PROMISING EVOLUTION OF FOURTH GENERATION BIOFUELS

Abstract

Microalgal biofuel has encouraging prospects for a sustainable economy. It has attained considerable quantity of heed all around the globe from researchers and academicians. They are a replacement to the fast depleting fossil fuels. Fourth generation biofuel mainly focusses on genetically modified algae and cyanobacteria biomass. It is found that fourth generation biofuel is compatible with engines and transport services. Biomass productivity and oil content of the algae are considered to be the most prominent factors related to cost in the production of fourth generation biofuel. In this regard, genetic engineering is the requisite for boosting the biomass yield and oil accumulation of the genetically modified strains of algae. Suitable mitigation strategies are crucial to overcome the concerns related to them, for the successful commercialized production of the fourth-generation biofuel. Because of the prospects of enhancing the biomass productivity by improving its quality and minimizing the production price, genetic engineering applications are flourishing in the area of biofuels.

Keywords: Algae; Biofuel; Biomass; Fourth generation; Genetic modification.

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I. INTRODUCTION

Biofuels are utilized to fabricate various fuels such as bioethanol, biodiesel, biobutanol, biomethane, biohydrogen, vegetable oil, gasoline, isoprene and aviation fuel [1]. Biofuels are categorized in four various generations viz. first, second, third, and fourth generation biofuels, taking into account the raw materials and manufacturing technique.

First generation biofuels are created from sugar and starch crops for instance maize, sugarcane etc., and oil-based plants. However, functionality of the first generation biofuels has been interrogated progressively because of the matters regarding the battle for feedstock and arable lands. To deal with these matters, second generation biofuels were launched as they make use of non food crop varieties. Second generation biofuel also make use of forestry and agricultural residues. But the manufacture of second generation biofuel would be unviable if battle for accessible land were to emerge. Third generation biofuels fabricated from algae has gathered huge awareness because of their elevated yield, carbon dioxide (CO_2) absorbance, and effortless processing comparatively. Algae can be cultured in different waters such as wastewater, brackish water and seawater, and also in uncultivable dry terrains and minimal croplands. Hence, they do not fight with food plants on fertile land or in freshwater ecosystem. The fourth generation biofuel (FGB) is fabricated from genetically modified (GM) algal feedstocks to attain improved biofuel manufacture. Enhancing photosynthetic efficacy, enhancing penetration of light, and minimizing photoinhibition are few general approaches employed in the microalgal genetic engineering [2]. The metabolic modification of microalgae is crucial enhancement of carbohydrate or lipid content. Maximization of carbohydrate and lipid are amidst the most fascinating parameters that can maximize the microalgal feedstock productivity efficiency.

The manipulation of GM microalgae needs a strict evaluation of the concerned threats and the feasible administration of the influences of the ecosystem [3]. The GM microalgae cultivation could be executed in both controlled and uncontrolled systems. However, the limitations of these methods are crucially separate from one another. The controlled system of cultivation has a more tightly contained state, while exposure to environment and contamination are reduced. In spite of providing more security, the operating cost of the controlled system of cultivation is immense. Uncontrolled system of cultivation are commonly raceway ponds. Uncontrolled system of cultivation has lower capital costs than controlled system of cultivation, but the probability for the GM algal strains to be diffused from these cultivation systems is immense, as uncontrolled system of cultivation are vulnerable to aerosol dispersal, interference of birds or animal, and leakage [4]. Other issues, such as environmental diffusion and safety of users, have made FGB to persist within research boundaries.

The first person to employ the expression "fourth generation of biofuel" to illustrate the fuel attained from GM algal species was Barrett in 2009. The proposed approach utilized synthetic biology to create microbes with remarkably elevated levels of CO_2 assimilation attributes. Large-scale manufacture of FGB is yet not feasible because of the risks relating to health and ecosystem, legitimacy issues, inadequate manufacture of biomass, and elevated production price [5]. Numerous algae species viz *Chlorella* sp., *Isochrysis galbana, Phaeodactylum tricornutum*, *Nannochloropsis* sp., displays elevated lipid, protein and carbohydrate content [6]. Still, few limitations do prevail with microalgae-based biofuels such as contaminations at the time of cultivation, harvesting problems and difficult downstream processing [7]. Latest evolutions in genetic modification and molecular biology approaches and techniques has permitted the discovery and assessment of novel biochemical trails in different model algae strains and have also furnished an effective and specific method to edit genomes with the intention of generating novel strains for optimum fabrication of biofuel [8]. Besides, with the aid of computational biology techniques and strategies viz. next-generation sequencing (NGS), genome-scale metabolic reconstruction, multiomic databases could swiftly aid in the identification of new pathways or target for development of new strains, in turn saving time and capital expenses [9]. The amalgamation of molecular biology, genetic engineering, and interdisciplinary physicochemical strategies to optimize and improve the yield of production of biofuel can be widely inferred as: Fourth generation Biofuels (FGB). FGB uses genetically engineered algal strains that cumulate excess lipid and carbohydrate yield to maximize biofuel content. There are several routes that can be exploited to generate the FGB such as- employing systems and computational biology-based strategies for metabolic engineering, genetic manipulation of photosynthetic microbes for elevated biofuel productivity, and the onging technology improvement of photobioreactors to improve organism growth and biofuel fabrication [10]. Figure 1 shows the FGB production and the concerned mitigation approaches for dumping of GMO.

