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Review

Cyanobacterial Biofuel: A Platform for Green Energy

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Abstract

Cyanobacteria have great potential as a platform for biofuel production because of their fast growth, ability to fix CO_2 gas, and genetic tractability. They also preserve the sustainability of an ecosystem without harming the environment. High-performance biofuels made from cyanobacteria can be utilized as a base for the production of green energy. Although a lot of studies have been conducted where plants and crops are used as the source of energy, cyanobacteria have been reported to have a more efficient photosynthetic process strongly responsible for increased production with limited land input along with affordable cost. The production of cyanobacteria-based biofuels can be accelerated through genetic engineering or genomics research, which may help to meet the global demand for these fuels on a large scale. Cyanobacterial strains that have undergone genetic modifications have been developed as part of a green recovery approach to transform membrane lipids into fatty acids to produce cheap and eco-friendly green energy. Cyanobacteria also produce different biofuels such as butanol, ethanol and isoprene. The four different generations of biofuel production to meet the energy requirement have been discussed in this review. This review presents a

comprehensive strategy for the commercial viability of green energy production utilizing cyanobacteria to achieve a price for biofuels that can compete with the present or future market.

Keywords
Cyanobacteria; biofuel; genetic engineering; green energy

1. Introduction

Natural organic fossil fuels such as oil, gas, coal and peat result from long-term geological accumulation of organic biomass containing carbon and hydrogen. Biofuels derived from biomass are the outcome of photosynthesis by photosynthetic organisms such as plants, eukaryotic algae, microalgae and cyanobacteria. Even after years of research, eukaryotic algae have yet to perceive their industrial potential, while artificial biology techniques for eukaryotic systems are restricting our aptness to improve and diversify these strains [1].

Cyanobacteria, a prokaryotic photosynthetic organism fix carbon dioxide as their chief carbon source, removing the need for a source of fermentable sugars as a carbon feedstock for biofuel production. The utility of cyanobacteria as a platform for biofuel production has achieved significant popularity as a resource that could probably avoid many issues [2]. Some cyanobacterial species such as *Anabaena muscorum, Anabaena doliolum, Anabaena cylindrica* and *Synechocystis* sp. have been investigated for their ability to produce biofuel.

Cyanobacteria are considered a possible feedstock for carbon-neutral biofuels owing to their high biomass production and quick growing capacity compared to other photosynthetic organisms [3]. They can manufacture and accumulate considerable quantities, approximately 13-14% of the dry weight of neutral lipids in the cytosol [4, 5].

Biofuels are a class of renewable energy sources derived from biomass that can be converted directly into liquid fuel. Biofuel production using cyanobacteria needs large-scale outdoor cultivation that can be attained with closed photobioreactors or open ponds. Nevertheless, costs linked to the large-scale outdoor cultivation, harvesting and downstream processing for biofuel production are very high which constitute menaces to the economic credibility of cyanobacterial biofuel production [6-8]. In addition, the steady reduction in the price of crude oil has created a competitive environment ahead of the commercialization of the biofuel industry. The unavailability of well-characterized host cells, instability of genetic constructs, limited performance of synthetic biology tools and toxicity of final products are major challenges in developing cyanobacteria as a fuel-producing factory. Success in commercial production will depend on developing synthetic biological techniques, systematically isolating versatile host strains and comprehensive insights into metabolic flux maps. Cyanobacteria are sources of extensive and precious bioactive compounds. Large-scale production of these bioactive compounds for industrial use could be united with biofuel production to diminish costs [9, 10].

In terms of photosynthetic efficiency, cyanobacteria dominate plants as well as other algae. Cyanobacteria contain lipids, chiefly present in its thylakoid membranes and also can convert lipids into fatty acids, which can be used as biofuel [11]. Cyanobacteria have already been engineered to

