth	Change in biochemical components	Possible reasons behind outcome	Remarks	References
Upregulation in Malic enzyme (ME) gene	<i>Phaeodactylum tricornutum</i>	Slight reduction in growth.	Accumulation of neutral lipid increased by 2.5-fold. Total lipid content reached to 57.8% of dry cell weight	Pyruvate metabolism and carbon fixation improved due to overexpression of the targeted gene.	Overexpression of <i>Phaeodactylum tricornutum</i> Malic enzyme (PtME) gene along followed by N-stress further enhanced total lipid content.	Xue et al. (2015)
Overexpression of glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase, (LPAT), and diacylglycerol acyltransferase (DGAT)	<i>Neochloris oleoabundans</i>	Insignificant effect on growth in case of single gene overexpression. Simultaneous expression of all three led to reduction in BP and PS activity.	Enhanced lipid production.	These genes are involved in Kennedy pathways catalyzes key TAG synthesis steps in algal cells. Their overexpression leads to higher accumulation of lipids in cells.	Under N-stress, higher lipid accumulation in transformed cells.	Muñoz et al. (2019)
Targeted knockout of gene Cre01.g000300 gene encoding ELT using CRISPR-Cas9	<i>Chlamydomonas reinhardtii</i>	Insignificant changes in cell concentration as compared to the wild strain.	Accumulation of lipid increased.	Suppression of catabolic enzymatic activity enhanced cellular growth and lipid productivity.	'Cre01.g000300' gene encodes for Esterase/lipase/thioesterase (ELT) family.	Nguyen et al. (2020)
Knockdown of a multifunctional lipase/ phospholipase/ acyltransferase gene, 'Thaps3_264,297'	<i>Thalassiosira pseudo-nana</i>	BP remains unchanged as compared to wild type.	Upto 4.1 folds higher lipid content as compared to wild type.	Disrupting lipid catabolism could increase lipid accumulation without negatively affecting growth.	Lipid catabolism involves the release of free fatty acids by lipases and the subsequent breakdown of these fatty acids through β -oxidation.	Trentacoste et al. (2013)
Overexpression of G3PDH, GPAT, LPAAT, PAP, and/or DGAT genes	<i>Chlorella minutissima</i>	—	Upto 2 folds higher lipid content as compared to wild type.	Overexpression of G3PDH, GPAT, LPAAT, PAP, and/or DGAT increased the storage lipid content	Multiple-gene approach can yield maximal enzymatic activity and thus maximal lipid synthesis.	Hsieh et al. (2012)
Overexpression of the enzymes thioredoxin reductase (TRXR), aldehyde dehydrogenase (ALDH), glutathione peroxidase (GPO), and glucose-6-phosphate dehydrogenase (ZWF)	<i>Schizochytrium</i> sp.	Upto 18.5% increase in dry cell weight as compared to wild type.	Lipid (80.9%) and DHA (114.5%) accumulation increased.	Increased level of Reactive Oxygen Species (ROS) and Reactive Aldehydes led to reduction of cell growth, and increase in lipid accumulation	Increment in generation of ROS and RA was due to oxidation and peroxidation of intracellular lipids.	Han et al. (2021)
Overexpression of diacylglycerol acyltransferase (DGAT) genes	<i>Phaeodactylum tricornutum</i>	Slight effect on algal growth.	More than 2 folds increase in TAG and total lipids.	Chloroplast and endoplasmic reticulum-derived diacylglycerol converted into TAGs due to overexpression of DGAT genes.	Hyper-accumulation of TAGs without any compromise in algal growth.	Zhang et al. (2021)
Overexpression of Carbonic anhydrase (CA)	<i>Chlorella sorokiniana</i> (CS) and <i>Chlorella vulgaris</i> (CV)	Biomass productivity increased.	2.2 folds increase in lipid accumulation.	CA facilitated enhanced rate of CO ₂ sequestration and their fixation.	Carbon flux was diverted, due to reduction in phosphoenolpyruvate carboxylase, towards lipid synthesis.	Lin et al. (2018)

penetration, photoinhibition, carbon assimilation, etc. In order to scale up microalgal production industrially, less than 6 million hectares of land which is less than 0.4% of arable land is required across the world for fulfilling the current demands of fuel (Popp et al., 2014). Recombinant

DNA technology can be used to introduce the gene of interest into the host cells through a transformation in order to manipulate physiological pathways or to produce commercially viable compounds. Currently, genetic manipulation of microalgae depends on random

mutagenesis accompanied by screening and selection (Godbole et al., 2021).