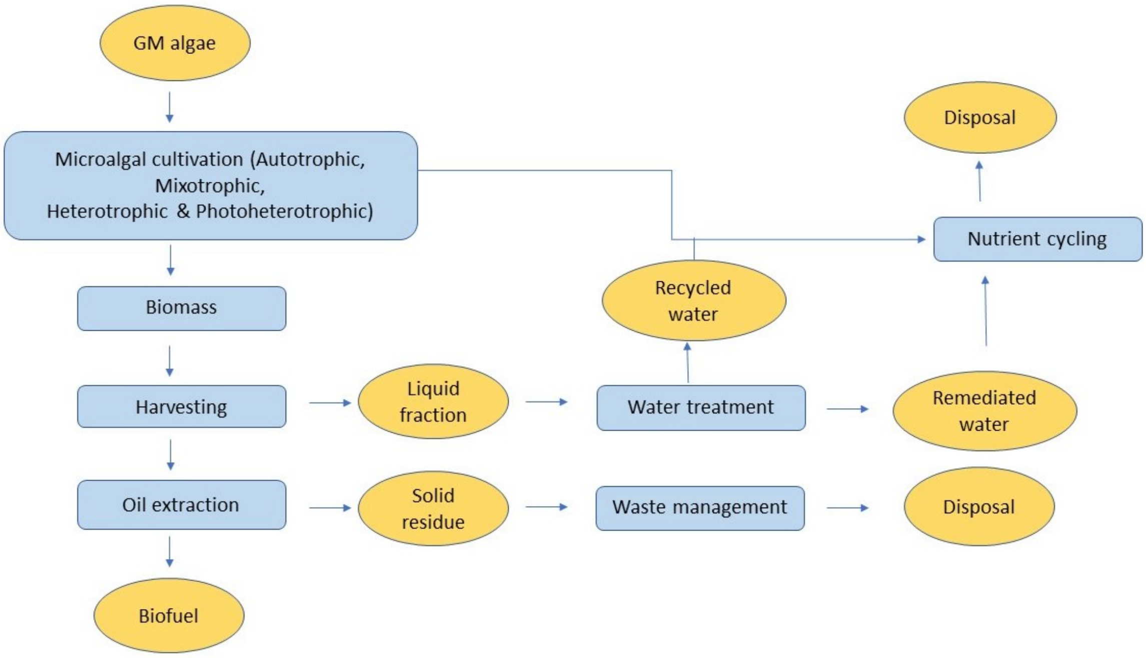

Figure 1: Schematic of the FGB production and the concerned mitigation approaches for disposal of GMO (Source: [11])

II. BIOFUEL GENERATIONS

Biofuels are categorized in four various classes relating to their biomass raw material [12].

1. **First Generation Biofuel:** First-generation biofuels use edible feedstock viz. starch (from corn, potato, barley and wheat) and sugar corps (from sugarcane and sugar beet). However, issues emerged regarding utilizing edible crops as biomass and the influences

on farmlands, food supply chain and biodiversity [13]. These biofuels are considered as 'Conventional biofuels'.

2. **Second Generation Biofuel:** The biomass here is lignocellulosic substances that comprises of the economical and ample nonedible raw materials obtained from plants [14]. A huge range of discarded substances could be employed as biofuel biomass such as agricultural waste, eucalyptus and willow, switchgrass, miscanthus, reed canary grass, poplar trees and wood residues and they substantially comprise of plant cell walls, whose primary compound is polysaccharides [15]. Non-food crops like Jatropha was also utilized. These polysaccharides have an excessive sugar amount that is favoured for biofuel fabrication. The biomass is called as lignocellulosic also, as it is obtained from cellulose, hemicellulose and lignin.

3. **Third Generation Biofuel:** Microalgae have various significant characteristics including high oil content, needing less space for its growth, the potential to cultivate in both natural and artificial habitats, and being environmentally safe [16]. Also, due to its higher quality, microalgal oil is recommended over lignocellulose-based oil. The third-generation biofuels are procured from microalgae through transesterification and hydrotreatment of the oil extracted from algae.

4. **Fourth Generation Biofuel:** Research on fourth generation biofuel has been implemented amid 2000 and 2020 [17]. The fourth generation biofuels are fabricated utilising genetically modified (GM) algal strains, electro-fuels and photobiological solar fuels [18]. The GM algal feedstock is efficient in fabricating biofuels via enhancing photosynthetic efficacy, and maximising penetration of light. The fourth-generation solar fuel raw biomass are extensively obtainable, cost effective and inexhaustible. Genome editing approaches and techniques viz. transcription-like effector nucleases (TALEN), zinc-finger nuclease (ZFN), and clustered regularly interspaced palindromic sequences (CRISPR/Cas9), are extensively utilised as the bioinformatics tools [19,20].

   Presently, various strategies have been embraced- engineering of pathways in indigenous producers (cultivation rates optimization, intending the metabolic flux in the direction of creation of biofuel, use of various carbon sources, and elevated production titers), and reformation of pathways recognized in innate producers in more genetically attainable hosts. A huge range of microbes could be utilized as model for the manufacture of biofuels, incorporating bacteria, cyanobacteria, fungi, yeast, and algae (both macroalgae and microalgae). The commonly used model organism is however microalgae. Proper containment approaches are however required to minimize the diffusion risk of GMO into the environment. CRIPSR/Cas9 is a commonly employed tool for genetic engineering, as it provides a simple layout with effective transfection and targeted gene disruption. Another option to targeted genetic manipulation is random mutagenesis, which is nothing but expeditious evolution. A newer experimental appeal to FGB is electrobiofuels production. These techniques are based on new-to-nature hybrid system thereby utilising renewable electricity and carbon sources to create biofuels hereby converting the solar energy to a liquid fuel which can be stored. This type of strategies can merge the higher photon efficiency of modern photovoltaic systems (compared to photosynthesis) with the viability of biofuel fabrication, enhancing potency of the overall process [21].

