2
Review of Literature

The energy crisis is happening due to the substantial reduction in fossil fuels, which has demanded to search for alternative energy sources. The emerging technologies should fulfill the criteria of net zero emissions, being affordable, and comparable to current oil prices [Ramos *et al*., 2022]. Most of the current energy requirements for the transportation sector are met with petrol/diesel. Therefore, biodiesel/bioenergy may be utilized as the alternative liquid fuel, which can be harvested through natural photosynthesis using plants, cyanobacteria, and microalgal oils. Microalgae has higher photosynthetic efficiency than plants, which utilizes 183 GT of CO_2 to produce 100 GT of microalgae biomass [Chisti, 2007]. Therefore, it not only provides the feedstock for biofuel but also helps reduce the CO_2 concentration in the atmosphere. Microalgal biofuels have received wide attention and have become the choice for alternative fuels [Milano *et al*., 2016]. Also, microalgae-based pigments are another crucial component when discussing microalgae-based biorefineries and circular bioeconomy.

Circular bioeconomy represents an integrated approach combining the principles of circular economy and bioeconomy. It involves the systemic utilization of biomass to drive economic development in a cascading manner. This approach optimizes the use of biomass, including waste by-products to generate high-value products such as food, bioenergy, and biomaterials. The advantages of embracing a circular bioeconomy encompass enhanced resource efficiency and, ecoefficiency, reduced greenhouse gas emissions, reduced dependence on fossil resources, and waste valorization. Consequently, the circular bioeconomy represents a low-carbon economy, showcasing its potential to foster a sustainable and environmentally friendly future. For example, microalgae-based circular bioeconomy offers enhanced CO_2 fixation, rapid growth rates, the capability to sequester carbon in the form of carbohydrates and lipids to produce biofuels like biodiesel and bioethanol, as well as the generation of Hydrogen (H_2) through anaerobic digestion. Additionally, it enables the extraction of valuable products such as polysaccharides, biopeptides, biopolymers, antioxidants, and pigments. Moreover, microalgal biomass can be utilized to produce biofertilizers. Furthermore, microalgae can utilize residues from various industrial processes (solid, liquid, and gaseous) to support their growth [Moreira *et al*., 2023].

2.1 MICROALGAE-BASED CIRCULAR BIOECONOMY

Algae are photosynthetic organisms, majorly classified into seven divisions, including Chlorophyta, Phaeophyta, Rhodophyta, Euglenophyta, Chrysophyta, Cyanophyta, and Pyrrophyta [Levring, 1969]. The eukaryotic microalgae are unicellular to multicellular photosynthetic microorganisms that offer a wide range of applications, including wastewater treatment, biofuel production, and therapeutics production. Recent developments have highlighted green microalgae's potential across domains like CO_2 capture, water treatment, bio-based product synthesis, and metal reclamation. Their broad distribution, efficient nutrition, remarkable photosynthetic capabilities, and production potential for low-volume, high-value products like food constituents (carbohydrates, proteins, oils, pigments), and eco-friendly polymers have amplified global interest in exploring microalgae-derived compounds. Consequently, the establishment of microalgae-based biorefineries is a promising avenue for circular bioeconomy [Udayan *et al*., 2022]. However, in the present scenario, microalgae-based products are not sustainable for low-value/high-volume industries, such as the bioenergy

industry. For instance, manufacturing microalgae-based biodiesel and bioethanol would be, respectively, €25.6/l and €33.3/Kg, which is too expensive for the fuels [Abreu *et al*., 2020].

Recent research studies are investing in high-value product generation from microalgae and studying their genetic modification to improve their baseline production. The selection of microalgal species is a critical consideration for biofuel and other metabolite production, which may alter under different operational conditions. Therefore, microalgae-based technology has the reliability to contribute to the circular bioeconomy, which requires sustainable microalgae biomass production, environmental protection, waste reduction, and reduction in upstream and downstream process requirements. Some of the desired characteristics for mass cultivation of microalgae are rapid growth rate, high productivity, ability to grow in extreme environments, wide tolerance of environmental conditions, ease in harvest, high CO_2 capture efficiency, tolerance of shear force, and no excretion of auto inhibitors [Kothari *et al*., 2017].

Table 2.1 : Biochemical Compositions of Various Microalgae Species used in Wastewater Treatment

S. No.	Species used	Biomass productivity (mg/l/d)	Lipid (%DCW)	Carbohydrate (%DCW)	Protein (%DCW)	References
1	*Botryococcus braunii*	-	25–75	17	48	[Mata *et al*., 2010]
2	*Chlorella vulgaris*	20–200	5–58	9–17	51–58	
3	*Hematococcus pluvialis*	50–60	15–25	27	48	
4	*Scenedesmus quadricauda*	190	1.9–18.4	NA	47	
5	*Spirulina maxima*	210–250	4–9	13 – 16	60–71	
6	*Chlamydomonas reinhardtii*	820–2000	16.6–25.3	17	48	[Kong *et al*., 2013]
7	*Chlorella* sp.	20–2500	10–48	12–17	51–58	[Cai *et al*., 2013]
8	*Scenedesmus obliquus*	4–74	11–55	10–17	11.8–56	
9	*Synechococcus elongatus*	1240	-	59	-	[Chow *et al*., 2015]
10	*Chlorella sorokiniana*	68.116 ± 14.5	43.3 ± 6	16.3 ± 0.5	-	[Hamidian Zamani, 2022]

It is observed from the literature studies that *C. reinhardtii* has a high biomass productivity along with high lipid, carbohydrate, and protein content, which make it a valuable species for biorefinery applications (Table 2.1). The following sections will discuss the *Chlamydomonas*-based biorefinery approach in depth, with a detailed discussion on PMFCs for wastewater treatment and resource recovery.

2.2 *Chlamydomonas reinhardtii* AND ITS CHARACTERISTICS

A unicellular, freshwater green alga, *Chlamydomonas*, is the model alga from the Chlorophyta class and was first described by Dangeard in 1888. It has a spheroid cell body with an approximate 10 µm diameter [Jeanneret *et al*., 2016]. The cellular features are shown in Figure 2.1. This chloroplast houses a single pyrenoid at the cell's base, which serves as the site for CO_2 fixation and predominant starch accumulation. The eyespot is a distinctive feature, consisting of two key components that involves a specialized region of the plasma membrane containing numerous copies of channelrhodopsin, a light-gated ion channel protein exhibiting high sensitivity within the 450 – 700 nm spectral range. The other component is the stigma which features the multiple stacks of carotenoid-rich granules and functions as a dielectric mirror. The

latter aids in concentrating light on rhodopsin when the eyespot faces the light source and screening rhodopsin when facing away [Harris, 1998].

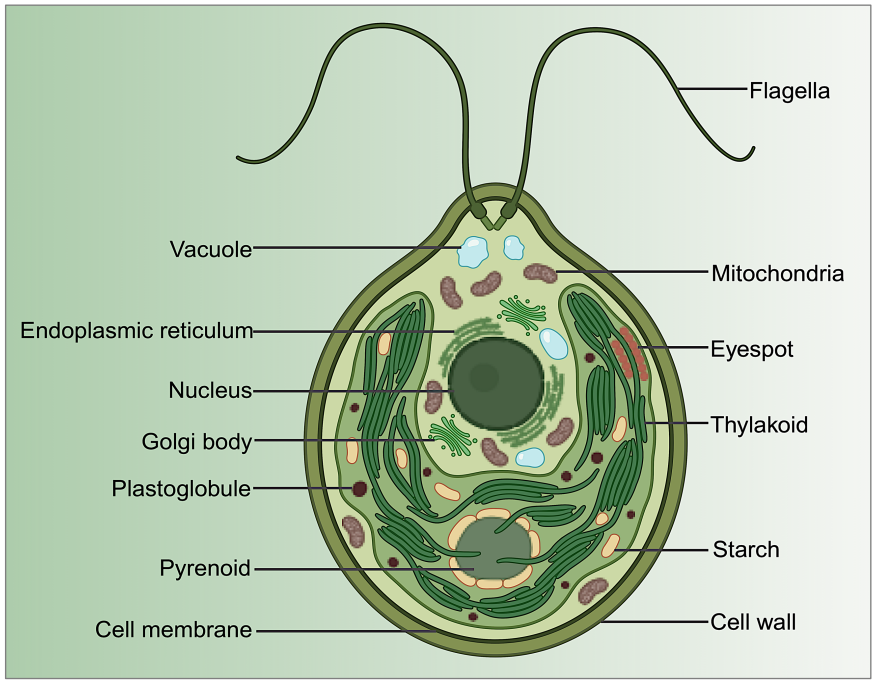

Figure 2.1 : The schematic representation of *Chlamydomonas* cell structure

C. reinhardtii is an ideal candidate for studying the photosynthetic mechanism, biosynthetic pathways, carbon concentrating mechanism, and H_2 production. *Chlamydomonas* cells can accumulate carbohydrates in the form of starch, lipids, and pigments by growing in organic and inorganic carbon sources [Nguyen *et al*., 2009; Choi *et al*., 2010]. Also, the loose cell wall structure offers ease in extracting intracellular metabolites with mild pre-treatments, reducing the steps in downstream processing. Therefore, it is suitable for biorefineries that aim to transform the microalgal biomass for biofuels and VAP production through a sustainable route [Banerjee *et al*., 2021]. Also, *C. reinhardtii* exhibits high biomass productivity and has acquired Generally Regarded As Safe (GRAS) status that approves the species for human consumption [Fields *et al*., 2020; Yadav *et al*., 2021].

2.3 DIFFERENT TROPHIC MODES FOR *Chlamydomonas* CULTIVATION

The microalgae species can be cultivated via autotrophic, heterotrophic, or mixotrophic approaches. In general, the high cost of microalgae cultivation is constrained due to the low biomass productivity in autotrophic cultivation, and high downstream processing and harvesting costs at large-scale reactors. Therefore, strategies are devised to produce high-value, low-volume compounds such as protein, and pigments, from microalgae which can provide valuable returns on investment costs. The cultivation of microalgae in different trophic modes is discussed below in detail concerning to *C. reinhardtii*:

2.3.1 Autotrophic Cultivation

In the autotrophic mode of nutrition, microalgae cells rely on inorganic carbon sources. Culturing *C. reinhardtii* in a photoautotrophic manner is the most economical by utilizing light energy, and CO_2. Moreover, these cultivation systems offer the advantage of remaining axenic and limit bacterial contamination in the cultivation unit [Ruiz *et al*., 2022]. However, the autotrophic system efficiencies are limited by the rate of natural photosynthesis to produce biomass energy [Ma *et al*., 2023].

2.3.2 Heterotrophic Cultivation

The heterotrophic cultivation mode is suitable for achieving high biomass productivity as the organic substrate utilization reduces the dependency on CO_2 and light energy for energy harnessing. Heterotrophic cultivation helps microalgae accumulate more lipids than autotrophically grown microalgae [Chandrashekharaiah *et al.*, 2021]. *C. reinhardtii* can grow heterotrophically by utilizing organic carbon sources such as acetate or glucose. Tris Acetate Phosphate (TAP) medium is widely utilized to grow *C. reinhardtii* in heterotrophic or mixotrophic cultivation modes [Schad *et al.*, 2023]. *Chlamydomonas* cells possess antibacterial compounds that limit the bacterial growth in the cultivation system [Little *et al.*, 2021]. However, some bacterial isolates have shown tolerance to these antibacterial compounds which may thereby reduce the microalgae productivity. Therefore, it is crucial to avert bacterial contamination in these cultivation conditions as bacterial species more readily utilize these organic substrates [Mitra *et al.*, 2021].

2.3.3 Mixotrophic Cultivation

In mixotrophic cultivation, microalgae can utilize the organic substrates for their metabolic processes, and these can grow autotrophically by using light energy through photosynthesis. The mixotrophic mode has been proven to be an economically viable alternative as it produces high biomass, lipids, and pigments. Two-stage cultivation mixotrophy has become the method of choice over autotrophy or heterotrophy alone. The strategy includes two phases of cultivation where phase I improves microalgal biomass production using heterotrophic growth while photoautotrophic growth occurs in phase II to increase biomass concentration and lipid production. This strategy has improved biomass production and lipid productivity in *Chlorella* over photoautotrophy alone [Cui *et al.*, 2017]. In the other two-stage cultivation approach, *C. reinhardtii* CC-125 was grown first in repeated fed-batch cycles, followed by high carbohydrate production in batch systems [Wang *et al.*, 2015]. Mixotrophic microalgae cultivation is also vital for bioremediation of organic wastes [Sharma *et al.*, 2021]. The mixotrophic mode is a commercially viable option, yielding significant biomass, lipids, and pigments [Demets *et al.*, 2023].

2.4 FACTORS AFFECTING *Chlamydomonas* GROWTH

Outdoor and indoor cultivation efficiencies vary due to factors such as abiotic (light, temperature, pH, N, and P concentration, salinity, Dissolved Oxygen (DO), CO_2 concentration, and presence of toxic chemicals), biotic (pathogens, bacteria, fungi, protozoa, and virus), and operational conditions (culture mixing rate, dilution rate, culture depth, and biomass harvesting). Therefore, the factors for optimal growth of microalgae may vary from the factors for high metabolite production. For example, under various stressors like nitrogen or sulfur deficiency, elevated temperatures, variable light conditions, light-dark cycles, or high salinity, microalgae respond by intensifying the production of valuable compounds such as carotenoids, starch, H_2, and lipids. For the production/recovery of specific compounds, reactor design, light, pH, and operational temperature are crucial parameters [Schad *et al.*, 2023]. These parameters are discussed in detail in the following sections:

2.4.1 Media Composition and pH

Standard microalgae culture media includes Blue Green-11 (BG-11), Bold's Basal Medium (BBM), Zarrouk's media, F/2 media, Tris Phosphate (TP), and TAP media [Chakraborty *et al.*, 2023]. Media composition is optimized for new microalgae isolates to achieve high cell-density cultures. The CO_2 concentration may alter microalgae cells' product formation, including lipids and carotenoids. Therefore, a trade-off is expected between two biosynthesis pathways competing for the same precursor, acetyl-coA, which diverges in various metabolic branches in the cells [Ma *et al.*, 2019; Abreu *et al.*, 2020]. The research studies may focus on extremophile strains to grow selective species for industrial applications. For example, *C. acidophila* has shown the highest productivity of lutein up to 10 g/Kg DCW when grown at pH 2–3 [Cuaresma *et al.*, 2011]. Also, the high pH of the culture medium in the presence of phosphates and bivalent cations

helps in the flocculation of *Chlamydomonas* cells. The high pH has been reported to negatively impact the contaminants in the culture and acts as a disinfectant agent [Gerloff-Elias *et al*., 2006].