Cyclotella cryptica and *Navicula saprophila*, two diatoms were genetically modified by *E. coli* neomycin phosphotransferase gene IZ (nptII) gene. Expression of nptII gene of bacterial origin in diatoms was done by using promoter and terminator sequences of ACC gene from *Cyclotella cryptica* T13L Reimann, Lewin and Guillard. Microprojectile bombardment was used for the introduction of vectors into *Cyclotella cryptica* and *Navicula saprophila* NAVICI Lange-Bertalot and Bonik. Putative transformants were screened and selected G418, and production of neomycin phosphor- based on their ability to grow in the presence of anti-transferase protein by transformed cells was confirmed by western blotting. It was observed that the foreign DNA got integrated into one or more random sites of the transformed algal cells, often in form of tandem repeats. This study was the first study on the reproducible, stable genetic transformation of a chlorophyll-c harboring alga (Dunahay et al., 1995).

In recent studies, it has been demonstrated that genetically engineered cyanobacteria have enhanced carbon-concentrating mechanisms (CCMs) which enables them to secrete heterologous carbonic anhydrases (CAs) (Dunahay et al., 1995). Genetic manipulations in eukaryotic microalgae are difficult due to the lack of stability in the expression of transgenes, the presence of highly efficient endogenous promoters, and the absence of complete genetic information (Lin et al., 2018). Researchers have demonstrated that CGI-58 homolog knockdown in *Thalassiosira pseudonana* resulted in increased polar lipid concentrations and membrane integrity during silicon starvation. This study elucidated the role of the CGI-58 enzyme in the turnover of the membrane during nutrient starvation-induced lipid accumulation. This enzyme is also beneficial for the conversion processes such as hydrothermal liquefaction. Similarly, CGI-58 homolog knockdown in *Arabidopsis thaliana* resulted in an increased concentration of polar lipids (Muñoz et al., 2019). It has been demonstrated recently that although about 60% of the accumulated TAGs under nutrient-deficient conditions are derived from *de novo* lipid synthesis pathways in the case of *Phaeodactylum tricoratum*, a considerable amount of phospholipids turnover also occurs (Xue et al., 2015). Scientists have also proposed a secondary TAG biosynthesis route involving turnover of intracellular membrane employing the enzyme phospholipid diacylglycerol acyltransferase (Han et al., 2021; Sharma et al., 2021). SYTOX staining results indicate that phospholipids of the plasma membrane may also act as a source of acyl groups required for TAG biosynthesis and lipid remodeling may also occur during lipid accumulation (Trentacoste et al., 2013).

Agrobacterium tumefaciens- mediated transformation has shown stability in the case of *Chlorella sorokiniana* as compared to other transformation methods which

demonstrate transient stability (Sharma et al., 2021). Overproduction of docosahexaenoic acid (of oxidative defense pathway) by means of genetic engineering in *Schizochytrium* sp. is known to promote DHA production, lipid yield, and cell robustness. Attempts have been made to enhance the oxidative stress defense system of *Schizochytrium* sp. to reduce or lower lipids and DHA oxidation (Han et al., 2021). There have been several attempts to express sequences encoding GPDH, GPAT, LPAT, PAP, DGAT, and PDAT, which can prove to be helpful in the manipulation of metabolic pathways leading to biodiesel production. These methods have been reported to increase storage lipid content by two-fold in *Chlorella minutissima* UTEX 2219 as compared to wild strains (Hsieh et al., 2012).

Drawbacks of GM microalgae

In recent times, there has been a switch towards genetic engineering approaches to escalate lipid and biomass productivity. The possibilities of non-GM approaches should not be forgotten due to strict regulatory procedures over GMOs, environmental safety concerns, commercial, and ethical constraints associated with GMOs. One of the major challenges associated with the use of GM microalgal strains is containment. GM strains must be prevented from their loss into the environment and to mitigate unknown consequences associated with GM strains such as the risk of transgene flow through horizontal gene transfer (HGT) to other species and loss of GM strains caused due to predation or competition with wild strains while mass culturing (Driver et al., 2014).