produce biofuel-related compounds such as bioethanol, isobutyraldehyde, etc. Isobutyraldehyde, an aldehyde, is produced by the hydroformylation of propane as a side-product. The cyanobacterium *Synechococcus elongatus* was engineered to achieve the former goal of producing isobutyraldehyde. Isobutyralaldehyde can be easily transformed into several hydrocarbons from petroleum such as isobutanol, isobutyric acid, acetal, oxime and imine, applying existing chemical catalysis (Figure 1) [2]. Cyanobacterium *Synechococcus elongatus* sp. strain PCC 7942 was the first successfully engineered cyanobacteria for biofuel production [10]. Genetic engineering has greatly improved cyanobacterial ethanol production by adding a pyruvate decarboxylase and an alcohol dehydrogenase, redirecting carbon from pyruvate [12-14]. Genetic engineering could create strains with flocculating capabilities, which could help drastically lower the harvesting cost (Figure 2). Future advancements in the field of genomics and metabolic engineering make cyanobacterial factories economically viable for increasing the yields of biofuel production.

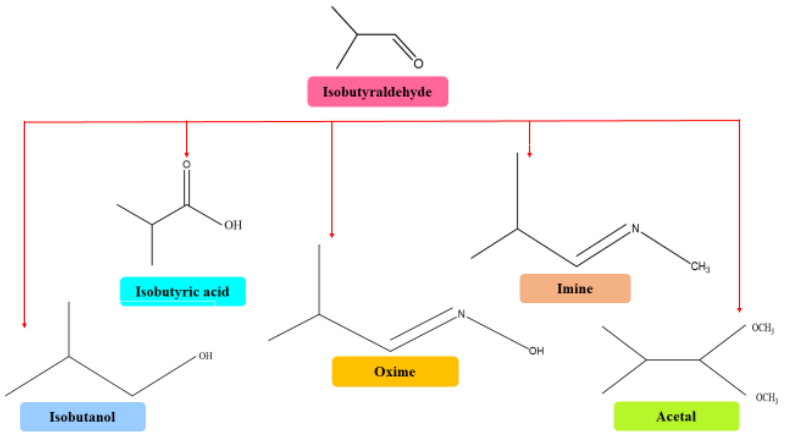

Figure 1 Conversion of isobutyraldehyde into various hydrocarbons.

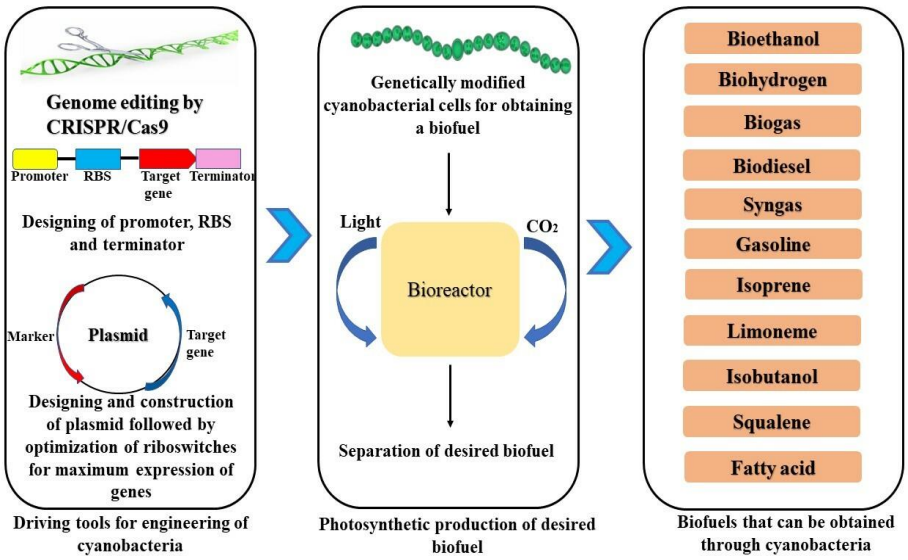

Figure 2 Driving tools involved in the engineering of cyanobacteria to obtain biofuels.

A biochemical outlook for producing substitute fuels implies biomass, which can be transformed into energy by thermal, chemical or biochemical conversion. The review mainly aims to highlight the potential of cyanobacteria in the field of biofuel production.