2.4.2 Temperature

Temperature affects the growth and cell division in microalgal cells. In addition, operational temperature influences metabolic activity, and high temperature can damage the cells by causing heat shock. The cell division time also changes with growth temperature. A recent study has shown that the cell cycle arrest, including the blocking of nuclear and cellular divisions, was the apparent outcome of the high-temperature exposure; however, the growth may remain unaffected till 39 °C [Zachledar *et al*., 2019]. Therefore, the temperature determines the bioproduct concentration and the compositional differences that occur [James *et al*., 2013]. A recently isolated psychrophilic strain from arctic sea ice, *Chlamydomonas* sp. KNM0029C has shown potential for biodiesel and bioethanol production which is crucial for the bioenergy sector as the strain can grow below 10 °C [Kim *et al*., 2020]. The biodiesel from cold regions grown microalgae could be useful owing to their high level of unsaturated fatty acids which is a desirable property to reduce the Cold Filter Plugging Point (CFPP). The CFPP is used to assess the fluidity of biodiesel at low temperatures. Therefore, the role of temperature is crucial in microalgae-based bioenergy applications.

2.4.3 Salinity or Salt Stress

The salt stress has also been proven to improve specific metabolite production; however, it also affects microalgae biomass production. For example, the salinity of the cultivation medium has affected the production of phytochemicals from *C. vulgaris* and *C. reinhardtii* where low salt stress increases the phenol content, while the flavonoids were increased under high salinity [Annamalai *et al*., 2016]. Also, the cells exposed to stress conditions may enter a temporary resting stage, for example, palmelloids in *Chlamydomonas*. These cells may show phenotypic alterations, including loss of flagella, cell clustering, high Exopolysaccharide (EPS) production, and cell wall thickening [Khona *et al*., 2016]. *C. onubensis* can be grown under acidic conditions and high salinity concentrations [Fuentes *et al*., 2016]. Moreover, the salt addition to the culture media improved biomass productivity and VAPs, including lutein and Polyunsaturated Fatty Acids (PUFAs) production previously [Bermejo *et al*., 2018].

2.4.4 Agitation and Dissolved Oxygen Concentration

For uniformity of suspension cultures, the algal PBRs or open ponds are equipped with agitators or paddles, respectively. However, the culture mixing causes the shear stress on the cells, causing cell biomass loss. Therefore, agitator design is another research domain that needs further exploration. In addition, DO concentration in microalgal cultivation needs to be monitored as the high oxygen concentration in the culture may reduce the microalgae growth through auto-oxidation and inhibit the cellular enzymes. It can also compete with the Ribulose-1,5-bisphosphate Carboxylase/Oxygenase (RUBISCO) enzyme for CO_2 binding and can, therefore, the photosynthetic machinery may stall. Thus, the cultivation should remove the additional oxygen from the culture [Kazbar *et al*., 2019]. Open pond cultivation has a high gas exchange that does not need oxygen ejection. The closed cultivation systems are equipped with membrane separators or a regulated supply of N_2 or CO_2 is recommended to replace the oxygen [Kishi *et al*., 2020]. Among the available cultivation systems, MFC cathodes can inherently deoxygenate the microalgae culture medium through an Oxygen Reduction Reaction (ORR) in the cathode chamber [Kakarla and Min, 2014]. The small-scale laboratory MFCs are however operated with manual stirring rather than using agitator-equipped complex reactors. A detailed discussion on MFCs is provided in the later sections.

2.4.5 Light

Microalgae require light for autotrophic cultivation. The optimum light for microalgae cultivation lies in the range of 26−400 µmol/m^2/s. Light energy can be supplied through sunlight or artificial illumination with lamps or LEDs. Sunlight is abundant and free, for cost-effective cultivation. However, it includes high variability in day/night cycles, changes in weather

conditions, and seasonal changes which may affect the microalgae productivity. The application of artificial light will reduce the variability but increase the production cost by 25.3 $/Kg of dry-weight biomass [Blanken *et al*., 2013].

Nevertheless, the microalgae growth and metabolite production are affected by the light availability [Maltsev *et al*., 2021]. For example, high light exposure for prolonged periods may lead to photo-oxidation of light-harvesting complexes. Therefore, a sequential adaptation strategy can be employed to change the optimum light regime of these photosynthetic species. When a WT *C. reinhardtii* CC-124 strain was subjected to extreme light conditions, it resulted in microalgae biomass's high carotenoid to chlorophyll ratio. Also, the *C. reinhardtii* has exhibited a high resistance to photoinhibition under very strong light exposure (3000 and 3500 µmol/m^2/s) which makes it a suitable candidate for bioenergy industries [Virtanen *et al*., 2021]. Hence, the selection of the optimum light in microalgae cultivation is useful to obtain high productivity as well as microalgal products for bioenergy production. Such as the red as well as blue light improves the microalgae growth. Also, the pulsed light exposure can stimulate the production of lipids and pigment especially carotenoids in microalgae cultures [Maltsev *et al*., 2021].

2.4.6 Cultivation Conditions

Microalgae cultivation depends on the cultivation strategies used, such as open or closed cultivation. These two offer distinctive features for microalgae biomass production. Open pond cultivation suffers from external contamination while the cost is relatively lower than the closed PBR. However, the closed cultivation systems offer better operational control for scaled-up applications. The closed cultivation systems may be economical for high-value products; however, these systems may be hindered by the cost and size limitations for biofuel production [Paul *et al*., 2021]. A detailed discussion on cultivation systems is provided in the later sections of the chapter.

2.5 WASTE VALORIZATION THROUGH *Chlamydomonas* CULTIVATION

Microalgae help to remove nutrients from wastewater by adsorption, accumulation, immobilization, and metabolizing the nutrients [Kaloudas *et al*., 2021; Yadav *et al*., 2021]. Therefore, microalgae cultures can be heterotrophically grown by supplying nutrients from wastewater which also reduces the water contaminants including nitrates, and phosphates. In addition, it also helps in reducing the economic burden of using synthetic media and minimizes environmental waste. A wide range of wastewater has been employed for microalgae cultivation, originating from dairy, housing societies, textile industry, agriculture fields, and cheese whey as shown in Table 2.2 [Chen *et al*., 2015].

Chlamydomonas strains were employed in various studies for wastewater remediation [Escudero *et al*., 2020; Zhou *et al*., 2022]. The biomass productivity also enhanced in the presence of N and P in wastewater. Moreover, microalgae cells also possess antibacterial properties that limit the pathogenic bacteria in wastewater [Shannon and Abu-Ghannam, 2016; Liakh *et al*., 2023]. In addition, several Microalgae Growth-Promoting Bacteria (MGPB) have been identified, and cultivated in the wastewater [Toyama *et al*., 2018]. A *Chlamydomonas* sp. was isolated and cultivated on anaerobic digester effluent wastewater, which resulted in high biomass productivity, and high-temperature cultivation. The isolated strain was effectively used for biomethane production under low-N conditions [Klassen *et al*., 2020]. Researchers have also used poultry manure for *Chlamydomonas* cultivation which improves biomass production (1.45 – 1.02 g/l) and reduces the dependency on chemical media [Radhakrishnan *et al*., 2021]. Therefore, the cultivation of microalgae in wastewater as a nutrient medium can be optimized for high biomass productivity.

Table 2.2 : Various *Chlamydomonas* Strains used in Waste Valorization

S. No.	Algae strain	Wastewater type	Biomass productivity/ Yield	Lipid productivity/ yield	Pollutant removal	References
1	*C. reinhardtii*	Municipal wastewater	2.00 ± 0.03 g/l/d	0.505 ± 0.02 g/l/d	N: 55.8 mg/l/d, P: 17.4 mg/l/d	[Kong *et al*., 2010]
2	*Chlamydomonas* sp. TAI-2	Industrial wastewater	1.5 g/l	18.4%	100% NH_4^+-N (38.4 mg/l) and NO_3^--N (3.1 mg/l), 33% PO_4^{3-}-P (44.7 mg/l)	[Wu *et al*., 2012]
3	*C. mexicana*	Piggery wastewater effluent	0.56 ± 0.35 g/l	0.31 ± 0.03 g/l (33 ± 3%)	N: 62%, P: 28%, Inorganic C: 29%	[Abou - Shanab *et al*., 2013]
4	*C. acidophila*	Pharmaceutical wastewater	-	-	9 mg/l/d NH_4, 3 mg/l/d PO_4	[Escudero *et al*., 2020]
5	*Chlamydomonas* sp. QWY37	Swine wastewater	9.9 g/l	-	COD: 81%, TN: 96% and TP: nearly 100%	[Qu *et al*., 2020]
6	*C. biconvexa* Embrapa|LBA40	Palm oil mill effluent	190.60 mg/l/d	11.3%	NH_4^+-N and NO_3^--N: 99%, $PO_4^{3—}$98%	[Pascoal *et al*., 2021]
7	*Chlamydomonas* sp. YC	Rare earth elements wastewater	1.57–1.64 g/l	32.98 mg/l/d	NH_4^+-N: 32.75–61.05 mg/l/d	[Zhou *et al*., 2022]

2.6 CO_2 MITIGATION THROUGH *Chlamydomonas* CULTIVATION

Microalgae are photosynthetic entities important for carbon fixation and the production of sustainable products. The CO_2 serves as a carbon source in photoautotrophic organisms. Two enzymes carbonic anhydrase and RUBISCO play important roles in the CO_2 concentration mechanism. Various factors, including light, pH, temperature, CO_2 concentration, and cultivation conditions can affect microalgal CO_2 uptake, thereby CO_2 mitigation also depends on these parameters. It becomes important when CO_2 sequestration through microalgae is integrated with wastewater treatment as biomass productivity, and pollutant removal complement each other. These processes can be employed in biorefineries to enhance biomass productivity and other microalgal products through mixotrophy as shown for *C. reinhardtii* UTEX 90 and CC 2656 [Banerjee *et al*., 2021]. Carbon fixation through microalgae is limited by the low availability of N and P concentrations which affect the microalgae growth. However, recently mutant strains of *C. reinhardtii* were produced using ultraviolet light treatment which have shown growth similar to WT strains by using less N and P which can improve CO_2 sequestration [Gopal and Sudhakar, 2016]. The high CO_2 levels are known to impose toxicity to *C. reinhardtii* cells by causing intracellular acidification due to the poor expression of plasma membrane H^+-ATPases. To overcome these challenges, *C. reinhardtii* strains were modified to increase the tolerance to high CO_2 levels by expressing these enzymes which resulted in high biomass productivity at high CO_2 levels (13%) [Choi *et al*., 2021]. For large-scale CO_2 mitigation studies, Life Cycle Analysis (LCA)

and sustainability analysis are required to evaluate the economic feasibility of the process and determine the technical infrastructure required.

2.7 MICROALGAE-BASED BIOREFINERY FOR BIOPRODUCT SYNTHESIS

Closed systems are useful in producing metabolites, biofuels, and CO_2 sequestration [Paul *et al*., 2021]. In addition to biofuels and oils, microalgae can produce high-value compounds, including carotenoids, flavonoids, protein, carbohydrates, PUFA, antioxidants, alginate, agar, vitamins, hormones, and phytosterols. These VAPs have numerous applications in bio-manufacturing sectors, including pharma, nutraceuticals, and personal care products [Okeke *et al*., 2022]. Some of the major microalgae biorefinery products are discussed in the below section.

2.7.1 Biofuels

Microalgae biomass is highly resourceful for biofuel/biodiesel production, which comes under third-generation biofuels. First and second-generation biofuels are derived from petro-crops and lignocellulosic waste biomass, respectively. Third-generation biofuels are a viable option as microalgae biomass can accumulate high lipids [Chowdhury and Loganathan, 2019]. However, there are still some challenges in implementing microalgae-based biofuels for industrial applications includes high initial capital expenditure, high cost for harvesting and extraction of oil, and contamination from non-target organisms. In addition, commercial prices and product yield should be high to ensure the profitability of biofuel production [Wiatrowski *et al*., 2022]. More research is required to lower the harvesting cost and increase the lipid productivity and high-value products from microalgae to revive the third-generation biofuels for commercial success.

2.7.2 Microalgal Lipids

The model green microalga *C. reinhardtii* is a preferred choice for biofuel research due to its rapid growth, short generation time, adaptability, and easy cultivation. The *C. reinhardtii* is not considered oleaginous; however, newer strains with enhanced lipid production capabilities have been isolated with a total fatty acid content reaching 25% [Morowvat *et al*., 2010]. Several stress conditions, including nitrogen and iron starvation, copper stress, heat stress, pH alterations, and high-light conditions, have been explored to induce effective lipid accumulation in microalgae [Devadasu and Subramanyam, 2021]. A notable combination treatment involving 100 mM NaCl and 100 mM $CaCl_2$ demonstrated the highest lipid content (73.4%) and lipid productivity (10.9 mg/l/d). This combined approach significantly upregulated key enzymes, including Glycerol-3-phosphate Dehydrogenase (GPDH), Lysophosphatidic Acid Acyltransferase (LPAAT), and Diacylglycerol Acyltransferase (DGAT), known to be involved in lipid accumulation in *C. reinhardtii* [Hang *et al*., 2020]. Genetic modifications have also been explored to enhance lipid production in C. *reinhardtii*. Co-regulation of three lipid-related genes (*DOF*, *LACS2*, and *CIS*) in C. *reinhardtii* CC849 resulted in a substantial increase in intracellular lipids and fatty acid content by 142% and 52%, respectively [Jia *et al*., 2022]. Additionally, phospholipase A2 knockout mutants, such as *C. reinhardtii* CC-4349, exhibited a remarkable enhancement in lipid productivity, reaching up to 64.25% (80.92 g/l/d) [Shin *et al*., 2019]. To improve the lipid productivity in microalgae, novel strains can also be isolated from natural habitats along with strain engineering and process optimization.

2.7.3 Hydrogen

Recently, biomass-based H_2 generation has gained much attention as a green and clean fuel. These processes are associated with many advantages, including a reduction in CO_2 emission, the substitution of fossil fuels, and solid residue decomposition through H_2 conversion [Mishra *et al*., 2023]. Microalgae biomass can be processed through thermochemical (gasification, supercritical water gasification, pyrolysis) or biological routes for H_2 production (direct biophotolysis, indirect biophotolysis, photo-fermentation, water-gas-shift reaction, and dark fermentation). Among available biological methods, dark fermentation using microalgae biomass, microbial electrolysis cells, and anaerobic digestion has emerged as the preferred

method for H_2 production [Banu *et al*., 2018; Goria *et al*., 2023]. The most common microalgae species used for H_2 production are genera *Chlorella, Chlamydomonas, Anabaena, Oscillatoria, Scenedesmus,* and their mutants. *C. reinhardtii* has been extensively studied for the photoproduction of bio H_2. *C. reinhardtii* due to its ability to utilize acetate as a respiratory substrate, can grow under low light conditions, and reach anoxia supporting H_2 production. It's a two-stage process, wherein at the first stage, biomass density is increased in the mixotrophic mode; after that, in the second step, the cultures are shifted to anaerobic conditions with N_2 supply where dark-fermentation takes place for H_2 production [Fakhimi *et al*., 2024]. However, it suffers from limitations such as seasonal variations in raw biomass availability, high handling cost, solar-dependent cultivation, and char production.