Biorefinery approach or zero waste approach

A biorefinery is established to produce biofuels and a variety of chemicals from algal biomass via bioprocessing and other cost-effective methodologies. It can utilize low energy and produce zero waste. Algal biomass can not only harness biofuel but also other economically viable products including pigments, polyhydroxybutyrate (PHB), and starch which can be used for different interventions (Chandrasekhar et al., 2022). Additionally, investigators have identified the bio-remedial properties of algae. Their capturing capability of heavy metals and toxic chemicals from wastewater makes them a great fit for bioremediation. Algae-based biorefinery has the potential to incorporate numerous technologies to produce green fuel including biodiesel, bioethanol, gasoline, methane, and other high-value compounds like fats, eicosatetraenoic acid and docosahexaenoic acid, dyes/pigments (β -carotene/lutein/astaxanthin), antioxidants and carbohydrates. Theoretically, biorefinery would cultivate algae on a farm followed by biodiesel, bio-oil, bioethanol, biomethane, and biohydrogen

TABLE 4 Biorefinery based algal products.

Algae sp.	Product	Applications	Synthesis/ any other information	Product features	References
<i>Pseudochoricystis ellipsoidea</i>	Fermentation nutrient source	high efficiency as to lactic acid and ethanol production	Hydrolysis (121°C for 20 min) + calcium carbonate. Hydrolysate: 15,000 rpm; 10 min	Sulphuric Acid is used for hydrolysis.	Gao et al. (2012), Rajeswari et al. (2022)
<i>Nannochloropsis salina</i>	Filler in biocomposite fabrication with PVA.	3D printing.	LEA + PVA (Mix at 400 rpm; 5 min) followed by sonication and in the end Heating:90°C at 350 rpm; 1 h.	enhanced the thermal stability	Tran et al. (2018)
<i>Ettlia</i> sp.	nutrient	Growth medium for microalgae	biomass + methanol (1:3 w/w) heated at 90°C. 1% sulfuric acid (v/v) was added, followed by oven dry (60°).	low-cost nutrient; sugar factory waste water and LEA make powerful growth medium	Moon et al. (2014); Mohammadi et al. (2021)
<i>Acutodesmus dimorphus</i>	Ag NP	Antioxidant Activity	10 ml LEA extract: 90 ml AgNO ₃ ; Room Temp; 24 h	2–20 nm/ Spherical	Chokshi et al. (2016)
<i>Saccharina japonica</i>	Ag NP	Antimicrobial Activity towards <i>E. coli</i> and <i>S. typhimurium</i>	50 ml LEA extract: 50 ml AgNO ₃ (1 mM); 200 rpm	14.77 nm	Sivagnanam et al. (2017)
<i>Micractinium</i> sp., <i>Scenedesmus</i> sp., <i>Chlorella</i> sp., pennate diatoms and <i>Chlamydomonas</i> sp.	Methane	Bioenergy	Anaerobic digestion	Hydrothermal pre-treatment and co-digestion enhance methane production	Bohutskyi et al. (2019)
<i>Chlorella</i> sp.	Antibiotic	Bioremediation	For Non-living biomass: light and air source is removed	71.19% removal efficiency for Cephalixin	Angulo et al. (2018)
<i>Auxenochlorella protothecoides</i>	Methane and Nutrients	Bioenergy and fertilizer	Semi-continuous Anaerobic digestion	Reduce the fertilizer cost up to 45% and enhances biofuel production	Bohutskyi et al. (2015)
<i>Scenedesmus obliquus</i>	levulinic acid	Chemical used for microalgae feedstocks and thermochemical conversion.	Acid-catalyzed thermochemical conversion	—	Jeong and Kim, (2020)
<i>Dunaliella tertiolecta</i>	Bioethanol	Bioenergy	Chemo-enzymatic saccharification and fermentation	Enzymatic saccharification: AMG 300L	Lee et al. (2013)
<i>Chlorella variabilis</i> and <i>Lyngbya majuscula</i>	Fertilizer	Biofertilizer	Ovendried de-oiled biomass in a muffle furnace at 575 ± 25°C	Fertilizer for <i>Zea mays</i> L.	Maurya et al. (2016a)
<i>Chlorella vulgaris</i>	Growth media and Lipid production.	Nutrient and Biofuel production	Cell disruption: cellulose Hydrolysis; 55°C for 10 h	Enhances productivity of lipid as well as <i>Chlorella vulgaris</i>	Ma et al. (2014)
<i>Lyngbya majuscula</i>	Growth Media for <i>Chlorella vulgaris</i>	Growth stimulating supplement	Residual biomass: Drying (80°C)	Replaces nutrient media BG-11; Enhances biomass productivity, lipid and pigments	Maurya et al. (2016b)
<i>Monoraphidium</i> sp. KMCA	Growth promoter and feedstock for bioenergy production	Bioremediation and Biorefinery	—	High-value bioenergy feedstocks	Mishra and Mohanty, (2019)
<i>Botryococcus braunii</i>	Gaseous product (CO + CO ₂ + CH ₄)	Renewable source of syngas or hydrogen.	Catalytic gasification	higher contents of CO and H ₂ ; Maximum hydrogen yield of 74.7 mmol g-biomass ⁻¹	Watanabe et al. (2015)
<i>Chlorella vulgaris</i> , <i>Nannochloropsis</i> sp. and <i>Nanostrum</i> sp.	Methane	Bioenergy	—	—	Zhao et al. (2014)
<i>Phaeodactylum tricorutum</i>	Lipid and Nutrient Production	Biodiesel Feedstock and Growth media.	—	Mixture of Lipid-extracted microalgal and molasses	Zheng et al. (2015)