In summation to genetic manipulation, few other fourth-generation strategies include gasification, pyrolysis (range of temperature between 400 to 600 °C), and solar-to-fuel pathways. However, these strategies are till now in premature progression phases [22]. Fig. 2 shows the different types of FGB.

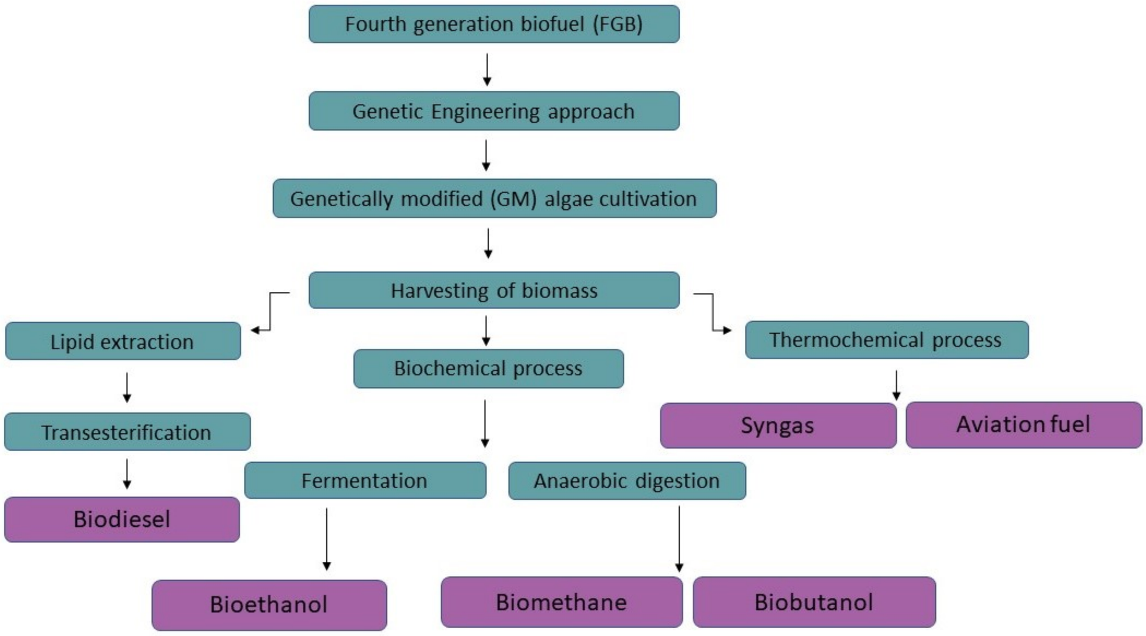

Figure 2: Different types of Fourth generation biofuels. (Source: [23])

III. ENVIRONMENTAL IMPACT OF BIOFUELS

Various genetic alteration methods have been launched to improve algal feedstock. These improvement approaches are primely built on the target genes for the direct biofuel biosynthesis, carbohydrate and lipid metabolism, enhanced nutrient utilization efficacy, hydrogen manufacture, enhanced photosynthesis efficacy, improved cell disintegration, higher stress resistance and bioflocculation. These processes can notably enhance the fabrication of algal biofuels. Improvement of yield and lipid cumulation is the simplest technique to minimize the price, nutrient consumption and water footprint. Broadly employed genome editing techniques for enhancement of the productivity and lipid yield of microalgae. Various types of genome editing techniques viz. zinc-finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced palindromic sequences(CRISPR/Cas9) are widely employed (Maeda et al., 2018). The first genome editing investigation in microalgae was documented on *Chlamydomonas reinhardtii* utilizing ZFN [24].

The applied genetic manipulation strategies for biofuel improvement may comprises of (1) altering the sequence of an available functional gene, (2) altering an existing regulatory sequence, or (3) reinstating a regulatory gene or sequence with one from other organism or strains [25]. The microalgae developed from the first two strategies (1 and 2) are liberated from any foreign DNA material and can be excused from the legislation of GMO (genetically modified organism) materials [26, 27].

1. **Health and Environmental Concerns:** Genetically modified (GM) microalgae can conquer effortlessly the surroundings because of their tiny shape, swift growth, and vastness. The prime ecosystem issues concerning the unguarded manipulation of GM algae concerns to battle among the introduced microalgae and indigenous species, alterations in natural terrain, horizontal gene transfer, and pathogenicity. Liberating toxic algal isolates into the surroundings can strike grave risks to humankinds' health.

   Lateral or horizontal gene transfer is stated as a technique where the genetic material of one strain is transmitted to other in a non-genealogical way [28]. The term 'genetic modification' is employed to address genetic manipulation strategies which are enforced to enumerate, delete, or alter particular chunk of a strain's genome. Therefore, natural replication approaches like random mutagenesis, can be excused from regulations of GMO.

   For the sustainability and marketing of FGB manufacture, the outdoor cultivation of GM microalgae is significant. However, prior to initiating the GM algal strains into the surroundings, risk investigations must be executed to diminish the potential ecological and safety issues regarding with the liberation of GM characteristics from cultivation, harvesting and processing provisions.