2.7.4 Value-added Products

Microalgae cultivation also generates VAPs such as pigments (chlorophyll and carotenoids), polysaccharides, EPS, recombinant proteins for therapeutics (including human vaccines, monoclonal antibodies, erythropoietin, human fibronectin, interferon, and growth factor), polyunsaturated fatty acids, lipids, and biomethane, which can significantly contribute to the circular bioeconomy success [Banerjee *et al*., 2021; Masi *et al*., 2023]. These products are synthesized in low volume; however, they contribute significantly to resource recovery. For example, microalgal cells can secrete polysaccharides into the extracellular medium during their growth. These EPS shows the potent activity of being biodegradable polymers, bio-flocculants, and bio-active compounds effectively utilized in anticancer, antithrombotic, and immunomodulatory agents. Production of these EPS compounds varies with the medium composition, such as C and N [Li *et al*., 2023]. Carotenoids are also important compounds produced from microalgae and act as potent health supplements. Current constraints hinder the distribution of microalgae-derived products within low-value, high-volume sectors such as the bioenergy industry, mostly due to elevated biomass production costs. Therefore, enhanced productivity of these VAPs would provide great potential to microalgae-based biorefineries. Genetic engineering strategies were employed to improve the production of VAPs, such as lipids and carotenoids (Table 2.3).

Table 2.3 : Production of Value-added Products through *Chlamydomonas reinhardtii* using Genetic Modifications

S. No.	Target product	Gene/target site	Approach	Results	References
1	Lipids	Diacylglycerol acyltransferase 2 (*DGAT*)	Heterologous expression	α-linolenic acid, an important omega-3 fatty acid, was improved by more than 12%	[Ahmad *et al*., 2015]
2		Phosphoenol pyruvate carboxylase (*PEPC1*)	Down regulation by CRISPRi/Cas9	Lipid content and productivity of 28.5% DCW and 34.9 mg/l/d	[Kao and Ng, 2017]
3		Malic enzyme isoform 2 (*ME2*)	Overexpression	Increased lipid production up to 23.4%	[Kim *et al*., 2019]
4		Phospholipase A2 (*PLA2*)	CRISPR-Cas9 knock out	Improved lipids' production up to 64.25%	[Shin *et al*., 2019]
5		Diacylglycerol acyltransferase (*HpDGAT2D*)	Heterologous expression	Increased TAG content by ~1.4-fold	[Cui *et al*., 2021]
6		Zeaxanthin epoxidase (*ZEP*) and ADP-glucose pyrophosphorylase (*AGP*) genes	CRISPR-Cas9 RNP-mediated knock-out	Increased oil productivity by 81%	[Song *et al*., 2022]
7	Carotenoids	*ZEP*	DNA-free CRISPR-Cas9, knock-out mutant	Increased zeaxanthin content and productivity by 56- and 47-fold, respectively	[Baek *et al*., 2018]
8		Deoxyxylulose 5-phosphate synthase (*DXS*) and deoxyxylulose 5-phosphate reductoisomerase (*DXR*)	Overexpressed via nuclear transformation	Increased lutein and β-carotene by 1.9-fold and 1.7-fold per cell, respectively	[Morikawa *et al*., 2018]
9		Carotene-ε-monooxygenase (*lut1*) and *zep*	High-intensity light induced repression of lut1 and zep	Increased lutein productivity (5.08 mg/l/d)	[Ma *et al*., 2019]
10		β-carotene ketolase (*CrBKT*)	Overexpression of the optimized *CrBKT*	Up to 50% of native carotenoids could be converted into astaxanthin	[Perozeni *et al*., 2020]
11		Bifunctional *PBS* gene	Heterologous expression	38% enhancement in β-carotene along with 60% increase in the lutein	[Rathod *et al*., 2020]

2.8 MICROALGAL CAROTENOIDS AND THEIR THERAPEUTIC APPLICATIONS

Carotenoids are accessory pigments that sense and provide the controlled light for photosynthesis. These pigments belong to the terpenoid group (unsaturated pigments) which are classified into two categories, carotenes (hydrocarbon) and xanthophylls (having one or more oxygen-containing functional groups) [Cordero *et al*., 2011]. These compounds are extensively studied for their properties such as antioxidants, anticarcinogens, dietary supplements, and nutraceuticals. Commercially, carotenoids are chemically synthesized from petrochemical compounds; hence, these cannot be utilized for human consumption. Microalgae species including, *Chlorella*, *Dunaliella*, *Nannochloropsis*, and *Scenedesmus*, produce a variety of light-

harvesting carotenoids and can respond to oxidative stress from their free radical scavenging abilities [Velmurugan and Muthukaliannan, 2022]. Therefore, the studies are directed at reducing the cost while increasing commercial-scale productivity.

2.9 CAROTENOID BIOSYNTHESIS IN MICROALGAE

The carotenoid biosynthesis is routed from the 2-C-methyl–D-erythritol-4-phosphate (MEP) pathway, where isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP) synthesis occurs in plastids through glyceraldehyde-3-phosphate (G-3-P). Following that, IPP (C_5) and its isomer DMAPP (C_5) produce geranylgeranyl pyrophosphate (GGPP), a C_{20} intermediate, by the reaction of GGPP synthase which produces a colorless intermediate phytoene (C_{40}). The condensation of GGPP forms phytoene, which later produces lycopene. Lycopene branches into two products, which are α-carotene and β-carotene, in a reaction catalyzed by lycopene-α-cyclase (*lcyE)* and lycopene β-cyclase (*lcyB*). β-carotene hydroxylase (*chyB* or *crt*) acts on α-carotene to produce lutein, which finally produces loroxanthin by loroxanthin synthase. In another pathway, β-carotene is converted to zeaxanthin or canthaxanthin by the action of β-carotene ketolase (*bkt*), which ultimately forms astaxanthin. The *chyB* or *crt* aids in the conversion of canthaxanthin to astaxanthin, as shown in Figure 2.2 [Tran and Kaldenhoff, 2020].

Extensive research has been conducted to study the microalgae cultivation strategies, harvesting methods, recovery processes, purification techniques, and extraction of microalgal compounds, focusing on their scalability and commercialization within the context of microalgal bioprocessing and biorefinery. However, the industrial production and economic feasibility of microalgal bioproducts remain limited [Sreenikethanam *et al.*, 2022]. Different strategies have been employed in the literature to improve carotenoid biosynthesis in microalgae, including optimization of culture parameters, adaptive laboratory evolution, mutagenesis, and genetic engineering approaches. These strategies are majorly categorized under two divisions: optimization of culture media/ operational parameters, and biotechnological approaches. These are discussed below in detail:

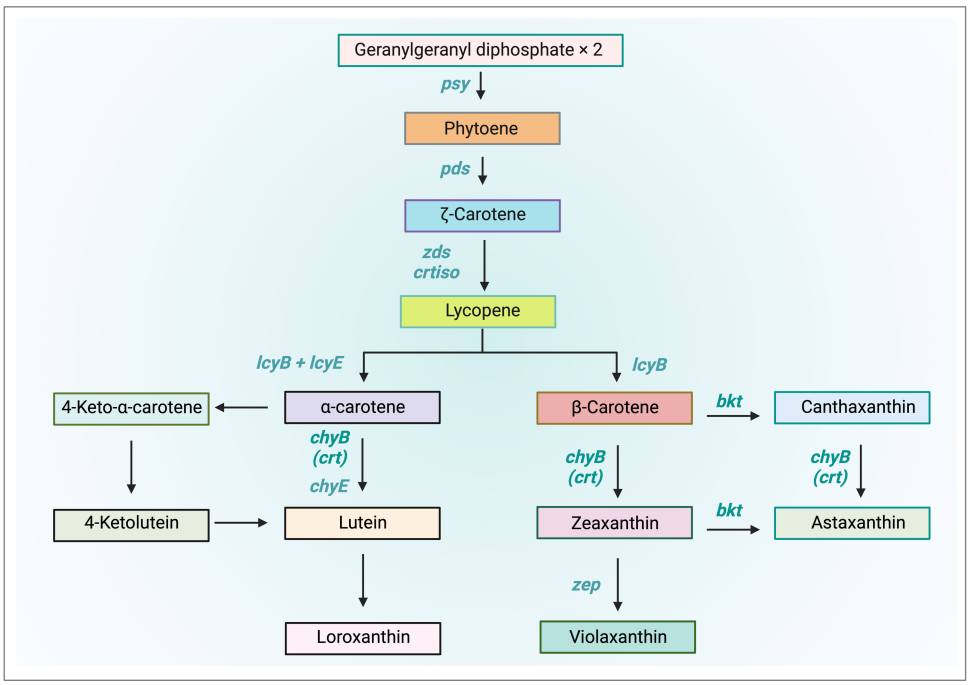

Figure 2.2 : Carotenoid biosynthetic pathway in *C. reinhardtii*. Here *psy*: phytoene synthase, *pds*: phytoene desaturase, *zds*: ζ- carotene desaturase, *crtiso*: carotenoid isomerase, *lcyB*: lycopene-β-cyclase, *lcyE*: lycopene-cyclase, *chyB* or *crt*: carotene-β-hydroxylase, *chyE*: carotene-ε-hydroxylase, *bkt*: β-carotene ketolase, *zep*: zeaxanthin epoxidase (*Source*: Tran and Kaldenhoff, 2020)

2.9.1 Optimization of Culture Media/ Operational Parameters

The common strategies for enhancing productivity are abiotic stress induction (light and salinity), media engineering, and two-stage cultivation as shown in Figure 2.3. For example, the astaxanthin production was increased from 3.53 mg/g to 17.7 mg/g in *H. pluvialis* when the culture was supplemented with 1% NaCl [Gao *et al.*, 2015]. The effect of high light induction under cold stress was used to improve the carotenoid production in the *C. reinhardtii* strain cultivated in TAP media under white light conditions for a 24: 0 h light: dark cycle [Potijun *et al.*, 2021]. In addition, salt stress is majorly employed in two-stage cultivation, wherein in the first stage, biomass production is maximized through optimal growth conditions, followed by accumulation of carotenoids under stress conditions [Li *et al.*, 2022]. Adaptive laboratory evolution undertakes the long-term cultivation of species under selective conditions which help in improving the phenotypes through spontaneous mutations. Therefore, different strategies can be combined to improve carotenoid production through microalgae cultivation.

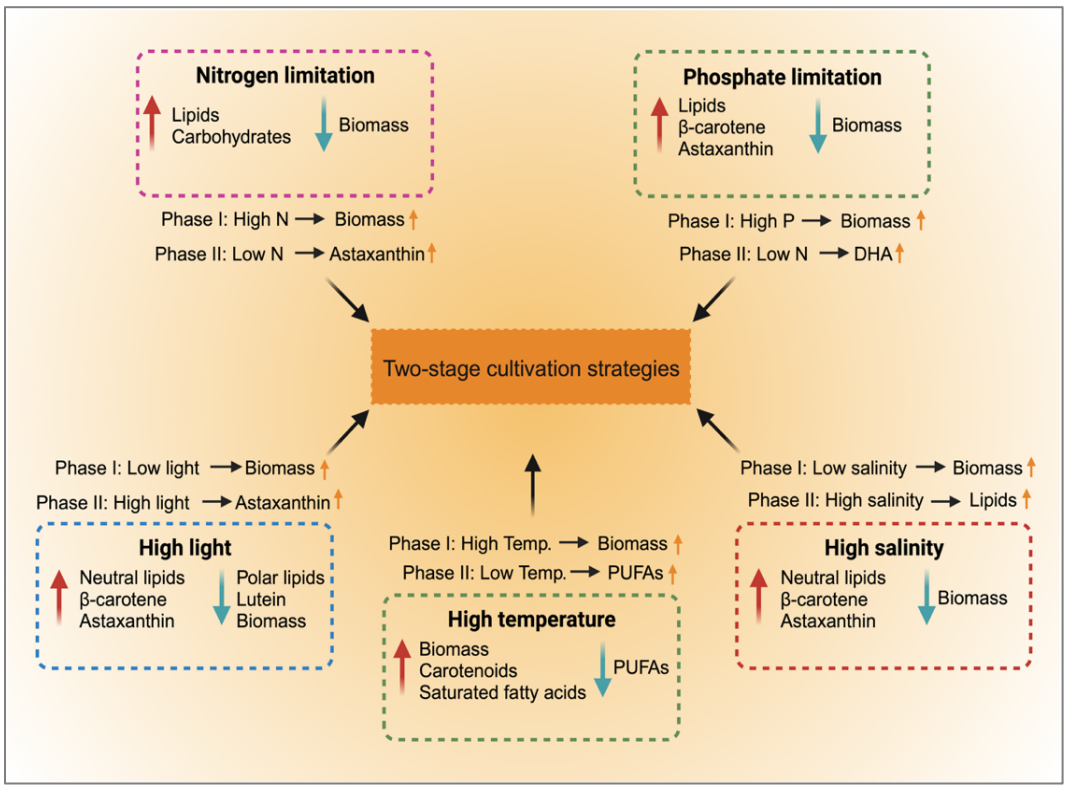

Figure 2.3 : Two-stage cultivation strategy employed for the synthesis of high-value products in microalgae (*Source*: Sun *et al.*, 2018)

2.9.2 Biotechnological Approaches

Among strain improvement methods, genetic/metabolic engineering is rapidly gaining attention to produce value-added compounds such as carotenoids, lipids, PUFAs, and other pharmaceutical proteins [Song *et al.*, 2020; Ganesan *et al.*, 2022; Yadav *et al.*, 2022]. These strategies include random mutagenesis and targeted genetic engineering. Astaxanthin and β-carotene are naturally produced using *H. pluvialis* and *D. virdis*, respectively; however, these microalgae's low productivity reduces the overall production. Therefore, strategies are devised to improve the growth of these microalgae through media engineering and gene alterations [Ren *et al.*, 2021]. Additionally, low carotenoid production strains were altered with exogenous genes from *H. pluvialis* for carotenoid overproduction using eukaryotic expression cassettes with constitutive promoters [León *et al.*, 2007; Zheng *et al.*, 2014; Tran and Kaldenhoff, 2020; Amendola *et al.*, 2023]. Along with this, gene origin is also crucial for determining a metabolite's expression and production, as shown for β-carotene production, where endogenous and exogenous *bkt* expression was studied [Chen *et al.*, 2023]. Further strategies can explore co-expression of these enzymes with robust constitutive promoters which may improve the product yield.

In addition, β-carotene production was increased by knocking down the autophagy-related genes using miRNA in *C. reinhardtii* [Tran *et al*., 2019]. Other strategies use post-transcriptional regulation for the controlled production of a metabolite. For example, Liu *et al*. have overexpressed the 18S rRNA methyltransferase gene to enhance the microalgae biomass and lutein in *C. reinhardtii* [Liu *et al*., 2023]. Moreover, the sequential co-expression of heterologous genes may achieve high production of isoprene, as shown by Yahya *et al*. where the *IbIspS* (*Ipomoea batatas*), *CrBKT* (*C. reinhardtii*), and *ScIDI* (Saccharomyces cerevisiae) genes were expressed constitutively from the nuclear genome of *C. reinhardtii* [Yahya *et al*., 2023].