2. Generation of Biofuels

Biofuels are represented by biogas or liquid biofuels resulting from biomass conversion. Solid biofuels are produced by burning agricultural waste biomass such as rice husks, wheat straws, coconut shells and corn cobs [15]. A mixture of gases (mainly methane and CO_2) called biogas is formed when biomass breaks down without oxygen. Hydrogen produced in the light by cyanobacteria and algae from water is known as biohydrogen [16]. Biodiesel and bio-alcohols (ethanol, butanol and methanol) are examples of liquid biofuels [17]. Around 10 billion liters of bioethanol were produced globally in 2011, primarily from corn and sugar beans and 281.5 billion liters are anticipated to be produced in 2020. A byproduct of the esterification of vegetable, microalgal, or other microbial-derived oils is biodiesel. Through distillation and cracking, triacylglycerides (TAGs) and diacylglycerides (DAGs) can also be transformed into gasoline (petrol) or jet fuel [18]. Different types of biofuels depend on the sources of raw materials and processing methods. Four different generations of biofuel production are shown in Figure 3.

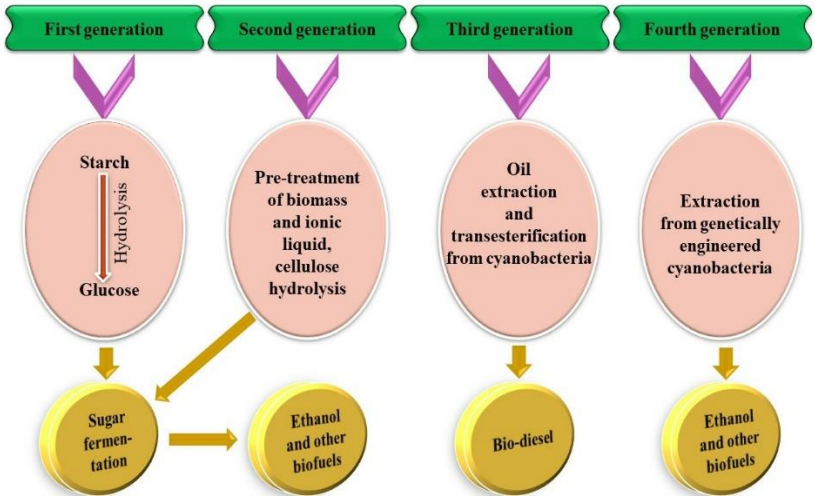

Figure 3 Diagrammatic representation of the four different generations of biofuel production.

2.1 First Generation Biofuels

The first-generation biofuel was produced by edible plants having a high oil, starch or sugar content which could then be converted into biodiesel or bioethanol [19]. First-generation biofuels include sugarcane, "corn" ethanol, starch-based biodiesel and pure plant oils. Vegetable oils [20, 21], bio-alcohols (most frequently ethanol) [22], biodiesel [23], biogas [24], and solid biofuels are examples of first-generation biofuel [25, 26]. Since producing biodiesel involves changing one kind of ester into another, it is known as transesterification [27].

2.1.1 Vegetable Oil

Vegetable oils have advantages in terms of energy, environment and the economy. About 90% as vegetable oils produce much heat as by diesel fuel. In some specific applications, waste cooking oil and vegetable oil are used as alternative fuels for diesel engines [27]. The engine recitation and

production in biofuel systems using vegetable oils as fuels in diesel engines were researched by Corsini et al. [27].

2.1.2 Biodiesel

Biodiesel comprises long-chain fatty acid-containing mono-alkyl esters derived from vegetable or animal fats. The primary areas of interest for current research on this subject include innovative biodiesel manufacturing and purification technologies, as well as affordable, plentiful feedstocks [28]. These feedstocks are primarily divided into waste or recycled oil, animal fats, edible vegetable oil and non-edible vegetable oil. It is less combustible than regular diesel. Because biodiesel is biologically degradable, it poses less of an environmental risk. Acid rain is mostly caused by sulfur, which is not included in this product. In many circumstances, biodiesel is suitable for catalytic converters. The engines that use biodiesel as fuel often have a longer lifespan. Biofuels contain more octane and have greater lubricating properties than pure diesel fuel made from petroleum. It can increase the machine's operating lifespan and engine efficiency. The accessibility of feedstock for biodiesel production is influenced by the country's agricultural history, local soil conditions, local soil environment and climate [29]. Biodiesel cannot be transported through pipelines. Upon combustion, it releases nitrogen oxide, which could pollute the environment.