   The ecological and health-related threats related with unanticipated or intentional diffusion of GM algal isolates and envisioning suitable mitigation approaches has a direct effect on the biofuel manufacture economy. The idea of genetic modification in algae strains is to enhance the quality and yield of FGB. Nevertheless, removal of GM algal species remained a crucial hindrance. The release of chromosomal or plasmid DNA might create horizontal gene transfer in microbes. Therefore, firm mandates are thrusted to stop the diffusion of GM products. Plasmid or chromosomal DNA in microbes stays potent even after their demise. OPR cultivations are the most vulnerable to contamination by wind, insects and birds. Administering secure genetic modification methods, viz. self-cloning and mutagenesis, might increase the sustainability and bio economics of FGB manufacture. Apart from utilising safe genetic manipulation, additional remission approaches such as integral biocontainment, and physical and chemical deposition techniques are utilized to demolish the microbes and the genetic constituents present in GM materials.

2. **Safe Genetic Engineering Techniques:** The biosafety mandate is built to terminate the unintentional diffusion of modified organisms to the ecosystem and interruption of the biotic equilibrium in the environment. Safe genetic manipulation strategies utilized for genetic engineering of algae species like mutagenesis and self-cloning do not hold foreign DNA sequences in their genome. Therefore, these mechanisms are eliminated from the biosafety mandate synthesis. Utilising a safe genetic modification approach is a dependable measure to increase the potentiality of FGB without hindering the biosafety-critical standards.

3. **Biocontainment of genetic materials:** Biocontainment is one of the most effective measures to reduce the threats conferred by the diffusion of genetically altered microbes into the surrounding. Major issue in the biocontainment of genetic material is to safeguard the cell genetic components from diffusing into the ecosystem even after cell demise.

Biocontainment could be attained by various approaches viz. auxotrophic method, toxin-antitoxin pair, toxin-based method and xenobiological constituents-based techniques [29].

IV. STRATEGIES AND METHODS FOR THE PRODUCTION OF FOURTH GENERATION BIOFUELS

1. **Genome Editing:** Alteration of the genes associated in triacylglycerol (TAG) and fatty acid biosynthesis pathways is a significant strategy to maximize the microalgal lipid yield. Genetic alteration techniques such as overexpression of genes linked to TAG and FASs or obstructing competing pathways are chief regulators for increasing microalgal lipid accumulation [30]. Microalgae is a sustainable origin of valuable chemicals such as carbohydrate, lipid and protein which is contemplated a renewable and ecofriendly option for fossil fuel. Algal feedstock cultivation for biofuel has numerous benefits such as CO_2 sequestration while being adept to be utilized as the biomass for manufacturing an extensive array of biofuels such as bioethanol, biogasoline and biodiesel.

2. **Genetic Engineering:** Genetic manipulation has been demonstrated as a pivotal technique in algae species generation for improved biofuel manufacture. In the past decade, comprehensive attempts have led to the progression of genetically engineered algal species for lipid creation. Few of the most regularly genetically engineered algal strains are *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, *Synechocystis* sp. PCC 6803, and several species of *Chlorella.* Several enzymes and metabolic pathways could be aimed for the fabrication of genetically engineered organisms. Nuclear transformation is a popularly employed method for the alteration of metabolic pathways. Nuclear transformation needs the utilization of algae cells deficient in cell wall for the random infusion of transgenes into the genome of the organism. Rochaix and Dillewijn attained the foremost occurrence of nuclear transformation in *Chlamydomonas reinhardtii* by utilizing polyethylene glycol. It is challenging to anticipate the steadiness of the transgene as genetic mutations, the motion of transposable elements and gene silencing, these procedures are controlled by various molecular techniques [10].

3. **CRISPR Technology For Microalgal Genome Editing:** Zinc Finger Nucleases (ZFN), RNA interference (RNAi) and Transcription factor-like effector nucleases (TALENs) were regularly employed earlier for genome editing in microalgal strains. Since the unearthing of the clustered regularly interspaced short palindromic repeats/associated protein 9 (CRISPR/Cas9) system in 2013, because of its cost effectiveness, adaptability, simple usage and to aim several genes at the same time, it has developed into the gold standard for genetic engineering [31]. The Cas9 protein gives rise to double-stranded breaks in the gene aimed by the synthetic guide RNA (sgRNA), in turn silencing the aimed gene. The foremost successful utilization of CRISPR/Cas9 for algae genome editing in the model strain *Chlamydomonas reinhardtii* was expressed by Jiang et al., 2014 [32]. But because of the toxicity of Cas9, the mutation rate was comparatively little. Shin et al., used a different strategy, Cas9-sgRNA ribonucleoprotein (RNP) to overcome this hindrance [33]. Efficient gene editing was attained by utilizing an optimized CRISPR/Cas9 vector in *Phaeodactylum tricornutum*. *Nannochloropsis* sp. has been tagged as a model strain for carbon sequestration and oil fabrication after fruitful genome editing by CRISPR/Cas9 [34].

V. SOCIOECONOMIC ANALYSIS

The equilibrium amidst social and economic factors is extremely vital for generation of a sustainable process. The triumph of a commercialized commodity can be affected negatively if an imbalance amidst these two factors arise, and subsequently it can lead to a crucial casualty in market price. Therefore, socio-economic factors should be evaluated before a product is released into the market. Profitability, social acceptability and well-being, and resource conservation, are the prime socioeconomic factors concerned to the FGB fabrication.

1. **Profitability:** The details on techno-economic inspection could be utilized to investigate and equate the price and advantages of various projects, techniques, or provisions. Data gathered from techno-economic inspection could be utilized to access if production goals are being attained, identification of substantial contributors to the price, and investigate the economic suitability of upscaling [35].