2.10 GENETIC TRANSFORMATION OF *Chlamydomonas* AND METHODS AVAILABLE

For studying the microalgae genetic function and metabolism pathways, *C. reinhardtii* is used as a model organism. *C. reinhardtii* cells were shown to take up exogenous bacterial DNA. Since then, the integration of exogenous genes into the algal genome and transgene expression has been established into routine techniques [Lin *et al*., 2019a]. *Chlamydomonas* cells have three genomes, including the nuclear genome, plastid genome, and mitochondrial genome, of which the nuclear genome is the major contributor to cell functions. The haploid nuclear genome is divided into 17 linkage groups. The genome size is about 111.1 Mb, with a high G+C concentration of roughly 64%. Notably, in vegetative cells, the nuclear genome is haploid, which facilitates the easy selection of gene mutations. On the other hand, the genomes of mitochondria and chloroplasts are found in numerous copies within a cell, which may result in heteroplasmy [Kong *et al*., 2023]. The genetic engineering in microalgae is affected by its cell wall composition and structure, which create a substantial barrier. Other factors affecting genetic engineering include genomic ploidy, selection agent sensitivity, target organelles (nucleus, chloroplast, and mitochondrion), and transformation method [Perozeni and Baier, 2023].

The first successful transformation in microalgae was accomplished using biolistic bombardment with tungsten/gold particles. The transformation method is effective for both nuclear and chloroplast transformation. In this method, DNA-coated gold microparticles are injected at high speeds at microalgae lawn to insert the DNA of interest into the cells, which results in the transformation efficiency of up to 100-250 transformants per micrograms of plasmid DNA. *Agrobacterium*-based transformation is also employed for microalgae genome engineering, which is a common practice for creating GM plants. Later, simple transformation methods such as electroporation and glass bead methods were introduced. A glass beads-based transformation method was proposed by Kindle, where microalgal cells were agitated in the presence of DNA, glass beads, and polyethylene glycol. It resulted in the nuclear transformation rates of approximately 10^3 transformants per microgram of plasmid DNA [Kindle, 1990]. Electroporation is the most widely used method for genetic engineering. In electroporation, an electrical field is applied to target cells, which opens the cell micropores and increases the membrane permeability, facilitating the delivery of foreign DNA into the cells. It is often used for nuclear transformation where high transformation efficiency can be achieved up to 10^5 transformants per microgram of plasmid DNA. High efficiencies can be obtained for electroporation and glass bead transformation when the microalgae cell walls are removed before transformation with enzymatic treatments [Mosey *et al*., 2021; Jeong *et al*., 2023]. The schematic illustration of these transformation methods is presented in Figure 2.4.

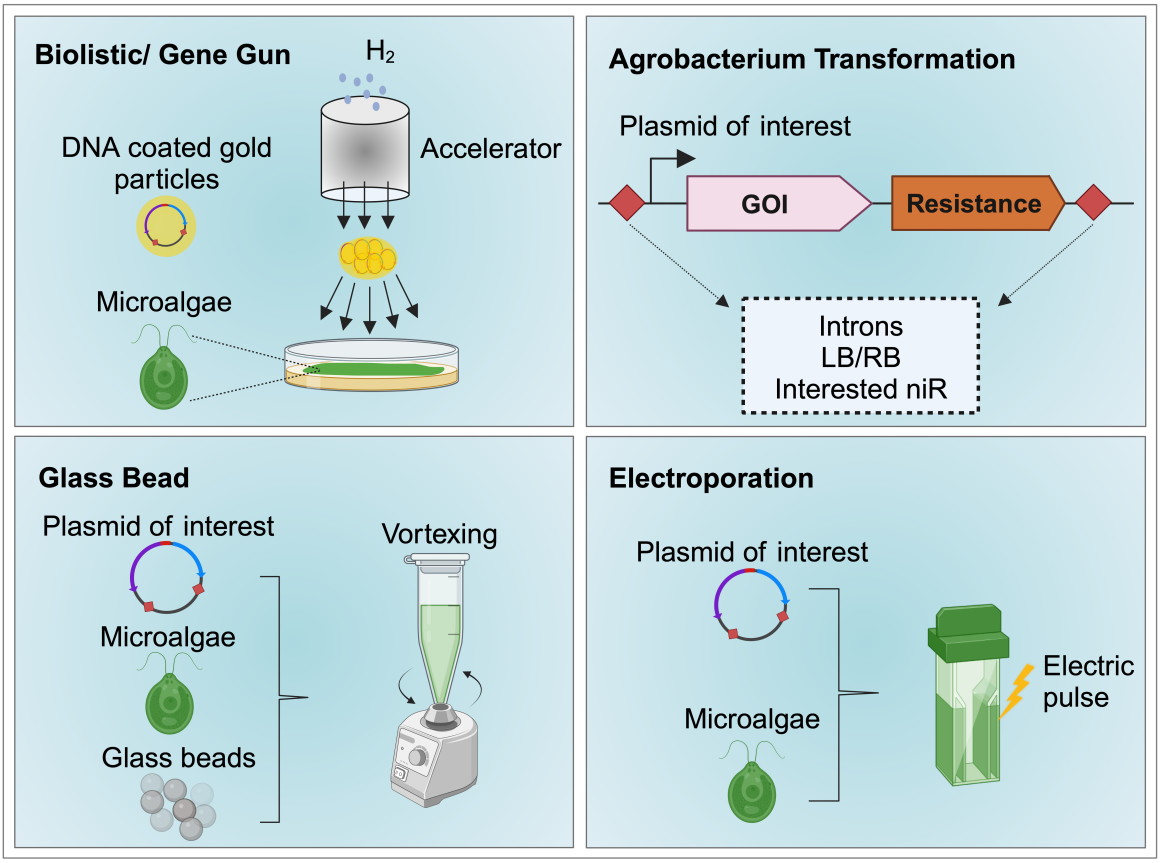

Figure 2.4 : Transformation methods employed for microalgae genetic engineering (*Source*: Lin *et al.*, 2019a)

2.11 MOLECULAR TOOLKITS FOR *Chlamydomonas* GENETIC ENGINEERING

Various molecular tools have been developed for genetic engineering in *C. reinhardtii*. These toolsets are necessary to target specific organelles, introduce and express foreign genes, and accomplish various engineering objectives. Here, we describe a few essential elements of the molecular toolbox, including promoters, reporters, selection markers, introns, and Untranslated Regions (3′ UTR).

2.11.1 Promoters and Terminators

Regulating the expression of genes requires a library of promoters. Both constitutive and inducible promoters are used in *C. reinhardtii*. Endogenous promoters like *Psa*D, *Rbc*S2, *Nit*1, *CA*1, and *Cyc*6, and chimeric promoters such as *Hsp*70A-*Rbc*S2 are frequently used. Exogenous promoters have also been studied in *C. reinhardtii*; however, these failed to establish stable transcription at a high level. Therefore, it is recommended to use endogenous promoters in *C. reinhardtii* [Mosey *et al.*, 2021]. Utilizing viral promoters such as *SV*40 or *CaMV*35S has also yielded a different level of effectiveness. The quest for new promoters and their optimization for robust gene expression is still ongoing [Jeong *et al.*, 2023]. Introducing suitable terminators is also crucial for expressional regulation as they stabilize mRNA products by the addition of polyadenylation or regulate the re-initiation of transcription. However, optimized terminators are still not ready for microalgae transformations. Recently, a Modular Cloning (MoClo) toolkit is developed extensively for *C. reinhardtii* which offers a streamlined and efficient gene expression. The details of this toolkit can be found elsewhere [Crozet *et al.*, 2018].

2.11.2 Reporters and Selection Markers

Auxotrophic mutants were saved in early *C. reinhardtii* transformations by using endogenous genes such as *arg*7, *nit*1, *oee*1, and *atp*C as selection markers. Antibiotic-resistant genes, including *shble*, *aad*A, *aph*VII, *aph*VIII, and synthetic *tet*X, were also integrated over time [Mosey *et al.*, 2021]. The ability of cells to endure in the presence of antibiotics or other selective

agents depends on these indicators. Reporter genes are used to represent and track gene expression visually. Although codon optimization was necessary for *C. reinhardtii* due to their low expression levels, genes producing fluorescent proteins such as green fluorescent protein have been utilized. Investigations have also been made into other fluorescent proteins with emission and excitation spectra tailored for photosynthetic events. Furthermore, non-invasive gene expression screening techniques based on enzymes, such as luciferase, ß-glucuronidase, and arylsulfatase, have been created, which produce fluorescence during expression [Sproles *et al*., 2021].

2.11.3 Introns and 3'-UTRs

Introns are the non-coding region of genes that may have the ability to serve as enhancers, increase transcript stability, and promote nuclear export of transcripts, all of which can have a favorable effect on gene expression. For example, introns such as *RbcS*2 intron 1 are found to increase gene expression in *C. reinhardtii*. Several 3'-UTRs have been effectively used for nuclear or chloroplast gene expression, including *RbcS*2, *Psa*D, and *Psb*A [Sproles *et al*., 2021].

2.12 ROADMAP FOR PRODUCING GM MICROALGAL LINES

For successful GM microalgae production, microalgae strain domestication is initially performed for newly isolated strains for industrial applications. Industrial cultivation calls for strains containing intact cell walls for enhanced resistance to mechanical shear stress for robust cultivation. It is crucial to study its genome widely using high-throughput technologies such as computational and bioinformatic analysis [Mosey *et al*., 2021]. The use of endogenous promoters has been successfully established for transformation events and routinely explored in nuclear transgene expressions such as *Rbc*S2 and *Hsp*70A. Moreover, terminators are introduced at the end of transgenes to regulate expressions. It is also crucial to consider that the *C. reinhardtii* strains have strong codon bias and their genome has high a GC content of up to 68%. Hence, codon optimization of transgenes is required to match the tRNA pool of the host for efficient protein translation in *C. reinhardtii*. Reporter and selection genes make the identification of successful transformation.

A roadmap to produce genetically engineered microalgae and their characterization are presented in Figure 2.5. Multi-omics approaches, including genomic and transcriptomic studies aid in designing and constructing the genes for transformation. Moreover, proteomic and metabolomic data assist in elucidating the biosynthetic pathways and offer functional insights for the biochemical products [Perozeni and Baier, 2023]. The metabolomic library is incomplete for most microalgae species; only the representative genera, including *Chlamydomonas*, have been compiled. The next step involves strain development and resource refinement using standard models and transformation methods. The high throughput transformants are further screened and selected for the desired trait using appropriate methods. Scaling up of these GM strains and their stability needs to be checked for the long-term success of the process [Kumar *et al*., 2020].

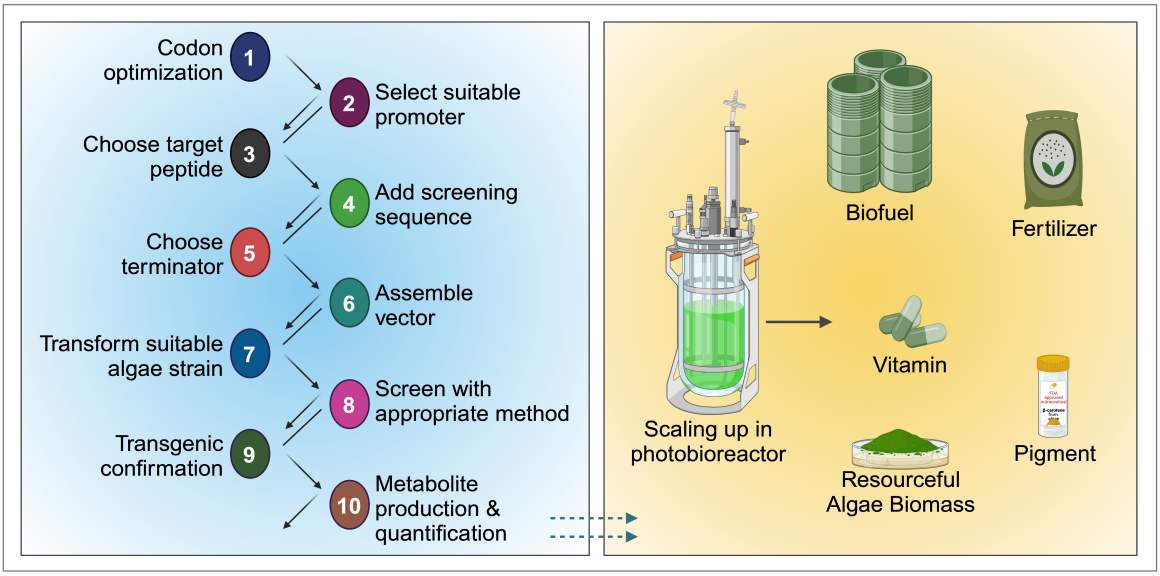

Figure 2.5 : The schematic shows a roadmap to GM microalgae production, characterization, and applications (*Source*: Mosey *et al.*, 2021)

2.13 METHODS FOR GM MICROALGAE CONFIRMATION

It is crucial to verify the effective integration and expression of foreign genes in GM microalgae to ensure the presence and functionality of the foreign gene. GM microalgae are confirmed through several techniques. The most common method is Polymerase Chain Reaction (PCR), which amplifies and identifies the gene's presence in the algal DNA using specific primers to target the introduced transgene. Southern blot analysis is another technique used to locate the transgene in the genomic DNA. This method involves the digestion of DNA using restriction enzymes, gel electrophoresis, and membrane transfer for hybridization with a transgene-specific labeled probe. If the transgene is present, the probe will adhere to it, giving a definite indication [Nawkarkar *et al.*, 2023].

Furthermore, assessing the transgene's expression can be studied through techniques such as Northern blotting or quantitative real-time PCR, which verify the transgene expression from DNA to RNA. Western blotting checks the protein production from the transformed microalgal cells. After gene integration and expression confirmation, these GM lines must be characterized for the phenotypic changes or production of target metabolites. Changes in growth rate, biomass production, and stress tolerance of microalgal lines can be analyzed; these are essential factors to consider for biofuel generation and environmental applications. Furthermore, metabolomics studies can be used to examine the impact of transgene integration on cell's metabolic pathways [Mosey *et al.*, 2021].

2.14 BIOCONTAINMENT OF GM MICROALGAE

The GM microalgae can be cultivated in open ponds or closed systems at an industrial scale. In either case, the industry should maintain the cultivation and handling conditions to avoid the transgene release into the natural environment. Horizontal gene transfer may pose the risk of transferring the gene to other WT species. Selection of a suitable biocontainment strategy would help effectively kill the cells outside the reactors [Sebesta *et al.*, 2022]. Moreover, the escape of foreign genes into the surrounding environment may be prevented by coupling the gene of interest with antisense or RNAi of genes that may increase the fitness of GM algae in the natural ecosystem [Gressel *et al.*, 2013]. Biosafety and regulatory aspects of GM microalgae need to be followed while operating these cultivation units. As per GM microalgae experts, modifying the photosynthesis, biomass, or lipid biosynthesis pathway is not expected to generate harmful strains concerning human health or perturb the surrounding natural ecosystem [Kumar, 2015].