2.1.3 Bio-Alcohol

Ethanol or bio-alcohol, is primarily produced through the fermentation of cane sugars and starches. During the generation of bio-alcohol, butanol and propanol are produced as byproducts. Ethanol is the major first-generation biofuel and extensively researched renewable energy source [30]. Most of the corn used to make gasoline-ethanol worldwide is produced in the United States [20]. Ethanol fuel spills are more easily biodegraded or diluted to non-toxic levels.

2.1.4 Bio-Gas

The primary phase in the digestion of waste materials to produce biogas (methane) and occasionally fermentation to produce ethanol is hydrolysis [31]. Biogas is produced during digestion under anaerobic conditions and its use as a practical method of providing continuous power generation is showing rapid signs of promise [32]. Without oxygen, organic substrates are broken down into methane through methanogenesis. Soon, biogas produced from wet organic materials such as animal manure, complete crop silages, wet food and feed wastes, etc., could account for more than 25% of all bioenergy [33]. Depending on the substrate, biogas composition varies greatly but commonly comprises 40–65% methane, 30–40% carbon dioxide and various contaminants such as hydrogen sulfide, ammonia and siloxanes [34, 35].

2.1.5 Advantages

The development of biofuels generates new market opportunities and aids in expanding agricultural products; as a result, farmers receive fresh income, improving their socio-economic condition. As a result, agriculture will continue to play a significant role in producing food and energy. Another benefit is the large-scale production of co-products, a necessary supplement, during the fermentation of sugar and starch plants.

2.1.6 Disadvantages

Feedstock is the primary source of first-generation biofuels. Feedstock mostly consists of food crops like corn and sugar beet. Costs have risen for food and animal feeds for several reasons [36, 37]. (a) Greater biofuel usage may lead to devastating effects on biodiversity such as water scarcity (b) First-generation biofuels require a lot of land to cultivate the biomass (c) They require significant amounts of energy to grow and accumulate. The supply of first-generation biofuels is constrained by restaurants' oil use (d) They are more expensive than gasoline and (e) Biodiesel comes from discarded restaurant cooking oils rather than virgin ones.

2.2 Second Generation Biofuels

Second-generation biofuels are obtained from non-food sources like grass, straw and wood which include lignin and cellulose [34]. These substances can be burned directly, pyrolyzed or transformed into flammable gases. Lignin is commonly used to produce second-generation biofuels because of its aromatic properties. Depending on the species, it comprises up to three distinct phenyl propane monomers. BioSNG, bioSNG, a synthetic gas, is another example of a second-generation biofuel [38].

2.2.1 Waste Vegetable Oil

Vegetable oil waste has no nutritional value; however, it may aid in lowering pollution levels. Certain diesel engines are designed to produce biofuels from this biomass without blending or refining [39]. Such a substance has some benefits, including not leaking sulfur into the environment, disturbing arable crops, and costing nothing to use the land. There are however some drawbacks, the fact that this biomass is hard to gather because it is dispersed throughout various locations and might harm diesel engines if it is not precisely cleansed before use.

2.2.2 Seed Crops

Although a large amount of this biomass can be grown on marginal land, its energy value is much lower than that of biofuels from soybean biomass.

2.2.3 Municipal Waste

Nowadays, this form of biomass is used to produce biofuels. It contains all forms of solid waste, such as leaf and grass clippings, human waste and landfill gas. Second-generation biofuels provide the following fuels.

2.2.4 Cellulosic Ethanol

It is produced by fermenting sugars extracted from lignocellulosic biomass's cellulose and hemicellulose components.

2.2.5 Biosynthetic Natural Gas (Bio-SNG)

Renewable natural gas can also be generated through gasification, catalytic methanation and purification. Anaerobic digestion using microorganisms can produce biogas. This gas is mainly composed of methane and carbon dioxide. Then, it can be put into an existing natural gas cylinder or used in automobiles as compressed natural gas (CNG) or liquefied natural gas (LNG) [40].

2.2.6 Pyrolysis Oils (Biocrude)

This is formed by ash pyrolysis, which involves heating to roughly 1,000 F and quickly cooling.