2. **Social Acceptability:** For an energy approach to be triumphant, it should strike equilibrium amidst the competitive interests of economic advancement and environment well being. The swift-growing industry of GM materials has ignited substantial resistance from public since its launch in 1994. The information on the harm to mankind and environment created by GM organisms must be obtainable by the people, as a way of dealing with issues regarding the social acceptability of FGB. This will enhance public's realization on the advantages and disadvantages concerned with GM feedstock.

3. **Social Well-Being:** Probable occupational damages particular to the FGB feedstock cultivation could be categorized in four classes- antibiotic resistance, carcinogens, allergies, and toxicity or pathogenicity. Engineered cells are revealed to antibiotics to shield them from foreign DNA at the time of introduction. As the cells persist to show the antibiotic-resistant gene, the antibiotics might transmit to other strains or into foodstuff ingested by mankind. Bacterial resistance might maximize because these series of occurrences. Carcinogenic materials particular to algae might develop the generation of cancerous tissue in an individual's body. GM strains could act as allergens themselves or create allergenic compounds [36]. Statistics shows that in 2004 and 2005 several farmers in India who came in contact with Bt cotton, experienced allergic symptoms. Besides, GM strains might initiate or enhance the existence of toxins or pathogens that might damage human health [37].

4. **Risk of Catastrophe:** The feedstock creation of FGB can create a catastrophe through its intended and unintended diffusion into the neighbouring land and water bodies. Probable catastrophe hazards concerned with the diffusion of GM algal strains can be categorized into four types- alterations in the innate ecosystem of preserved strains, battle with the indigenous strains, pathogenicity, and horizontal gene transmission [38].

5. **Cultivation Methods:** Photobioreactors (PBR) and open raceway ponds (ORP) are two dominant cultivation methods used in commercialization of microalgae feedstock manufacture. Confined cultivation methods provide optimal control and diminish the threat of contamination; however, they are extremely expensive. GM algae can improve the quality and yield of biofuel, ensuing in the commercial viability of manufacture of FGB. PBR demonstrated inflated productivity and photosynthesis efficacy and hence, had

less manufacture value. Nonetheless, the investment price of PBR are more than of ORP. Wind and leakage were the prime components accountable for transmitting the algal strains from the ORP cultivation system. Various control alternatives should be contemplated when constructing ponds for cultivating GM algal strains to minimize spreading risk. Employing lining to prevent leakage and utilizing air-supported plastic hoop greenhouses are few of the control measures that has been taken to minimize this hazard [11].

6. **Water Supplies and Recycling:** Water footprint (WF) is evaluated by estimating the variety of pollution and the polluted water proportion. Minimizing the WF in the manufacturing procedure is the foremost objective of a biofuel method's sustainability [39]. In 2012, Wu et al. were the first to propose a life cycle water assessment framework utilizing a standardized WF technique to investigate green water, blue water, and agricultural grey water discharge in the fabrication of biofuel biomass. Green and blue WF are respectively the consumed volumes of rainwater and groundwater utilized. Grey WF is determined through estimating the freshwater needed to dilute polluted water in the freshwater quality standard. Vital factors in the WF of the biofuel production are- biomass utilized for biofuel [40], the energy extraction procedure, and the final product, which is biofuel [41]. The residue from the culture medium of GM algal feedstock cultivation could strike crucial risks to health and ecosystem. Hence, definite remediation should be undertaken before the diffusion of wastewater into the neighbouring environment. Freshwater consumption can be minimized from recycling and reusing discharged wastewater effluents from the harvesting stage. Hence, nutrient-recycling this discharged wastewater can improve the economic efficacy by reducing material input. Utilizing wastewater as a replacement of freshwater is an optimistic approach to cultivate microalgal strains because of its elevated levels of nutrients. Employing wastewater to grow algal strains could make the creation of FGB suitable and viable.

7. **Diffusion Risk:** Diffusing pathogenic microalgal strains into the ecosystem can have adverse societal consequences and shatter the welfare of humankind and flora and fauna. Horizontal gene transmission is the system through which components from one strain to another are transmitted in a non-genealogical way. Wind was quoted as the main invador of the genes into the ecosystem. The transfer of DNA amidst entities of several strains by horizontal gene exchange is one of the prominent issues concerned to embrace GM feedstock for the manufacture of FGB. Hence, the remnants attained from the energy extraction technique and water diffused from harvesting of the GM feedstock must be discarded attentively. Doing so would avert horizontal gene exchange by transferring chromosomal DNA or transgenic plasmid.

VI. REGULATIONS AND MITIGATION STRATEGIES RELATED TO THE FOURTH GENERATION BIOFUELS

Restricted number of explorations and evaluations are present on the outdoor large-scale cultivation of GM microalgae and its influence on the ecosystem. A significant restriction halting the administration of largescale trials is the rigid laws and regulations enforced through authorities [42]. Most of the commercially fabricated microalgal strains are grown in outdoor open-pond process. The improvement of lipid content in GM algal strains by administering the suitable cultivation conditions could crucially impact the potentiality of biofuel production. Even though employing an outdoor pond is the finest option for algal cultivation, it is not without limitations. Mutant breakout can strike a severe menace to the biodiversity of a local surrounding, potentially maximizing the risk of an algal bloom formation. The infringement of a wild variety of microalgal strain in a cultivation pond could affect its yield [43]. De Mooij et al., 2016 [44] simulated an outdoor mass culture of engineered *Chlorella sorokiniana* algae to access the battle amid the mutant and wild types. The mutants were quickly overgrown, ensuing into productivity loss, in a surrounding contaminated with the wild variety. Aravanis et al. [45] launched an end-to-end algal biofuel manufacture technique utilized a genetically modified organism *Chlamydomonas reinhardtii*. Szyjka et al., 2017 [46] accessed the environmental threat of the open pond cultivation of genetically modified algal strain. The assessment displays that outdoor cultivation did not develop any detrimental consequences on the neighbouring indigenous algal population or the ecosystem. The utilization of the genetic or allied biological manipulation of microalgae is extensively recommended for the feasibility of FGB creation. Nevertheless, for the large scale open-pond cultivation of the GM microalgae to be potent, it is required to be economically feasible. Another hindrance in manufacturing FGB concerns to the removal of remnants that generates from this energy-extraction procedure.