2.15 TYPES OF MICROALGAE CULTIVATION SYSTEMS

It is imperative to select a suitable cultivation system as productivity is highly dependent on the employed cultivation method. The two primary categories, agricultural (open ponds), and industrial (closed cultivation systems), offer distinct advantages. Open ponds, including raceway and circular forms, are structurally simple and easily supervised yet struggle with maintaining pure cultures, while closed PBRs address this limitation by mitigating external contamination risks, enabling precise operational control, and facilitating year-round, high-density biomass production [Narala *et al*., 2016]. Among the available cultivation methods, the two major categories are open ponds and closed PBRs which are shown in Figure 2.6. These will be elaborated in detail in the following sections:

2.15.1 Open Ponds

Open pond cultivation offers a simple, low energy demand, cost-effective, and easy-to-scale-up cultivation strategy that is industrially adapted. The pond depth is generally kept shallow for light penetration. The open ponds include raceway ponds and circular forms, which offer ease of supervision and structural ease and can be either agitated or inclined [Narala *et al*., 2016; Tan *et al*., 2020]. The open pond cultivation is used majorly for photoautotrophic nutrition mode for *Chlorella* sp., *Spirulina* sp., *Haematococcus* sp., *Dunaliella salina,* etc. One major limitation of open pond cultivation is poor mixing of the biomass, resulting in the thickening of biomass on bottom lines. Also, the heterotrophic cultivation in open ponds is not a viable option as these struggle to maintain the axenic cultures [Chandrashekharaiah *et al*., 2021]. Additionally, the seasonal variations result in changes in diurnal temperature, light intensity, and evaporation losses, the major factors affecting the open pond performance. Moreover, environmental issues such as rainwater runoff may affect the growth condition for microalgae such as salinity, pH, and erosion of banks that result in lower biomass productivity [Tan *et al*., 2020]. Care should be taken while cultivating GM microalgae in open cultivation systems, which have the possibility of any spillage and the risk of horizontal gene transfer in other related organisms, including cyanobacteria. It is suggested to create an open pond surrounded by a buffer zone to minimize the escape of transgenes into the environment. Also, it is important to have regular surveillance to reduce the potential risks in scaled-up applications [Kumar, 2015].

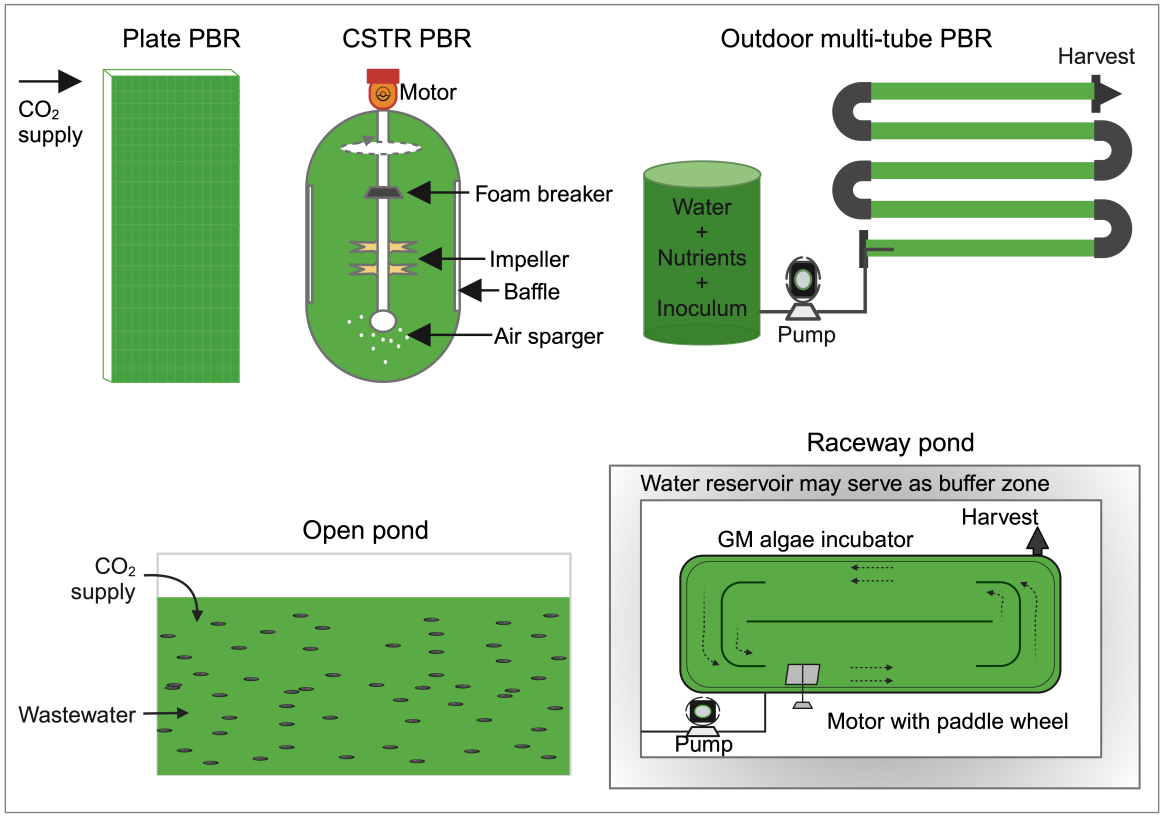

Figure 2.6 : Various microalgae cultivation systems employed for large-scale applications (*Source*: Kumar, 2015)

2.15.2 Enclosed Systems and Photobioreactors

Photobioreactors (PBRs) are the specialized bioreactor systems used to cultivate phototrophs, such as microalgae and cyanobacteria, in an enclosed system that does not allow a direct material exchange between the culture and environment. Closed PBR offers better operational control, production of high-density biomass throughout the year, and limits external contamination [Tan *et al*., 2020]. A closed PBR supports GM microalgae cultivation by preventing contact between the enclosed microalgae and the environment. However, scaling up is difficult in closed systems [Kumar, 2015].

It supports heterotrophic cultivation, such as *C. reinhardtii*, which is grown in TAP media. The most employed PBRs include flat-plate, horizontal plastic plates, tubular, vertical PBRs, attached /biofilm PBRs, airlift, bubble column PBRs, and multi-stage continuous flow reactors. Microalgae culture is circulated through the pump and returned to a reservoir. In the closed PBRs, a turbulent flow is maintained using mechanical or airlift pumps [Pathak *et al*., 2018]. In the helical PBRs, circular tubes are coiled over a glass tube where microalgae culture is circulated to improve the photosynthetic efficiency through effective light penetration due to the high surface area, which results in high biomass productivity. Attached/biofilm-based microalgae cultivation, such as plate PBR, allows the surface cultivation of microalgae, which also helps to reduce the cost and energy-intensive harvesting processes. The closed systems excel in producing metabolites, biofuels, and CO_2 sequestration [Paul *et al*., 2021]. Nonetheless, their costliness relative to open ponds, and the need to eliminate DO from the liquid to prevent cell auto-oxidation pose challenges, often addressed through membrane systems or nitrogen infusion [Kishi *et al*., 2020].

2.16 BOTTLENECKS ASSOCIATED WITH EXISTING MICROALGAE CULTIVATION SYSTEMS

The primary focus of industrial microalgae cultivation is to enhance the productivity of high-value compounds and biofuels to maximize the microalgae biorefinery outcomes. The current scaled-up applications of biorefineries are executed through raceway ponds or tubular PBRs. Nevertheless, current strategies for cultivating microalgae suffer from several drawbacks

that must be addressed to make microalgal products viable [Chi *et al.*, 2022]. Therefore, the microalgae cultivation units must be restructured for higher production efficiencies. A detailed comparative assessment of available microalgae cultivation systems is presented in Table 2.4 which suggests the usefulness of PMFCs for scaled-up applications and microalgae-based biorefineries. Hence, PMFCs have now been explored as the choice of reactor to enhance microalgae production in an integrated wastewater remediation and microalgae biorefinery system [Khandelwal *et al.*, 2018; Das *et al.*, 2023; Sharma *et al.*, 2023].

Table 2.4 : Comparative Assessment of Various Microalgae Cultivation Systems [*Source*: Kothari *et al.*, 2017; Khandelwal *et al.*, 2023]

S. No.	Parameters	Open systems	Closed systems	PMFCs
1	Algal species	Restricted	Flexible	Flexible
2	Contamination control	Difficult	Easy	Easy
3	Operation regime	Batch or semi-continuous	Continuous	Semi-continuous/continuous
4	Area/volume ratio	Low	High	High
5	Main criteria for species selection	Growth competition	Shear-resistance	Shear- resistance
6	Cultivation period	Limited	Extended	Extended
7	Evaporative losses	High	Low	Low
8	CO_2 sequestration	Low	High	Very high
9	Temperature control	None	Excellent	Excellent
10	Most expensive	Mixing	Oxygen control, temperature control	Temperature control, reactor fabrication
11	Degasification	Not required	Required	Inherent deoxygenation
12	Process control	Difficult	Easy	Easy
13	Scale-up	Easy	Difficult	Difficult
14	Light utilization efficiency	Poor	Excellent	Excellent
15	Energy recovery	Low	Low	High
16	Biomass productivity	Low	High	Excellent
17	Harvesting efficiency	Low	High	High
18	Investment	Low	High	High
19	Operation costs	Low	High	High

2.17 MFC PRINCIPLE AND COMPONENTS

These systems exploit microbial metabolic redox reactions to convert the chemical energy present in organic compounds/wastes into electrical energy. The electrons harvested from the degradation of organic matter are then transported to the cathode electrode via an external circuit, where they combine with a terminal electron acceptor (often oxygen), producing an electric current. Electroactive bacteria residing in the anode chamber of MFCs play a pivotal role

in generating electrons [Logan *et al.*, 2006]. The power output from MFC is affected by several factors, which include MFC configuration, electrode material, anode/cathode catalysts, membrane material, electrode spacing, microbial characteristic/biofilm properties, and operational conditions in both chambers, including inoculum source, substrate type, and pH [Du *et al.*, 2007; Do *et al.*, 2018]. The MFC basic components are shown in Figure 2.7, and discussed in the below sections:

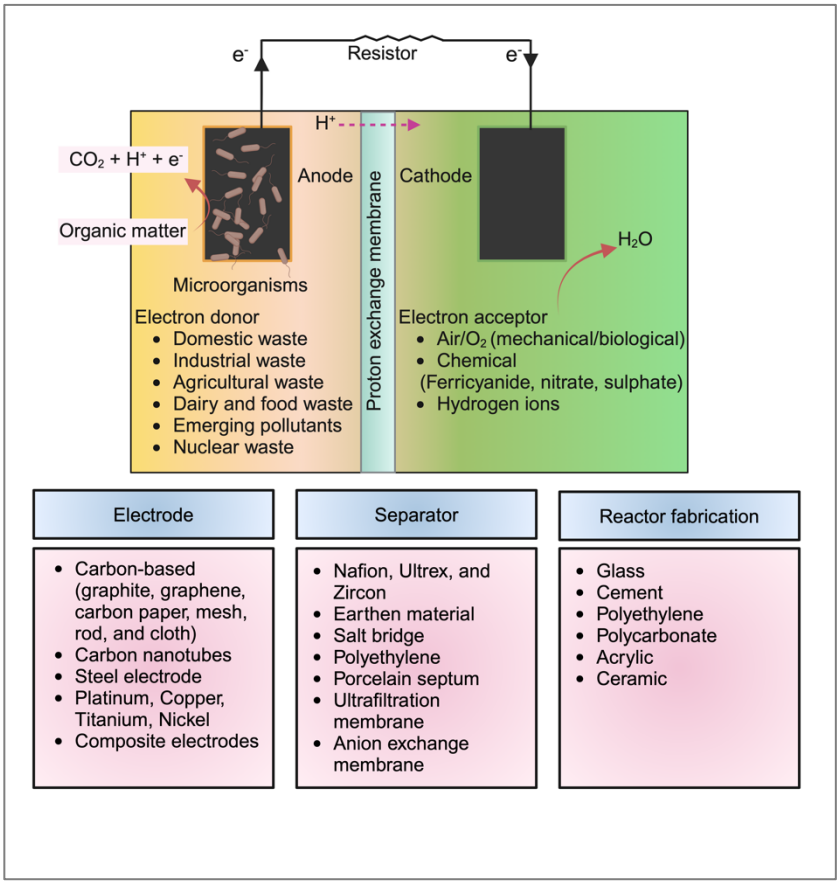

Figure 2.7 : A basic dual chamber MFC fundamentals, and components (*Source*: Logan *et al.*, 2006)

2.17.1 Electrochemically Active Microorganisms

MFC applications are majorly governed by electroactive organisms, which can extracellularly donate their electrons from cells to external electron acceptors. Common exoelectrogenic bacteria include *Shewanella sp., Geobacter sp., Pseudomonas sp.,* and *Rhodoferax sp.* There are three different mechanisms reported for electron transfer in MFC, namely direct electron transfer, use of nanowires, and indirect electron transfer using soluble mediators or shuttles as shown in Figure 2.8 [Reguera *et al.*, 2005; Logan *et al.*, 2006; Santoro *et al.*, 2017]. These soluble mediators can be added to the anode/cathode chamber or can be produced during bacterial metabolism. These mediators undergo a cyclic oxidation-reduction cycle to transfer electrons to electrodes [Logan, 2009]. Direct electron transfer is aided by cell outer membrane cytochromes or using conductive pili or nanowires (cellular extension) [Kim *et al.*, 1999; Bond *et al.*, 2002].

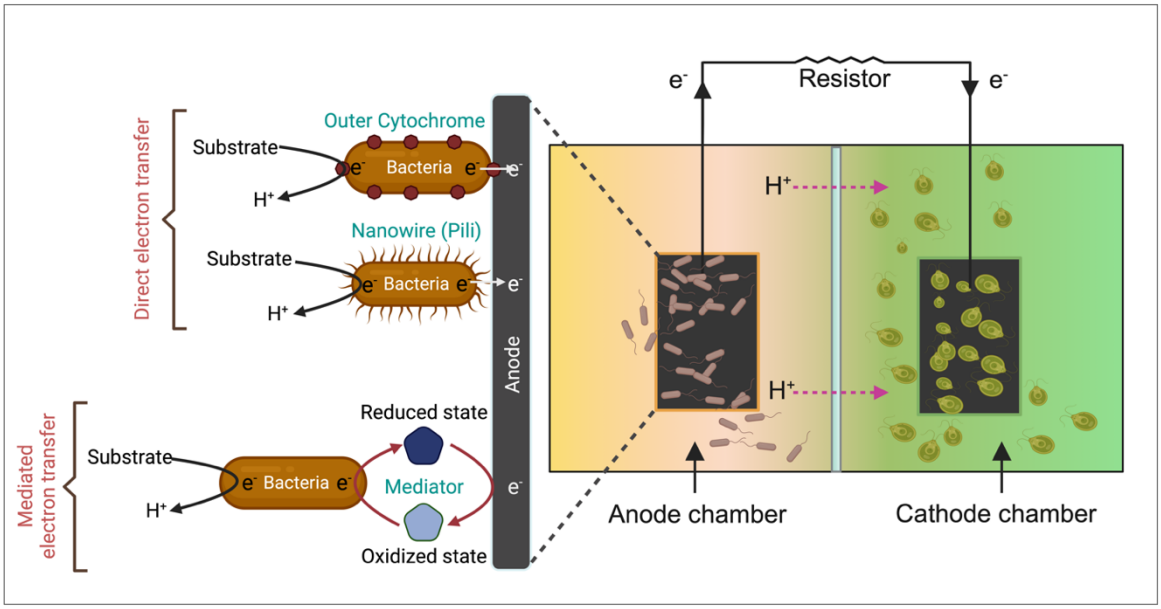

Figure 2.8 : The schematic shows the electron transfer mechanism at anode chamber in MFC (*Source*: Santoro *et al*., 2017)

2.17.2 Anode

The anode electrode is maintained in an anaerobic chamber. The oxidation reactions occur at the anode surface, acting as an electron acceptor. Anode material should have high electrical conductivity, corrosion resistance, biocompatibility, high surface area, mechanical strength, chemical stability, and cost-effectiveness for scaling up applications [Wei *et al*., 2011]. Carbon-based electrodes are the most preferred choice as electrode material [Zhou *et al*., 2011]. Recently composite electrode materials have been prepared for cost-effectiveness and long-term operations [Santoro *et al*., 2017].