2.2.7 Hydrotreated Vegetable Oil

It is utilized as a diesel substitute because it contains qualities that are greatly in demand, such as high cetane, no odor and no sulfur.

2.2.8 Advantages

Second-generation biofuels are profitable because they utilize a non-food feedstock (like lignocellulosic biomass material, such as earth crops residues, forest products residues, or fast-growing devoted energy crops). The fuel can be used in existing cars without blending and is a call-on substitute for traditional petroleum-based fuels. Second-generation biofuels are less harmful to the environment and emit fewer greenhouse gases. They don't produce byproducts like animal feed. They use less area, so they compete less with other agricultural fields for available land and require less water and food fiber.

2.2.9 Disadvantages

There is currently no commercial production of second-generation fuels due to the high production costs and lack of technical validation of this technology. The current technologies for harvesting, storing and transporting biomass are insufficient for handling and distributing it widely. The necessity to produce biomass feedstock from residues and crops implies a considerable change in the current business model, as well as trade-in feedstock and biofuel.

2.3 Third Generation Biofuels

Third-generation biofuels are derived from microalgae (unicellular algae), which can be cultivated in open ponds or closed photobioreactors [41]. Microalgal triacylglycerols (TAGs), isolated from cells and utilized to produce biodiesel, are typically referred to as third-generation biofuels [42]. It yields more than 30 times as much energy per acre than conventional land crops like soybeans. Algae typically include many prokaryotic and eukaryotic species [43]. The technologies used to convert algal biomass into energy sources mainly fall into three categories: biochemical, chemical and thermochemical conversion, as well as the construction of an algal biorefinery [44]. Biodiesel, butanol, gasoline, methane, ethanol, vegetable oil and jet fuel are examples of third-generation biofuels produced from algae.

2.3.1 Advantages

(a) It has the potential to grow year-round, (b) there is a higher tolerance for high carbon dioxide content, (c) water consumption is very low, (d) algal farming does not require the use of herbicides or pesticides, (e) it can grow in insensitive environments like saline, brackish water and coastal seawater, which have no impact on the productivity of conventional agriculture [44], (f) one advantage of biofuels over other fuel types is that they are biodegradable, which makes them relatively harmless for the environment in the event of a spill, (g) algal biofuels were chosen as the best resource to replace liquid petroleum fuel because they have a high oil content with high productivity, a good yield per acre (up to 10 times greater than other biofuels).

2.3.2 Disadvantages

(a) the higher cost of agricultural demands as compared to that of other traditional crops, (b) the harvesting of algae requires comparatively more energy input, which accounts for roughly 20–30% of the total cost of manufacture, (c) various techniques, including flocculation, floatation, centrifugation, sedimentation and filtration, are typically used for producing and concentrating the algal biomass, (d) despite being able to grow in wastewater, algae still require a significant amount of water.

2.4 Fourth Generation Biofuels

Fourth-generation biofuels combine the properties of third-generation biofuels with the advantage of genetic optimization of their producers. The raw materials required for fourth-generation biofuel production are comparatively cheaper and easily accessible [45]. This biofuel is produced by converting vegetable oil and biodiesel into gasoline [20].

2.4.1 Advantages

Due to the higher yield and higher lipid content of fourth-generation algae compared to third, they are more suitable for producing biofuels. Compared to other biofuels, it has a higher capacity for CO_2 collection and a higher production rate.

2.4.2 Disadvantages

The high rate of initial investment is the primary drawback of algae production. The early stages of algae production research are now being conducted.

Due to the depletion of oil and gas supplies, rising prices for these resources and the need to assure energy security, biofuels are becoming more popular. According to Angermayr et al. [46], cyanobacteria are suitable sources of renewable liquid biofuels that contain hydrocarbon chains and can take the place of petroleum hydrocarbons in the production of fuels, lubricants, polymers and other products. Three types of diesel fuels are currently characterized by their origin and processing methods [47, 48]. The diesel fuel that is made from petroleum is known as petro-diesel (Standards EN 590 in the EU and ASTM D975 in the USA).