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TABLE 4 (Continued) Biorefinery based algal products.

Algae sp.	Product	Applications	Synthesis/ any other information	Product features	References
<i>Chlorella vulgaris</i>	Lipid and Nutrient Production	Biodiesel Feedstock and Growth media	enzymatic hydrolysates and 5% CO ₂	enhances Lipid (335 mg/L/day) and <i>Chlorella</i> sp. (5.58 g/L) production Biomass: 3.83 g/L and Lipid: 157 mg/L/d	Zheng et al. (2012)
<i>Chlorella vulgaris</i>	Microbial fuel cell	Power generation	Dual chamber MFC reactors: Graphite felt (Anode and Cathode)	(LEA) used as an anodic substrate; Power density of 2.7 W m ⁻³ (260% Increase as compared to MFC)	Khandelwal et al. (2018)
<i>Thraustochytrid</i> sp.	Lipid and algal Biomass	Nutrient recycling strategy	—	Enhances thraustochytrid biomass and Lipid production	Lowrey et al. (2016)

extraction from the harvested biomass. While, the leftover biomass can be processed to harness other value-added products including livestock feed, organic fertilizer due to the presence of high N:P ratio, food additives, bio-adsorbents, growth regulators, and bioactive compounds for pharmaceutical industries (Chugh et al., 2022; Wang et al., 2008; Karpanai Selvan et al., 2022). Some of the biorefinery-based products are discussed in Table 4 below.

Lipid extracted algal biomass-based fertilizers, nanomaterials, biochar and other products for various agricultural, biomedical, and environmental applications

LEA based fertilizers and bio-stimulants

Modern agriculture is totally dependent on the use of fertilizers for the desired growth. Therefore, extensive research is going on in this field to develop sustainable fertilizers that are non-toxic to the environment. According to some recent investigations, a variety of algae species act as bio-stimulants and enhance crop yield (Win et al., 2018; Abusweireh et al., 2022). Blue-green algae (cyanobacteria) are the most promising source due to their nitrogen metabolizing capability. Additionally, they solubilize phosphates and fix nitrogen anaerobically (Chittora et al., 2020). The nitrogen fixation mechanism is switched on during the deprived conditions that are below 40 ppm. Besides, reduced fertility along with rice-mustard-mung crop rotation was preferable for cyanobacteria-based nitrogen fixation in comparison to rice-wheat-maize rotation (Jha et al., 2001). Recently, *Chlorella variabilis* and *Lyngbya majuscula* were investigated as potential biofertilizers for *Zea mays* L. The pot experiments were successful in reproducing

recommended rate for fertilizer by algae-based biofertilizer which was nearly 65.16 g per plant.