Genetic manipulation targets to increase the algal biofuel manufacture. The phenomenon of microbes remains active even after their demise. This mechanism might cause plasmid or chromosomal DNA diffusion into the environment. The unintended or deliberate diffusion of DNA at specific concentrations can develop in horizontal gene transference via transformation; therefore, there are firm laws and rules for the removal of these substances.

Waste disposal methods are targeted at diminishing the consequences of ecosystem related to GM remnants and at the substitution of hazardous by-products with increased environmental-friendly options. Various approaches can be enforced to minimize waste residues (e.g., waste separation and concentration, energy/material recovery, minimization of waste, incineration/treatment, secure land disposal and waste exchange). Composting is one of the most dependable alternatives for the secure removal of these residues [47]. Two categories of composting are commonly utilized for the degeneration of GMO isolates- enclosed bioreactor systems and open turned windrow systems. Utilizing an enclosed bioreactor is suggested as it has a remarkably quick composting time while making sure that the substances are composted in an optimal manner [48]. Recycling the wastes of algae feedstock after its carbohydrate, lipid, and protein content processing is a widespread result for eradicating issues concerning the horizontal gene transference [49]. Ueda et al., 1996 [50] recommended flaming the biofuel extraction procedure dried residues to regain energy and CO_2. Hence, the recommended technique has three benefits- decline in waste, the energy recovery, and the CO_2 recovery and reuse. After the processing stage, the solid residues withdrawn from recycling the culture medium could be employed as a secondary origin of nutrients in cultivation methods [51].

Thermal treatment is a notably employed technique for the degeneration of DNA in GMOs. Hrnčírová et al., 2008 [52] evaluated the consequences of thermal degeneration on the amount of the DNA extracted. The outcomes demonstrated that maximizing the temperature crucially minimize the content of DNA in a time-dependent way in GMOs. The consequences of thermal processing on transgenic and non-transgenic DNA was investigated by Bergerová et al., 2010 [53]. The magnitude of the matrices of the extracted DNA was found to be a vital criterion in DNA degeneration.

Consumption of water throughout the FGB production is another problem that requires to be handled to ensure the sustainable utilization of biodiversity and resources. Harvesting is the most significant stage in the biofuel manufacture procedure. There are two foremost viable options concerning the WF (water footprint) of algal biofuel: (1) culture with recycling water and (2) culture with the residue of the water that is harvested. Recycling the diffused water from the harvesting stage could minimize the water quantity absorbed throughout biofuel manufacture up to about 90.2% [54].

VII. FUTURE PROSPECTS AND CONCLUSION

Algae biofuel is an encouraging option for fossil fuel beholden to its benefits such as elevated energy yield, less discharge, and eco-friendly nature. Nonetheless, manufacture of the algae biofuel is not feasible economically because of its less yield and elevated manufacturing price. Utilizing open-pond cultivation is the required attempt for triumph in industrial-scale FGB fabrication.

The future of GM algae biofuel relies on enhancing the competence of its growth, improving the strains of algae that is being used, and encouraging the industrialization of biofuel manufacture. These developments could be attained through minimizing the price and enhancing the yield of cultivation processes in addition to initiating increased coherent genetic engineering or associated strategies to maximize the output and product quality. However, the commercialized production of GM algae is presently hindered by the threats associated with the intended and unintentional diffusion of the genetically manipulated isolates into the ecosystem. The facets regarding legislation matters and ecosystem threats associated with the manufacture of GM algae feedstocks are two significant issues that requires more advanced recognition. Vital matters in this area comprises the threat of the entrance of GMO species into the natural ecosystem, the big capital expenditures needed, and the excessive functional price of the photobioreactors. These cons restrict the commercialized utilization of FGB. Hence, studies need to be conducted to conquer these limitations and moving forwards toward the commercialized sustainable fabrication of FGB. Maximum yield can be attained by enhancing reactor designs, utilizing high-throughput genetic manipulation techniques, and constructive management of waste. The future is optimistic for FGB, and genetic engineering is a required fundamental component for an effectual transformation from fossil fuels to biofuel as the prime energy origin in the 21st century and its beyond.

For the cultivation of algal feedstock, Open-pond is the most favourable choice for microalgal biomass fabrication as they are the most economical large-scale bio reactors. However, executing an open-pond reactor cultivation system without conducting a thorough and detailed risk evaluation of the procedure can lead to a consequential menace to the ecosystem as well as mankind. Preventive methods should be contemplated all through the design and production procedures to minimize the risk of diffusion by horizontal gene transfer generated through diffusing chromosomal DNA or plasmid into environment. The evolution and implementation of feasible and cautious genetic engineering strategies which do not require foreign DNA are anticipated to minimize biosafety issues, thereby making genetic modification approaches more admissible to the public and policymakers.