2.17.3 Cathode

The cathode electrode can be biotic and abiotic based on the applications. Abiotic electrodes consist of chemicals/electrode catalysts for reduction reaction, while biotic electrodes may consist of microalgae/bacteria, which may produce terminal electron acceptors, such as nitrate and oxygen. Among biotic electrodes, denitrifying bacteria and microalgae are commonly employed [Liu *et al*., 2014; Sharma *et al*., 2023]. Platinum is the most extensively utilized cathode catalyst in the MFC due to its high catalytic activity for oxygen reduction [Wei *et al*., 2011]. However, its application in microalgae MFC is limited due to toxicity, and it is inactivated by wastewater ionic species such as phosphate, sulfate, and chloride [Bard and Faulkner, 2001; Logan *et al*., 2006]. Recently, non-platinum catalysts, including transition metals, metal oxides, and carbon nanostructures, have also been explored due to added advantage and cost-effectiveness [Chaturvedi and Kundu, 2021; Khandelwal *et al*., 2021; Liu *et al*., 2022].

2.17.4 Membrane/ Separator

MFC uses membranes or separators to maintain the redox gradient across the anode and cathode chamber [Li *et al*., 2011]. A separator should be impermeable to oxygen and organic acids, resistant to biofouling, and, have high ionic conductivity [Logan, 2012; Leong *et al*., 2013]. The most used proton exchange membrane is Nafion which is not recommended for scaled-up reactors due to high cost and biofouling in long-term operation. Recent studies have identified new separator materials, including Zircon, ceramic membranes, ultrafiltration membranes, J-cloth, nylon fibers, and glass fibers. Three-chamber MFCs also require anion exchange membrane and cation exchange membranes. Moreover, the membrane-less configurations, such as sediment MFC, use the column height to maintain the redox gradients [Santoro *et al*., 2017].

2.18 MFC PERFORMANCE AND POWER LOSSES

The maximum theoretical obtained cell voltage in MFC is in order of 1.1 V based on an acetate oxidizing anode (HCO_3^- = 5 mM, CH_3COO^- = 5 mM, pH = 7) and an oxygen-reducing cathode [Schröder, 2007]. The overall cell voltage for the above reactions can be described as:

Anode: $CH_3COO^- + 2H_2O \longrightarrow 2CO_2 + 7H^+ + 8e^-$ (E_{An} = -0.28 V)

Cathode: $O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$ (E_{Cat} = +0.82 V)

The overall cell voltage is:

$$E_{Cell} = E_{Cat} - E_{An}(+1.1\,V) \quad (2.1)$$

The ideal cell voltage or Open Circuit Voltage (OCV) is a thermodynamic value that does not consider internal losses and is measured in the absence of current. However, the system encounters several losses, which lowers the cell operating voltage. Different voltage/power losses can be quantified using a polarization curve (current v/s voltage), showing three significant regions: activation losses, mass transfer losses, and Ohmic losses [Larminie and Dicks, 2003].

The activation losses at the electrodes show a rapid fall in the voltage. These activation polarization losses on the anode and cathode are represented as $\eta_{act,An}$, and $\eta_{act,cat}$, respectively. Activation energy is required for any electrochemical reaction (reduction/ oxidation); however, the losses occur during the electron transfer process from/ to a compound to the electrode surface. These compounds can be redox mediators in the solution or on the bacterial surface. These activation losses can be minimized by adding external mediators to improve the electron transfer process, using efficient catalysts, increasing the surface area of electrodes, promoting biofilm enrichment on the electrode surface, and optimizing the operating temperature [Logan *et al*., 2006]. The following region, where there is a linear drop in the voltage with an increase in the current, is constituted by Ohmic losses. These Ohmic losses (η_{Ohm}) occur due to the resistance in the flow of ions present in the electrolyte and the flow of electrons from the electrode.

The Ohmic drop is represented by *IRi*, where *I* is the current while *Ri* is the internal resistance in the system. The ohmic resistance can be lowered by improving the solution's conductivity and reducing the electrode spacing [Zhao *et al*., 2009; Santoro *et al*., 2017]. The mass transfer /concentration losses are shown by a rapid decrease in the voltage with the current, which happens due to the concentration polarization between the anode and cathode chamber.

Concentration losses occur due to the slow mass transfer when the substrate concentration becomes a limiting factor, limiting the current production through the MFC system. Concentration losses can be minimized by increasing the mass transfer rate from/to the reactive species. The stirring and bubbling of the solution can help in reducing the concentration gradient in the bulk solution [Logan *et al*., 2006].

2.19 TOOLS AND TECHNIQUES TO STUDY MFC PERFORMANCE

There are various tools and techniques to study the MFC performance. These techniques involve the electrochemical assessment, biochemical properties, and microbial community profiles. Some of these techniques are briefly described below:

2.19.1 Voltage Measurement

The performance of MFC can be evaluated by measuring voltage and power generation. The cell voltage is defined as a potential difference between the anode and cathode. The ideal cell voltage is calculated using Eq. 2.1. The Open Circuit Voltage (OCV) is the cell voltage in the absence of the current when the anode and cathode are not connected by any load. The OCV is a measurement of cell voltage at infinite resistance. Theoretically, the OCV should approach the cell emf, which is equal to cell voltage, without accounting for internal losses [Larminie and Dicks, 2003; Zheng *et al*., 2020].

The potential of anode/cathode, also called open circuit potential can be calculated by monitoring the voltage against the reference electrode. The examples include Standard Hydrogen Electrode (SHE) (0 V), silver-silver chloride (Ag/AgCl) electrode (+0.197 V v/s the SHE), and saturated calomel electrode (0.242 V v/s the SHE) as reference electrodes.

2.19.2 Polarization Curve and Power Density

Polarization curves and power density curves are essential tools for evaluating the performance of MFCs [Zhao *et al*., 2009]. The polarization curve shows the relationship between voltage and current density, as shown in Figure 2.9. This curve can be made for the anode, cathode, or whole cell using a potentiostat or a multimeter. The curve helps to elucidate the optimum load a system can take up based on its kinetics. The power of the MFC system can be calculated by connecting the anode and cathode via the appropriate load/resister using the given equations:

$$P = I\, E_{Cell} \tag{2.2}$$

Where E_{cell} is the voltage of the system and I current is calculated using Ohm's law:

$$I = {}^{E_{Cell}}/_{R} \tag{2.3}$$

Where R represents the external resistance applied to the MFC system.

The power density is calculated by normalizing power with either electrode projected surface area or reactor volume. The unit of power is Watt (W), and power density is represented by either W/m^3 or W/m^2 [Logan, 2012]. The voltage and current response at variable resistances or through potentiostat are measured and plotted. It shifts the system away from equilibrium during polarization. At higher resistance, the voltage value is increased while the current is low. Also, the low resistance supports the low voltage and high current. The three losses (mentioned in section 2.18) can be estimated by analyzing the polarization curve [Zhao *et al*., 2009].

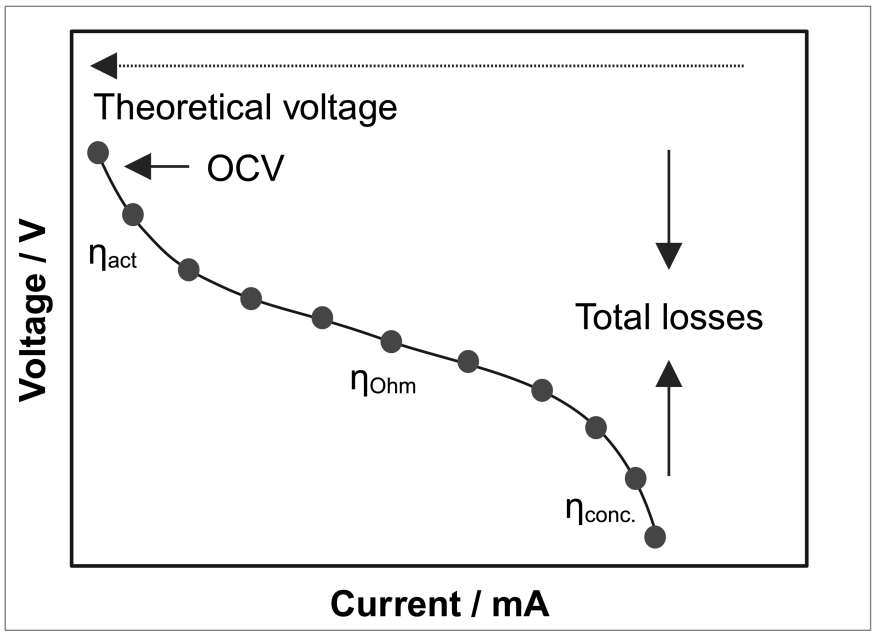

Figure 2.9 : A polarization curve showing various power losses (*Source*: Zhao *et al*., 2009)

A power density curve is derived from the polarization curve and focuses on power output. In the power curve at a particular resistance value, the voltage and current are at optimum value while the power is at maximum. The maximum power is shown as the midpoint on the hyperbolic curve, which provides insights into the maximum power that an MFC can produce at various operating conditions. These curves provide a comprehensive understanding of the electrical characteristics of the MFC, which helps in the optimization of design and

operation for different applications, including wastewater treatment, biofuel production, and power generation [Logan, 2012].

2.19.3 Cyclic Voltammetry

Cyclic Voltammetry (CV) is a non-destructive, highly informative potentiodynamic electrochemical technique used to study redox reactions at the electrode surface. CV is based on measuring the current response as a function of applied potential (voltage) over time [Sánchez *et al*., 2020]. During a CV experiment, the working electrode's potential (typically the anode in the case of an MFC) is swept linearly between two end points, typically referred to as the starting and ending potentials. The potential is swept back and forth at a constant scan rate, and the resulting current is recorded. The CV curves are obtained by plotting the current against the applied potential, as shown in Figure 2.10. The CV can help to characterize the electron transfer reaction between microorganisms and electrodes. Different mediators involved in electron transfer reactions can be identified through the CV curve based on peak potentials [Santoro *et al*., 2017]. New catalyst materials can be studied based on their ability to enhance anodic/cathodic currents using the CV curves [Logan, 2012].

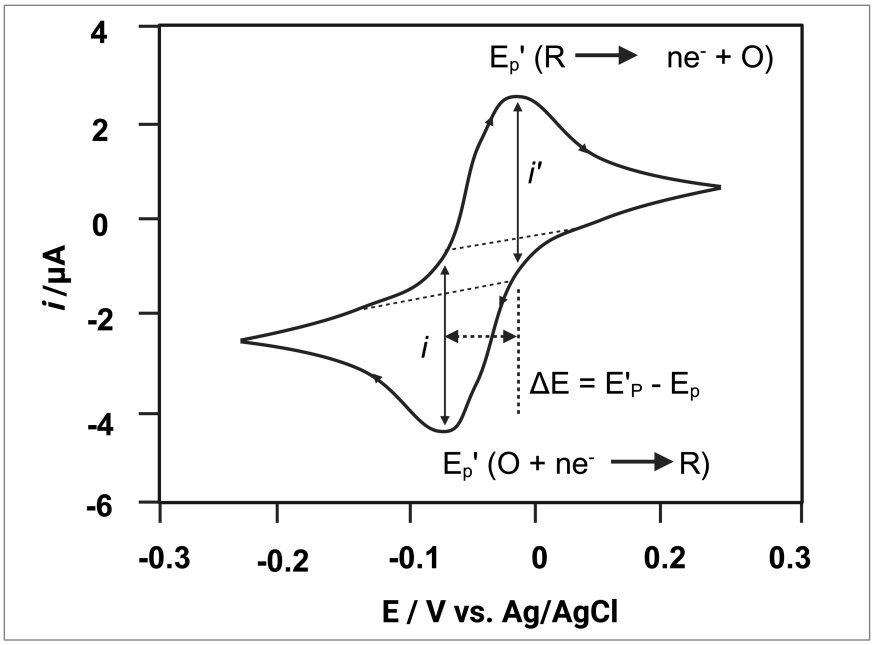

Figure 2.10 : A cyclic voltammogram showing reversible redox couple showing peak potentials, peak currents and the potential difference ΔE between the reduction and oxidation peak (*Source*: Zhao *et al*., 2009)

2.19.4 Electrochemical Impedance Spectroscopy

Electrochemical Impedance Spectroscopy (EIS) is used to investigate the internal resistance, including Ohmic losses, mass transfer, and charge transfer losses within MFCs. This includes applying a small AC voltage amplitude on the system (either electrode or complete cell) and measuring the corresponding current response through the cell in terms of its magnitude and phase shift [Wagner *et al*., 1998]. Figure 2.11 shows the difference between the charge transfer parallel *RC* impedance as kinetic control (semicircle at medium-high frequencies), which can be distinguished clearly from the mass transfer derived impedances (sloped line at low frequencies).

EIS measures the impedance (*Z*) of an MFC as a function of the applied frequency. Impedance is a complex quantity consisting of resistance (*R*) and reactance (*X*). Resistance (*R*) represents the real part of the impedance and is associated with the ohmic resistance within the MFC. It includes resistance from the electrolyte, electrode materials, and other conductive paths. Reactance (*X*) represents the imaginary part of the impedance and is associated with the capacitive and inductive effects in the system. The EIS data can be represented using the Nyquist plot (Figure 2.11) and the Bode plot. In the Nyquist plot, the x-axis and y-axis are the real part

(Z_{Re}) and the imaginary part (Z_{lm}) of the impedance ($Z_\omega = Z_{Re} + jZ_{lm}$), respectively. EIS is analyzed using the most appropriate equivalent circuit, which may comprise the resistor, capacitor, and Warburg elements in series/parallel. However, interpreting impedance spectra is complex, which involves modeling and fitting the data using equivalent electrical circuits [Zhao *et al.*, 2009].