LEA based nanomaterials

Nano-based products have dimensions in the range of 1–100 nm and are classified as natural, incidental, or engineered. Due to the presence of hydrophilic surface groups, such as sulfate, carboxyl, and hydroxyl nano-based materials are successful in the fabrication of products possessing antioxidant, antimicrobial, antibacterial, cell imaging, and dye degradation properties. Parameters include pH, temperature, incubation time, intensity of light, and precursor effects the synthesis rate (Rahman et al., 2020; Kumar et al., 2022b). For instance, *Acutodesmus dimorphus* synthesized silver nanoparticles depict 79% ROS scavenging at 25 µg/ml concentration making it antioxidant (Chokshi et al., 2016). Similarly, *Gracilaria corticata* based ZnSe nanoparticles showed 77.9% oxidant activity. Additionally, ZnSe NPs possess an anticancer effect on the lung as well as breast cell lines (Mirzaei et al., 2021). *Trichodesmium erythraeum* was also studied to depict 77.1% of antioxidant activity. Most of these algae-derived NPs are successful against bacteria. Such as, ZnSe NPs elucidated antibacterial effect on *S. aureus* (gram-positive) and *K. pneumoniae* (gram-negative) bacteria. Similarly, *Botryococcus braunii* extract was also effective against *S. aureus* and *K. pneumoniae*. While *Chlorella* sp.-based nanoparticles have been inhibiting *E. coli*, *Klebsiella pneumoniae*, *B. sphaericus*, and *P. mirabilis* respectively (Mirzaei et al., 202; Borah et al., 2020; Kashyap et al., 2019; Sathishkumar et al., 2019). Moreover, AgNPs can do dye degradation and doped NPs can be of use in imaging root and guard cells (Zhang et al., 2017; Borah et al., 2020). Therefore, nanotechnology has the ability to not only revolutionize human health but also offer detection and disease prevention products. Some of the

TABLE 5 Green synthesis of Nanomaterials from Algae and lipid extracted algal (LEA) biomass.

Nanomaterials	Algae sp.	Morphological	Synthesis conditions	Applications	References
		Features			
		Size/shape			
Ag NP	<i>Acutodesmus dimorphus</i> (LEA)	2–20 nm/ Spherical	10 ml LEA extract: 0 ml AgNO ₃ ; Room Temp; 24 h	Antioxidant Activity: 79% ROS scavenging at 25 µg/ml conc.	Chokshi et al. (2016)
Ag NP	<i>Saccharina japonica</i> (LEA)	14.77 nm	50 ml LEA extract : 50 ml AgNO ₃ (1 mM); 200 rpm	Antimicrobial Activity: MIC value: (5 µg/ml AgNP) <i>E. coli</i> - 6 ± 1.5 µL/ml <i>S. typhimurium</i> : 14 ± 4.2 µL/ml	Sivagnanam et al. (2017)
Ag NP	<i>Chlorella ellipsoidea</i> (LEA)	17.7 nm/ Quasispherical	1 g extract powder: 100 ml AgNO ₃ (0.001 M); Room Temp; 12 h	Dye Degradation: MB: blue to colorless in 30 min s MO: orange to pale yellow in 30 min s Antimicrobial Activity: ZoI: <i>E. coli</i> : 9 mm at 5 µg ml ⁻¹ conc. Of AgNP <i>Klebsiella pneumoniae</i> : 10 mm at 20 µg ml ⁻¹ conc. Of AgNP	Borah et al. (2020)
ZnSe NP	<i>Gracilaria corticata</i>	50–250 nm/ spherical	30 ml algae extract: 10 ml sodium selenite, 10 ml zinc nitrate; Constant stirring; Room temp; 48 h	Antioxidant activity: DPPH Assay: 30 µg/ml of ZnSe NP showed 77.97% Antibacterial activity: MIC: <i>K. pneumoniae</i> —150 µg/ml and <i>S. aureus</i> 125 µg/ml Antitumor activity: MTT assay: ZnSe NP 28.42 mg/ml IC50 epithelial kB cell line.	Mirzaei et al. (2021)
Ag/AgCl Hybrid NP	<i>Chlorella sp.</i>	10–20 nm	10 ml extract: 90 ml AgNO ₃ (0.5 mM); 5 h	Antimicrobial Activity: ZoI: <i>B. sphaericus</i> -4.9 mm <i>B. subtilis</i> -8.6 mm <i>B. pasteurii</i> -11.25 mm	Kashyap et al. (2019)
Ag NP	<i>Trichodesmium erythraeum</i>	26.5 nm	:100 ml AgNO ₃ pH-7; Sunlight irradiation; Constant stirring	Antioxidant Activity: DPPH assay: 77.01% at 500 µg AgNP Antibacterial Activity: 100 µg/ml AgNP – <i>S. aureus</i> -11 mm ZoI <i>P. mirabilis</i> - 10 mm ZoI	Sathishkumar et al. (2019)
N-S doped Carbon dot	<i>Nannochloropsis</i>	2.5–6 nm/spherical	80°C; pH 7; 30 min	Root and guard cell imaging: Blue light emitted at 450 nm.	Zhang et al. (2017)
Cu NP	<i>Botryococcus braunii</i>	10–70 nm	5 ml extract: 50 ml Cu (CH ₃ COO) ₂ ; Constant stirring; 1 day incubation;	Antimicrobial Activity: <i>Staphylococcus aureus</i> ZoI-22 mm <i>Klebsiella pneumoniae</i>	Arya et al. (2018)