Residue discarding is another vital concern which needed to be regarded in manufacture of FGB and suitable mitigation approaches must be employed to lessen the hazard of ecosystem and health problems. Disposal techniques that eliminate the microbes and the genetic element are the most fruitful alternatives to diminish the lateral gene transfer risk. The cultivation media utilized to make biomass culture could be recycled back into the operation and reused up till four times. The residual water from the harvesting procedure of the FGB creation carries genetic constituents and required to be treated with care. The treatment procedure should be done parallelly with the nutrient recycling to certify that resources are feasibly utilized in justification. The bioeconomics of the FGB could be remarkably enhanced by genetic manipulation approaches for improving biomass yield and lipid accumulation. However, employing an open pond can maximize the risk of diffusion of manipulated strains in the environment through wind and animals. Utilizing plastic hoop air-aided greenhouse enveloping is recommended as a measure initiative to arrest the discharge of the GM species in the surroundings. The economic execution of FGB manufacture can be enhanced through recycling water and nutrients, and reusing released residue. Apart from the incurred prices, socioeconomic parameters prevail amongst the most crucial factors in market valuation and commercialization of GM algae feedstock.

REFERENCES

[1] Bajracharya SK, Vanbroekhoven CJ, Buisman DP, Strik TB, Pant D, Faraday Discuss 2017; 202:433–449.
[2] Tandon P, Jin Q. Microalgae culture enhancement through key microbial approaches. Renew Sustain Energy Rev 2017;80:1089–99.
[3] Beacham TA, Sweet JB, Allen MJ. Large scale cultivation of genetically modified microalgae: a new era for environmental risk assessment. Algal Res 2017;25:90–100.
[4] Hannon M, Gimpel J, Tran M, Rasala B, Mayfield S. Biofuels from algae: challenges and potential. Biofuels 2010;1:763–84.
[5] Shokravi H, Shokravi Z, Heidarrezaei M, Ong HC, Koloor SSR, Petrů M et al. Fourth generation biofuel from genetically modified algal biomass: Challenges and future directions Chemosphere 2021;258:131535.
[6] Kumar R, Biswas K, Singh PK, Singh PK, Elumalai S, Shukla P et al. Lipid production and molecular dynamics simulation for regulation of accD gene in cyanobacteria under different N and P regimes. Biotechnol Biofuels 2017;10.
[7] Mehmood M. The global trend in biofuel science; challenges and opportunities. Protein Pept Lett 2019;26:468–470.
[8] Jagadevan S, Banerjee A, Banerjee C, Guria C, Tiwari R, Baweja M et al. Recent developments in synthetic biology and metabolic engineering in microalgae towards biofuel production, Biotechnol Biofuels 2018;11:185.
[9] Banerjee C, Singh PK, Shukla P. Microalgal bioengineering for sustainable energy development: recent transgenesis and metabolic engineering strategies. Biotechnol J 2016;11.
[10] Godbole V, Pal MK, Gautam P. A critical perspective on the scope of interdisciplinary approaches used in fourth-generation biofuel production. Algal Research 2021; 58:102436.
[11] Abdullah B, Muhammad SAFS, Shokravi Z, Ismail S, Kassim KA, Mahmood AN, Aziz MAA. Fourth generation biofuel: A review on risks and mitigation strategies Renewable and Sustainable Energy Reviews 2019;107:37–50.
[12] Dutta K, Daverey A, Lin J-G, Evolution retrospective for alternative fuels: First to fourth generation, Renewable Energy 2014;69:114-122.
[13] Alalwana HA, Alminshid AH, Aljaafari HAS, Promising evolution of biofuel generations. Subject review. Renewable Energy 2019;28.
[14] Trabelsi ABH, Zaafouri K, Baghdadi W, Naoui S, Ouerghi A. Second generation biofuels production from waste cooking oil via pyrolysis process. Renew Energy 2018;126:888–896.
[15] LeBauer D, Kooper R, Mulrooney P, Rohde S, D. Wang D, Long SP et al. BETYdb: a yield, trait, and ecosystem service database applied to second-generation bioenergy feedstock production. GCB Bioenergy 2018;10:61–71.
[16] Yi-Feng C, Wu Q. Production of biodiesel from algal biomass: current perspectives and future, Biofuels 2011;399–413.