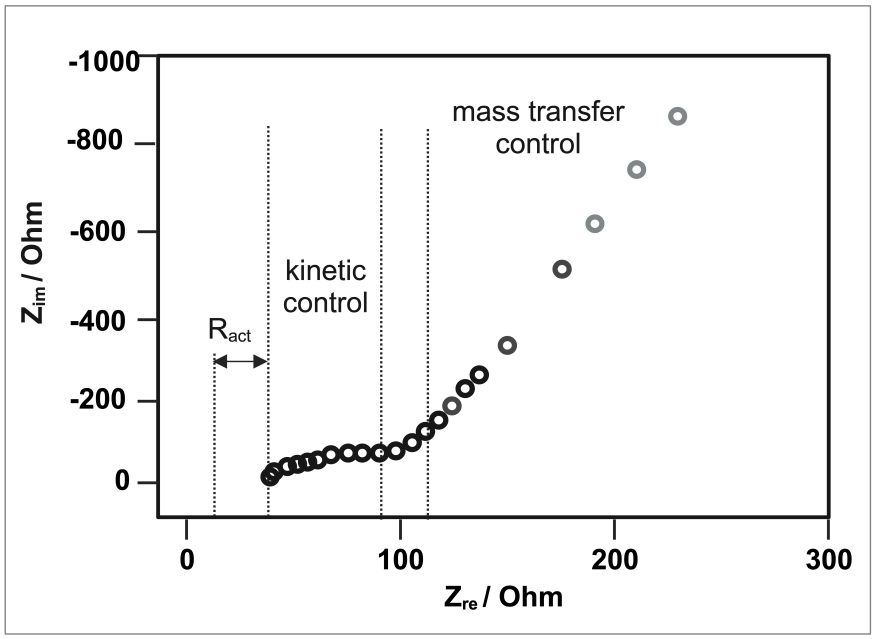

Figure 2.11 : A typical Nyquist plot of the impedance spectrum measured for single chamber MFC with two-electrode configuration under open circuit voltage (*Source*: Zhao *et al*., 2009)

2.19.5 Coulombic Efficiency

The Coulombic Efficiency (CE) measures the ratio of the total coulombs transferred from substrate to anode to the maximum possible coulombs if all substrate degradation resulted in current production. The total coulombs can be determined by integrating the current over time or measuring the area under the curve for the current vs time curve. For a fed-batch run MFC, CE is determined over a period t, which is calculated by the given equation [Logan, 2009]:

$$\mathrm{CE} = \frac{M \int_0^t I dt}{FnV_{An}\Delta COD} \tag{2.4}$$

Where, where M = 32 (molecular weight of oxygen), F is Faraday's constant, n = 4 (number of electrons exchanged per mole of oxygen), V_{An} is the volume of the anode chamber, and ΔCOD is the difference in COD over a period.

2.19.6 Analytical Chemistry

Gas Chromatography and High-Performance Liquid Chromatography (HPLC) quantify substrates, metabolites, and organic pollutants in influent and effluent samples. These techniques are essential for evaluating the degradation of organic matter, monitoring byproduct formation, and tracking the transformation of specific compounds. UV-vis spectroscopy assesses the absorption of ultraviolet and visible light by compounds. It provides information on chemical composition, concentration, and compound transformation [Sekar and Ramasamy, 2013].

2.19.7 Microalgae Growth Kinetics

Industrial microalgae cultivation is assessed based on two important parameters: microalgae growth rate and biomass productivity. These parameters help to estimate the microalgae production at scaled-up systems [Khandelwal *et al*., 2018]. The specific growth rate (μ) of the microalgae was calculated using the equation:

$$\mu = \frac{LnN_2 - LnN_1}{t_2 - t_1} \tag{2.5}$$

Where μ is the specific growth rate, and N_1 and N_2 are the number of cells at time 1 (t_1) and time 2 (t_2), respectively.

Similarly, the biomass productivity (P, g/l/d) is calculated by the given equation:

$$P = \frac{N_2 - N_1}{t_2 - t_1} \quad (2.6)$$

Where N_1 and N_2 are defined as the biomass at time 1 (t_1) and time 2 (t_2), respectively.

2.19.8 Enzyme Assays

Enzyme assays determine the enzymatic activity of microorganisms within the MFC. By measuring the activity of specific enzymes involved in metabolic pathways, researchers gain insights into microbial processes responsible for organic matter degradation and electron transfer [Logan, 2012].

2.19.9 Microbial Community Profiling

Identifying new bacterial species is important for MFC application which is done using standard microbiology procedures. Also, microbial profiling can be done using 16S rRNA-based gene clone libraries, and denaturing gradient gel electrophoresis, which shows differences between communities. Moreover, pyrosequencing can be employed to probe more deeply into the community members [Zhi *et al*., 2014]. This data reveals microbial diversity, their roles in electron transfer and substrate degradation, and the impact of changing operational conditions on microbial community structure [Logan, 2012].

2.19.10 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) techniques provide high-resolution images of the microbial biofilm on MFC electrodes. These images can reveal the spatial organization of microorganisms, biofilm thickness, and interactions between microorganisms and electrode surfaces, which are critical for understanding biofilm development and performance [Slate *et al*., 2019].

2.20 FACTORS AFFECTING MFC PERFORMANCE

MFC performance is a collective efficiency of its reactor design, electrode material, catalysts utilized, and microbial community responsible for electron transfer. Anodic and cathodic biofilm can also be modified to enhance the MFC performance. Common strategies, include manipulating key enzymes in bacteria to enhance electron transfer, modulating redox mediators, and incorporating foreign traits to improve biofilm performance. Researchers are also exploring microbial communities from different niches to study their role in power production under various operational conditions [Logan, 2012]. Also, studies are ongoing to develop innovative reactor designs, alternative cathode catalysts, and novel composite/nanomaterial-coated electrodes to improve MFC performance. However, to reduce MFC systems' capital and operational expenditures, their integration with complementary technology would be imperative for making it self-sufficient [Santoro *et al*., 2017].

2.21 ECONOMIC ASSESSMENT OF THE MFC SYSTEMS

The PMFCs are becoming promising technology for electricity generation, wastewater treatment, biomass generation and CO_2 sequestration. For the successful implementation of PMFCs on a large scale, it is essential to evaluate their economic feasibility and other key parameters [Sonawane *et al*., 2024]. These include capital cost, operation and maintenance costs, revenue, net present value, benefit-to-cost ratio, internal rate of return, discounted payback period, and sensitivity analysis. These parameters are described in Table 2.5. Assessing these factors before scaling up will aid in the successful deployment of the technology. Since MFCs are not yet employed in microgrids, comprehensive data on scaled-up capital, operating, and maintenance costs is lacking. Additionally, the long-term useful life of MFCs remains

undetermined due to the corrosive fumes from wastewater, which can rapidly deteriorate metal infrastructure and necessitate extensive maintenance over time. Among the various parameters, capital expenditure for reactor and electrode fabrication is a major cost factor. Therefore, developing low-cost strategies and enhancing the durability of reactors and electrodes are crucial. PMFC systems that incorporate microalgae have not yet been scaled up for bioenergy applications and VAPs production. Therefore, scaling up these systems is necessary to perform a comprehensive LCA of the process [Khan *et al*., 2023].

Table 2.5 : Cost and Components for Economic Assessment of PMFC Systems [*Source*: Khan *et al*., 2023]

S. No.	Components	Description
1	Capital cost	The initial investment required based on the market value of equipment, including reactor material, electrodes, chemical supplies, and electrical connections.
2	Operation and maintenance	Running expenses such as feedstock arrangement costs, maintenance, monitoring, post-treatment water transportation, and administrative costs.
3	Revenue	The amount generated from selling electricity and clean water, after excluding transportation costs. It also includes revenue from algae biomass and related bioproducts.
4	Net Present Value	The return on the initial investment, considering the time and value of money.
5	Benefit-to-cost ratio	Indicates the immediate benefit relative to the cost for decision-making.
6	Internal rate of return	The rate at which the investment and costs are equal.
7	Discounted payback period	Indicates the period needed to recover the initial investment.
8	Sensitivity analysis	Identifies the impact of changes in different variables.

2.22 APPLICATIONS OF MFC

MFCs were initially applied for their potential to produce electrical energy. However, the power output resulting from MFC applications ($< 6W/m^2$; $\leq 500W/m^3$) is relatively meager compared to chemical fuel cells (140 kW/m^3). As a result, these systems cannot function as standalone energy sources [Arends and Verstraete, 2012]. The MFC applications can become more sustainable when coupled with high-value applications such as wastewater treatment, microalgae cultivation, powering remote sensors, biosensors, carbon capture and sequestration, H_2, and VAP production [Santoro *et al*., 2017]. The prominent applications of MFCs are shown in Figure 2.12 and discussed briefly in the following sections.

2.22.1 Wastewater Treatment and Metal Recovery

Conventional wastewater treatment is an energy-intensive technology but integrating it with MFC technology can offset the total energy requirements of the treatment process. About 1.5% of the total electricity is invested in wastewater treatment alone. About 0.5 kWh/m^3 of energy is required for the aeration process, which is significantly high for scaled-up systems [Logan, 2008]. It is reported that wastewater contains approximately 9.3 times more energy than is required to treat water [Shizas and Bagley, 2004]. For example, domestic, animal, and food wastewater contains 17 GW of power [Logan, 2008]. Different types of wastewater, including domestic wastewater, dairy wastewater, textile dye wastewater, industrial wastewater, brewery wastewater, and petroleum hydrocarbon wastewater, have been treated in the anode chamber [Do *et al*., 2018]. The MFC cathode chamber also supports wastewater treatment using the cathodic reduction reaction or the use of biological phenomena employing bacteria or microalgae [Wang *et al*., 2023].

2.22.2 Bioremediation

Several studies have proven the applicability of MFC for bioremediation applications. MFC offers oxidation at the anode chamber while reduction at the cathode chamber, which helps remove pollutants from the wastewater. Metals such as mercury, arsenic, chromium, and ions such as nitrate and sulfate can be reduced and precipitated at the cathode. Among all these compounds, nitrate (NO_3/N_2 +0.74V), iron (Fe^{3+}/Fe^{2+} +0.77V) and manganese (MnO_2/Mn^{2+} +0.60V) have a high redox potential but sulfate (SO_4^{2-}/H_2S -0.22V) has relatively lower redox potential [Thauer *et al*., 1977; Rhoads *et al*., 2005]. The use of electron acceptors other than oxygen at the cathode also eliminates the risk of oxygen diffusion at the anode, thereby imparting high CE to the process [Al-Mamun *et al*., 2016]. Cathodic denitrification is another primary research domain in MFC, which has shown enormous potential [Vijay *et al*., 2019]. Another advantage associated with using MFC is that the bacterial sludge formed at the end of the treatment process is considerably less when compared to activated sludge. The success of the procedure depends on the effectiveness of the microbial consortia, which can be acclimatized for the supplied pollutant.

2.22.3 Electricity Generation

MFCs can convert chemical energy in organic matter into electrical energy with the aid of microorganisms. Efforts are underway to enhance the device's power output and the CE. The CE as high as 97% is obtainable in MFC as opposed to other thermodynamic systems. The reports of CE values in the order of 80%, 89%, and 97% exist in the literature [Chaudhuri and Lovley, 2003; Rabaey *et al*., 2003; Rosenbaum *et al*., 2005]. The overall power output from MFCs is meager for large-scale applications. The electricity produced from MFC can run low-power sensors or recharge batteries, such as a capacitor [Shantaram *et al*., 2005]. The EcoBot series of robots is an excellent example of MFC-powered batteries used to perform sensing, information processing, communication, and actuation [Greenman *et al*., 2021]. MFCs can be powered using human body fluids such as blood glucose and can be used as self-powered bioelectronic implants for diabetes monitoring [Maity *et al*., 2023].

2.22.4 Microalgae Cultivation and CO_2 Sequestration

MFCs can be integrated with microalgae systems to provide electron donor substrates at the anode or oxygen at the cathode for the oxygen reduction reaction. Microalgae biomass is a low-cost, readily available electron donor substrate at the anode. In addition, the microalgal biomass is harvested for bioenergy generation [Ahirwar *et al*., 2023]. Microalgae also treat water contaminated with nitrates and phosphates at the cathode [Kannan and Donnellan, 2021; Sharma *et al*., 2021]. The CO_2 produced at the anodic respiration can be fed to the cathode chamber for capture by microalgal photosynthesis [Khandelwal *et al*., 2018]. Recently, PMFCs were employed to capture CO_2 from petrol-engine flue gas, where bicarbonates generated from flue-gas scrubbing were utilized by microalgae growth. This process was accompanied by high power output [Sharma *et al*., 2023].

2.22.5 Biosensors

MFCs have been conventionally used for wastewater treatment and power generation. However, due to limited power production, recent research also looks at these devices for sensing applications, primarily miniaturized devices, which can run using low power [Do *et al*., 2020]. MFC-based biosensors are used for monitoring the organic matter, heavy metals, and toxicants in online mode, and different alarming systems have also been deployed on the field based on MFCs [Sharma and Chhabra, 2023].

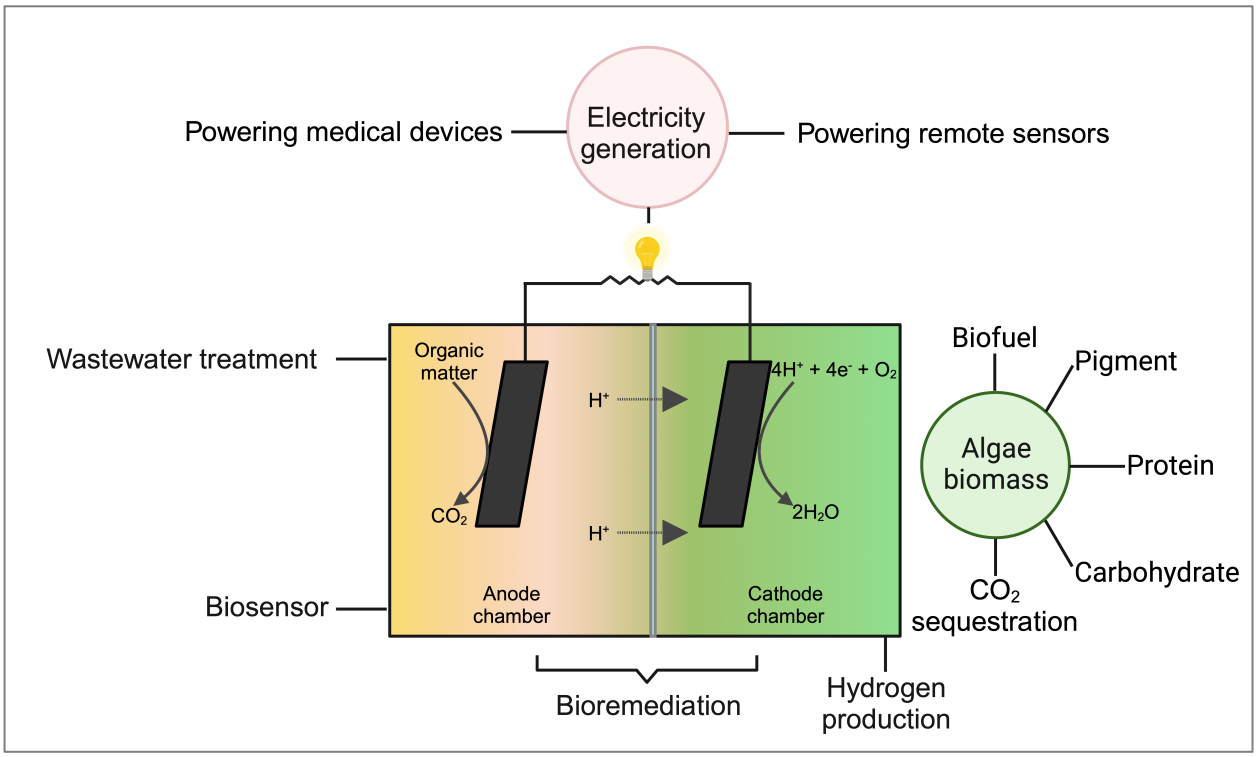

Figure 2.12 : The schematic showing various applications of MFC

2.23 MFCs INTEGRATION WITH MICROALGAE CULTIVATION

MFCs are cutting-edge bio-electrochemical systems that capitalize on the metabolic activity of microorganisms to generate electricity while offering sustainable solutions for wastewater treatment. The microalgae cultivation in the PMFC cathode chamber aids in oxygen production for ORR. Microalgae can also efficiently remove contaminants, such as N and P, and help in phyco-remediation [Kannan and Donnellan, 2021; Ahirwar *et al.*, 2023]. PMFCs also show significant promise in biofuel production [Khandelwal *et al.*, 2020]. Hence, PMFCs exemplify a multifaceted technology that simultaneously addresses wastewater treatment, biofuel production, and clean energy generation, making them a vital component of sustainable and environmentally friendly systems today.