(Continued on following page)

TABLE 5 (Continued) Green synthesis of Nanomaterials from Algae and lipid extracted algal (LEA) biomass.

Nanomaterials	Algae sp.	Morphological	Synthesis conditions	Applications	References
		Features			
		Size/shape			
Ag NP		40–100 nm	100°C	ZoI-19 mm	
			5 ml extract: 45 ml	<i>Staphylococcus aureus</i>	
			AgNO ₃ ;		
			Constant stirring;	ZoI-22 mm	
			25°C;	<i>Klebsiella pneumoniae</i>	
			3 h	ZoI-21 mm	

applications of nanoparticles have been discussed in Table 5 below.

LEA based biochar

Biochar is defined as a carbon enriched material usually produced *via* thermochemical decomposition of biomass at extremely high temperatures (500–1,000°C) in an O₂ deficient environment. It is prominently produced from the leftover biomass after bio-oil extraction. Recently, different strategies are developed to produce biochar including conventional techniques like slow and microwave-assisted pyrolysis as well as some newly developed methodologies like hydrothermal carbonization and torrefaction (Yu et al., 2017). It is charcoal that can adsorb heavy metal impurities from wastewater, pharmaceutical metabolites, phenols, and chemical intermediates, perform dye degradation, reduce soil erosion as well as improve crop yield. Due to high nutrition content and ion-exchange capacity, they are successful in replenishing soil (Karthik et al., 2021). Moreover, recent investigations reveal that biochar can enhance soil productivity, prevent greenhouse gas emissions and enhance the degradation speed of organic matter in the soil making it an ideal solution for composting (Wu et al., 2019). More recently, N-doped biochar has been utilized as an energy absorbent and promotes energy conservation. Super capacitors, batteries, fuel cells, and biochar-based composites are a successful result of this N-type doping (Xia et al., 2020). Therefore, for over a decade the algae-derived biochar can be of great importance in energy storage as well as carbon sequestration.

LEA based biomedical applications

Carotenoids are the bioactive compounds extracted from algal biomass and are of great importance for drug preparation. According to the investigators, pigments including β -carotene, lutein, fucoxanthin, violaxanthin, astaxanthin, and phycobiliproteins possess antitumor potential. Besides, they are also a promising source of antioxidants. For instance, *Haematococcus pluvialis* extracted astaxanthin provides UV

protection, improves immune response, and reduces tumor formation as well as inflammation (Mota et al., 2022). Phycocyanin obtained from *Spirulina* possesses fluorescence and is utilized for labeling antibodies, receptors, and other biological molecules for diagnostics. Numerous brown algae including Wakame, Kombu, Mozuki are a rich source of fucoxanthin which has anti-cancer as well as anti-obesity effects. It also improves lipid metabolism (Das, 2016). Additionally, algae machinery can be used for recombinant protein production due to the easy transformation of both plastids as well as nuclear genomes and offers advantages such as cheap production, easy scale-up, lack of pathogens, and easy assembling of complex proteins (Rosales-Mendoza et al., 2012). Hence, chloroplast can be a site for antibiotics fabrication (Purton et al., 2013). The pure algal extract also possesses some antimicrobial effects on gram-positive and gram-negative species such as *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Proteus mirabilis* (Soltani et al., 2011).