[17] Vignesh P Kumar ARP, Ganesh NS, Jayaseelan V, Sudhakar K. Biodiesel and green diesel generation: an overview. Oil & Gas Science and Technology – Rev. IFP Energies Nouvelles 2021;76:6.
[18] Sharma D, Singh D, Soni SL, Sharma S, Sharma PK, Jhalani A. A review on feedstocks, production processes, and yield for different generations of biodiesel. Fuel 2019; ISSN 0016-23611165539.
[19] Maeda Y, Yoshino T, Matsunaga T, Matsumoto M, Tanaka T. Marine microalgae for production of biofuels and chemicals. Curr Opin Biotechnol 2018;50:111– 120.
[20] Mahapatra S, Dilip Kumar D, Singh B, Sachan PK. Biofuels and their sources of production: A review on cleaner sustainable alternative against conventional fuel, in the framework of the food and energy nexus. Energy Nexus 2021; 4:100036.
[21] Cavelius P, Engelhart-Straub S, Mehlmer N, Lercher J, Awad D, Bruck T. The potential of biofuels from first to fourth generation, Plos Biology 2023;21(3):3002063.
[22] Sikarwar VS, Zhao M, Fennell PS, Shah N, Anthony EJ. Progress in biofuel production from gasification. Prog Energy Combust Sci 2017; 61:189–248.
[23] Malode SJ, Sayeed Aaqueeb MG, Kamble PJ, Nalwad AA, Muddapur UM, Shetti NP. Recent evolutionary trends in the production of biofuels Materials Science for Energy Technologies 2022;5:262–277.
[24] Sizova I, Greiner A, Awasthi M, Kateriya S, Hegemann P. Nuclear gene targeting in Chlamydomonas using engineered zinc-finger nucleases. Plant J 2013;73:873–82.
[25] Henley WJ, Litaker RW, Novoveská L, Duke CS, Quemada HD, Sayre RT. Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation. Algal Res 2013;2:66–77.
[26] Glass DJ. Regulation of the commercial uses of microorganisms. Encycl Environ Microbiol 2003.
[27] Bergeson LL, Auer CM, Hernandez O. Creative adaptation: enhancing oversight of synthetic biology under the Toxic Substances Control Act. Ind Biotechnol 2014;10:313–22.
[28] Goldenfeld N, Woese C. Biology's next revolution. Nature 2007;445:369.
[29] Wang F, Zhang W. Synthetic biology: recent progress, biosafety and biosecurity concerns, and possible solutions. J Biosaf Biosecurity 2019;1:22–30.
[30] Wase N, Black P, DiRusso C. Innovations in improving lipid production: algal chemical genetics. Prog Lipid Res 2018;71:101–123.
[31] Jeon S, Lim J-M, Lee H-G, Shin S-E, Kang NK, Park Y-I et al. Current status and perspectives of genome editing technology for microalgae, Biotechnol. Biofuels 2017;10.
[32] Jiang W, Brueggeman AJ, Horken KM, Plucinak TM, Weeks DP. Successful transient expression of Cas9 and single guide RNA genes in *Chlamydomonas reinhardtii*. Eukaryot Cell 2014;13(11):1465.
[33] Shin S-E, Lim J-M, Koh HG, Kim EK, Kang NK, Jeon S et al. CRISPR/Cas9-induced knockout and knock-in mutations in *Chlamydomonas reinhardtii*. Sci Rep 2016;6.
[34] Wang Q, Lu Y, Xin Y, Wei L, Huang S, Xu J, Genome editing of model oleaginous microalgae *Nannochloropsis* spp. by CRISPR/Cas9, Plant J 2016:88.
[35] Slade R, Bauen A. Micro-algae cultivation for biofuels: Cost, energy balance, environmental impacts and future prospects. Biomass Bioenergy 2013;53:29–38.
[36] Menetrez MY. An overview of algae biofuel production and potential environmental impact. Environ Sci Technol 2012;46:7073–7085.
[37] Snow AA, Smith VH. Genetically engineered algae for biofuels: a key role for ecologists. Bioscience 2012;62:765–768.
[38] Hewett JP, Wolfe AK, Bergmann RA, Stelling SC, Davis KL. Human health and environmental risks posed by synthetic biology R&D for energy applications: a literature analysis. Appl Biosaf 2016;21:177–184.
[39] Quiroz D. Geographical Assessment of Algal Productivity and Water Intensity Across the United States. 2021.
[40] Holmatov B, Hoekstra, AY, Krol MS. Land, water and carbon footprints of circular bioenergy production systems. Renew Sustain Energy Rev 2019;111:224–235.
[41] Amundson MJ. An economic comparison of high moisture feedstock biofuel production. 2016.
[42] Hamilton ML, Warwick J, Terry A, Allen MJ, Napier JA, Sayanova O. Towards the industrial production of omega-3 long chain polyunsaturated fatty acids from a genetically modified diatom *phaeodactylum tricornutum*. PLoS One 2015;10:0144054.
[43] Russo DA, Beckerman AP, Pandhal J. Competitive growth experiments with a high-lipid Chlamydomonas reinhardtii mutant strain and its wild-type to predict industrial and ecological risks. AMB Express 2017;7:10.
[44] De Mooij T, Schediwy K, Wijffels RH, Janssen M. Modeling the competition between antenna size mutant and wild type microalgae in outdoor mass culture. J Biotechnol 2016;240:1–13.

[45] Kuiken T, Dana G, Oye K, Rejeski D. Shaping ecological risk research for synthetic biology. J Environ Stud Sci 2014;4:191–9.
[46] Szyjka SJ, Mandal S, Schoepp NG, Tyler BM, Yohn CB, Poon YS, et al. Evaluation of phenotype stability and ecological risk of a genetically engineered alga in open pond production. Algal Res 2017;24:378–86.
[47] Singh A, Billingsley K, Ward O. Composting: a potentially safe process for disposal of genetically modified organisms. Crit Rev Biotechnol 2006;26:1–16.
[48] Epstein E. The science of composting. Routledge 2017.
[49] Heilmann SM, Valentas KJ, Von Keitz M, Schendel FJ, Lefebvre PA, Sadowsky MJ. et al. Process for obtaining oils, lipids and lipid-derived materials from low cellulosic biomass materials. 2013.
[50] Ueda R, Hirayama S, Sugata K, Nakayama H. Process for the production of ethanol from microalgae. 1996.
[51] Möller K, Müller T. Effects of anaerobic digestion on digestate nutrient availability and crop growth: a review. Eng Life Sci 2012;12:242–57.
[52] Hrnčírová Z, Bergerová E, Siekel P. Effects of technological treatment on DNA degradation in selected food matrices of plant origin. J Food Nutr Res 2008;47.
[53] Bergerová E, Hrnčírová Z, Stankovská M, Lopašovská M, Siekel P. Effect of thermal treatment on the amplification and quantification of transgenic and non-transgenic soybean and maize DNA. Food Anal Methods 2010;3:211.
[54] Feng P-Z, Zhu L-D, Qin X-X, Li Z-H. Water footprint of biodiesel production from microalgae cultivated in photobioreactors. J Environ Eng 2016;142:4016067.