2.23.1 Role of Microalgae in PMFC

Microalgae can be employed as a cathodic species for oxygen production or as the anodic substrate for power generation. The production of electron acceptors within the system reduces the dependence on mechanical aeration [Rosenbaum *et al.*, 2010]. Certain studies have also utilized microalgae at anode chamber for their role as electrogenic species. Microalgae cultivation at the cathode chamber is undertaken primarily for oxygenation, wastewater remediation, microalgae biomass production for lipids and biofuel production. Therefore, PMFC functions may be affected based on light, CO_2, diurnal cycle, DO, salinity, and other factors [Schad *et al.*, 2023]. This configuration allows for a net zero emission technology for bioelectricity generation. Similarly, the cathode chamber plays a pivotal role in CO_2 sequestration, which positively impacts voltage and power generation [Sharma *et al.*, 2023].

2.23.2 PMFC Configurations for Microalgae Cultivation

Various studies have been done on PMFCs [Rosenbaum *et al.*, 2010; Khandelwal *et al.*, 2018]. MFC configurations depict the arrangement of different components of MFC. MFC configurations that have the least internal resistance and offer the best performance to the required application are desirable for scaled-up studies. The research focus is to lower either the internal resistance or the cost associated with the design of MFC systems. Some design considerations involve increasing the electrode surface area, reducing the anode and cathode

spacing, mixing requirements, separator position and conductivity, membrane placement, number of chambers, effective volume, and ion transfer efficiency [Fan *et al*., 2007]. The most prominent MFC configurations are single-chamber, dual-chamber, sediment MFC, H-type MFC, clayware MFC, MFC coupled with microalgae PBR, and three-chamber MFC [Logan *et al*., 2015].

Single-chamber MFCs are a basic configuration useful for COD removal and power generation. The two-chamber or H-type MFC is the most common configuration where a proton exchange membrane, like a Nafion, salt bridge, physically separates both chambers. However, the internal resistance is a significant limitation in the H-type configuration. Another configuration is sediment MFC, which may/may not have a separator membrane, as the column height maintains the redox gradient between the anode and cathode chamber. These systems are operated in up-flow mode, where polluted water enters the bottom of the anode chamber and flows upwards while passing through the anodic and cathodic biofilms. Three-chamber MFCs are commonly employed for desalination. Saltwater is fed into the middle chamber to facilitate the movement of positive (towards the cathode chamber) and negative ions (towards the anode chamber) with the help of anion and cation exchange membranes, respectively. For scaling up applications, low-cost MFC reactor configurations have been developed for robust operation, using earthenware as an anode chamber and separator membrane [Khandelwal *et al*., 2020; Sharma *et al*., 2023]. Different MFCs can be stacked in series/parallel to enhance the power output of the systems [Aelterman *et al*., 2006; Mukherjee *et al*., 2022]. A novel equipment was designed for membrane-free PMFC which optimized the most expensive components of the MFC, including separator and cathode. The study incorporated a revolving algae-bacteria biofilm cathode in a photosynthetic membrane-free MFC of 12 l working volume for electricity generation, pollutants removal and algal biomass production [Zhang *et al*., 2022]. Some of the common PMFC configurations are shown in Figure 2.13.

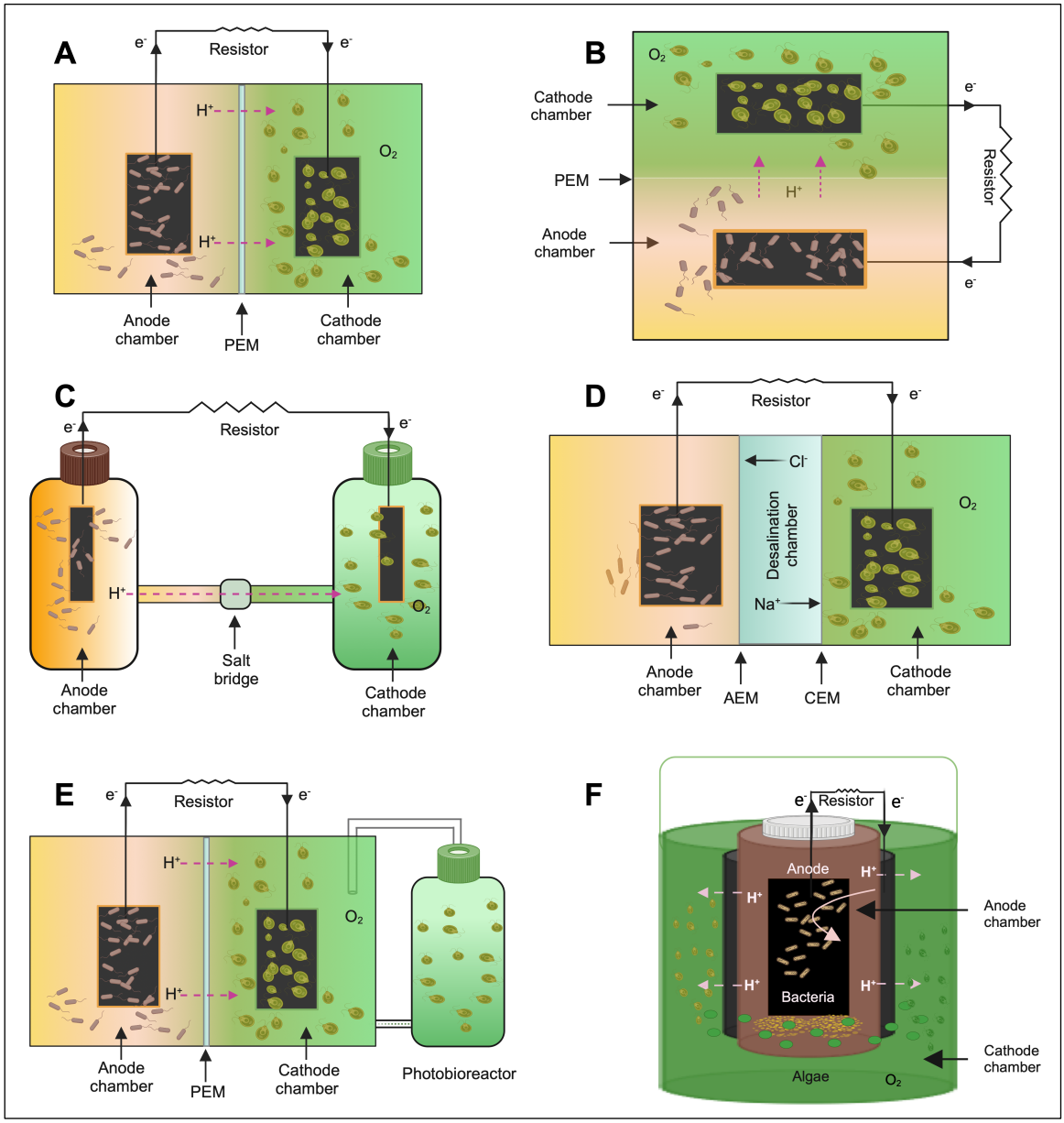

Figure 2.13 : Various configurations of PMFCs. A) dual chamber MFC, B) single chamber/sediment MFC, C) H-type MFC, D) three-chamber microbial desalination cell, E) MFC-coupled with microalgae PBR, and F) earthenware-based two-chamber MFC (*Source*: Ahirwar *et al.*, 2023; Sharma *et al.*, 2023)

2.23.3 Microalgae Strains Employed at PMFC Cathode Chamber

The PMFCs use organisms like microalgae, plants, or photosynthetic bacteria to produce oxygen in the cathode chamber. Previous studies employed various microalgae species at the cathode chamber based on the applications. These include *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Scenedesmus obliquus*, *Scenedesmus acutus*, *Desmodesmus sp.*, *Microcystis aeruginosa*, *Spirulina*, and *Golenkinia sp.* [Reddy *et al.*, 2019; Apollon *et al.*, 2021]. Mixed algal cultures were also utilized in the PMFC cathode chamber, which maintained high power output [Kakarla *et al.*, 2015; Neethu and Ghangrekar, 2017; Nguyen *et al.*, 2017; Kakarla and Min, 2019]. These systems, with mixed microalgae communities, were applied to utilize bicarbonate or CO_2 for carbon removal applications. However, their role in the biorefinery perspective was not studied. Also, it is rather difficult to control the cathodic activities when employing mixed algal communities. It was observed from the literature studies (Table 2.6) that the type of microalgae strain employed at the cathode chamber plays a key role in governing the PMFC performance [Ahirwar *et al.*, 2023].

Table 2.6 : Summary of Prominent Microalgae Strains used at PMFC Cathode Chamber and their Performance

S. No.	Algae strain	MFC type	Cathode materials	Culture conditions	Algae specific growth rate/ productivity	Maximum power density	References
1	*C. vulgaris*	Dual Chamber (Plexiglass)	Toray carbon cloths 10% Teflon	CO_2 from anode chamber	-	42.98 mW/m2	[Del Campo *et al.*, 2015]
2	*C. pyrenoidosa*	Dual Chamber (H-type)	Graphite rod	-	-	30 mW/m^2	[Xu *et al.*, 2015]
3	*C. vulgaris*	Dual Chamber (H-type MFC)	Copper sheet	15% CO_2	100 mg/l/d	19151 mW/ m^3	[Hou *et al.*, 2016]
4	*Chlorella* sp.	Dual Chamber (Airlift type)	Carbon fiber cloth	10% CO_2 in airlift mode	0.406 d^{-1}	0.14 mW	[Hu *et al.*, 2016]
5	*C. vulgaris*	Dual Chamber (H-type)	Copper sheet	-	-	400 mW/m^3	[Hou *et al.*, 2017]
6	*C. vulgaris*	Dual Chamber (H-type)	Graphite plates	-	-	34.2 mW/ m^2	[Commault *et al.*, 2017]
7	*Spirulina sp.*	Single and Dual Chamber (H-type)	Carbon cloth/ activated carbon-PTFE	-	0.1 g/l/d	0.8–1 W/m^2	[Colombo *et al.*, 2017]
8	*Golenkinia* sp.	Dual Chamber (H-type)	Graphite plate	-	45 mg/l/d	327.67 mW /m^2	[Huarachi-Olivera *et al.*, 2018]
9	*C. vulgaris*	Dual Chamber (Acrylic)	Graphite felt	CO_2 from anode chamber	0.275 ± 0.02 d^{-1}	2.7 W/m^3	[Khandelwal *et al.*, 2018]
10	*C. vulgaris*	Dual chamber (Tubular)	Carbon cloth	-	0.94 ± 0.03 g/l	2.8 W/m^3	[Pei *et al.*, 2018]
11	*Chlorella* sp.	Dual Chamber (H-type)	Nickel foam/graphene electrode	-	-	36.4 mW/ m^2	[Zhang *et al.*, 2018]
12	*C. vulgaris*	Dual Chamber (Plexiglass)	Carbon fiber cloth	CO_2 from anode chamber	-	3720 mW/ m^3	[Zhang *et al.*, 2019]
13	*Synechococcus* sp.	Dual Chamber	Noncoated graphite electrodes	-	0.547 d^{-1}	95.63 mW/m^2	[Lakshmidevi *et al.*, 2020]

In light of prior investigations, it has been observed that the specific microalgae strain utilized in the PMFC cathode significantly influences power generation and bioenergy production. Consequently, it is imperative to investigate PMFC functionality with diverse microalgae species and compare their performance metrics with existing studies. To enhance PMFC efficiency, research efforts can be directed toward the selection and engineering of microalgae strains tailored for PMFC application, aiming at simultaneous power generation, VAP production, wastewater treatment, and carbon capture within the PMFC cathode chamber. Such

PMFC-based biorefineries contribute to efficient waste management and resource utilization, aligning with global sustainability goals. Despite these advancements, the specific impact of the PMFC environment on the performance of GM microalgae remains unexplored.

The engineering of microalgae strains opens up new possibilities for circular bioeconomy through enhanced synthesis of valuable bioproducts. Various genetic engineering approaches, as outlined by Fayyaz *et al*. (2020) and Ma *et al*. (2019) and detailed in Table 2.3, have been explored to enhance the production of these compounds. Among microalgae transformation methods, electroporation stands out as a quick and efficient technique with high transformation efficiency. The targeted delivery of genes/enzymes for over-expression is pivotal, considering the varying success of nuclear or chloroplast transformation and the molecular tool kits employed for expression. Thus, the selection of efficient expression cassettes is crucial for augmenting product synthesis through GM microalgae.

However, previous studies report that the growth of GM microalgae is hindered compared to their WT counterparts. Strategies should be devised to alleviate the growth rates to enhance overall product yield. In this order, cultivating GM microalgae strains at PMFC cathodes could positively impact product recovery through high biomass productivity. Therefore, the incorporation of GM microalgae at the PMFC cathode is suggested, offering a promising avenue for a potentially cost-effective process in the production of high-value products such as pigments and lipids. This represents an unexplored research domain, as understanding the interplay between MFC conditions and GM microalgae behaviour holds the potential to establish microalgae-assisted MFC-based biorefineries. However, the scaling up of these systems always require preventive measures to avoid horizontal gene transfer of transgenes from GM microalgae into the environment [Kumar, 2015]

In consideration of existing literature, an attempt was made to establish PMFCs operating within a biorefinery framework for high-value microalgal pigment production through GM microalgae cultivation, coupled with wastewater treatment and power generation.