LEA based other products

Apart from biodiesel, bioethanol, biohydrogen, biomethane, and bio-oil synthesis leftover biomass can be used to reproduce products including food, feed, natural colors, and polymers. Microalgae such as *Spirulina*, *Chlorella*, *Scenedesmus*, *Dunaliella salina*, and *Aphanizomenon flos-aquae* have promising nutrients for not only humans but also animals. For instance, *Chlorella pyrenoidosa* comprise of 65% protein, 5–10% lipid, 10–20% hydrocarbons, 200–500 mg kg/L vitamin C, 120–300 mg kg/L of vitamin A and antioxidants (Shen et al., 2008). *Dunaliella*-based proteins are used in the baking industry while its biomass is utilized for fish and animal feed (Das, 2016). Several vitamins (vitamins A, B1, B2, B6, B12, C, E, niocinate, folic acid, and pantothenic acid) and minerals are also enriched in species (Koyande et al., 2019). Some freshwater species have a higher prebiotic index and modulate intestinal microbiota by increasing *Lactobacillus-Enterococcus* and *Bifidobacterium*

(desired bacteria) while inhibiting *Prevotellaceae-Bacteroidaceae*, *Clostridium histolyticum*, and *Eubacterium rectale Clostridium coccoides* (undesired bacteria) (de Medeiros et al., 2021). Algae-derived protein not only has essential amino acids (isoleucine, valine, lysine, tryptophan, methionine, threonine, and histidine) but is higher than conventional sources such as meat, fish, and dairy products. For instance, 7 g dried biomass of *Spirulina maxima* contains 4 g of protein, 1 g of fat (omega-3 and omega-6 fatty acids) 11% Vitamin B1, 15% vitamin B2 and 4% vitamin B3, 21% copper, and 11% iron of required daily allowance (RDA) (Milledge, 2011). While, magnesium, manganese and potassium are reported in trace amounts (Christaki et al., 2011). The inclusion of microalgae biomass in the animal feedstock can improve meat quality in ruminants, pigs, poultry, and rabbits (Madeira et al., 2017). For example, the addition of *Arthrospira platensis* in poultry diets increases average daily gain but negatively affects the feed conversion ratio. Similarly, *Schizochytrium sp.*, improve fatty acid composition in poultry animals, due to the presence of a high amount of DHA. Therefore, adding algae biomass into the feed can be a cost-effective measure of fuel production. Due to the presence of chlorophylls, carotenoids, xanthophylls, and phycobiliproteins in several algal species natural colors can be harnessed as additives in ice-creams, candies, non-alcoholic beverages, and dietary foods as well as clothes (Azeem et al., 2019) (Das, 2016). According to some recent studies, species of algae are also capable of producing bioplastic which can be harnessed to produce numerous products including biodegradable plastic bags, slippers, buckets, etc. Additionally, red algae can produce some gelling polysaccharides like agar, agarose, and carrageenan possessing sulphated galactans. For instance, *Hydropuntia cornea*-produced agar can be blended at different concentrations with polyvinyl alcohol to produce biodegradable films for the packaging industry (Das, 2016). Therefore, numerous applications of algal species are possible by implementing a biorefinery.

Conclusion

Over the decade, genetic and metabolomic engineering coupled with omics has played a prominent role in improving lipid yield. While, more recently integrating biomass-based biorefinery with a waste-water treatment setup has proved to

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reduce the production cost of algae-based fuel. However, more innovation is required to overcome the issues of strain selection, cultivation, harvesting, drying and fuel conversion methodologies. Additionally, environmental risk assessment, resource organization, and life cycle assessment (LCA) of algal strain to biofuels is required. More recently biorefineries have been established that not only helps to produce biofuel but also several high-value-low-yield biomolecules for pharmaceutical and nutraceutical interventions. Additionally, several environmentally important products such as biofertilizers, biochar, bio-stimulants, nanomaterials, etc., are also generated. However, research on various aspects of algal-based bioactive compound extraction is in nascent phase and requires bioprospecting of high yielding native algal species, development and deployment of mass cultivation strategies, process optimization for harvest and cell disruption techniques followed by efficient biomolecule extraction procedures to make algal biorefinery sustainable and commercially viable in nature.

Author contributions

LK, LM, and RA- wrote the original draft, Concept. VJ and MC- Review and edited the manuscript. NB- Concept, and Supervise.

Conflict of interest

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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