1. A sustainable fuel known as biodiesel, which complies with specifications and industry standards, is made up of mono-alkyl esters of long-chain fatty acids obtained from vegetable oils or animal fats (standards ASTM DD6751 and EN 14214).
2. Renewable diesel can also be produced from biomass using the same ASTM D975 and EN 590 standards for petrodiesel, a catalytic reaction of fatty acids and fatty acids esters with hydrogen, hydrodeoxygenation or cracking and pyrolysis of biomass [49, 50].

We are currently on the verge of developing and utilizing fourth-generation biofuels based on a resource that is genetically designed, rapidly renewable and fast-growing prokaryotic cells of cyanobacteria. Cyanobacteria have a strong potential for producing cyano-diesel due to their numerous beneficial characteristics.

- Cyanobacteria grow to biomass rapidly.
- Because cyanobacteria can grow on non-fertile wasteland, they don't compete with fertile lands.
- Cyanobacteria directly fix atmospheric CO_2 for which the minimal requirements are sunlight, water and certain inorganic trace elements for growth. The surplus CO_2 (the emissions we want to reduce) can be handled by cyanobacteria and converted directly into hydrocarbons for biofuels.
- Since cyanobacterial metabolism is flexible, lipid production in managed photobioreactors is possible.
- Many cyanobacterial strains can be quickly and successfully transformed. Therefore, the genetic modification of metabolic pathways can be done on them, providing a simple platform [51]. Some scientists also prefer cyanobacteria because, unlike eukaryotic algae, which produce fuel inside the cell, engineered cyanobacteria excrete or secrete their fuel outside the cell.

3. Biofuels

Biofuel is defined as fuel obtained from biomass, which includes plant, algal, or animal waste. Due to their non-toxic, sulfur-free, biodegradable and derivation from renewable sources, biofuels are considered an alternative to fuel [52-58]. Energy and environmental security are among the major global concerns, necessitating substantial study to find and implement economical and sustainable biofuel production methods. While it is hard for biofuels to completely replace petroleum-derived fuels, even a small amount of diesel substitution with biofuels might delay the depletion of petroleum resources [30]. The following is a list of the official biofuel targets for the world.

(a) Brazil showed a 40% increase in ethanol production between 2005 and 2010, with mandatory ethanol blends of 20–25% in gasoline and at least 3% biodiesel in diesel by July 2008 and 5% (B5) by the end of 2010.
(b) Canada aims to have 5% renewable content in gasoline by 2010 and 2% diesel fuel by 2012.
(c) European Union –10% in 2020 (biofuels); target set by European Commission in January 2008.
(d) UK–5% by 2020 (biofuels, by energy content).
(e) USA–25% ethanol production by 2020.
(f) Japan aims to have 10% biofuel production by 2030.

Over the past 30 years, cyanobacteria have been widely studied for producing biofuels, especially concerning their ability to quickly accumulate biomass through photosynthesis and synthesize lipids.

3.1 Production of Butanol by Cyanobacteria

Butanol is a four-carbon structure having straight-chain alcohol and its chemical formula is $C_4H_{10}O$. Due to its low hygroscopicity and energy content (27 MJ/L), which is about the same as gasoline's energy (32 MJ/L), it is considered an alternative to diesel fuel and gasoline. It is a byproduct of microbial fermentation processes and can be produced industrially from the petrochemical feedstock propylene. Butanol is less volatile, highly viscous and has high heating value and inter solubility. It is safely used at high temperatures and has fewer ignition problems. So, it overcomes the limitations caused by low-carbon alcohols [59].

Butanol and isobutanol are not produced by cyanobacteria naturally because biosynthetic pathways responsible for butanol production and necessary genes involved in this pathway are not present in cyanobacteria [60, 61]. Cyanobacteria have been designed to make photosynthetic butanol in the presence of water, carbon dioxide and sunlight. The genes from *Clostridium acetobutylicum*, *Treponema denticola* and *E. coli* that generate 1-butanol without oxygen have been added to the *S. elongatus* PCC 7942 genome [35]. Nowadays, *Synechocystis* can produce up to 0.9 g L^{-1} in 46 days and 4.8 g L^{-1} in 28 days [62, 63] of isobutanol and 1-butanol, respectively, during long-term cultivation. The corresponding maximum rates for isobutanol and 1-butanol are 43.6 mg L^{-1} day^{-1} and 302 mg L^{-1} day^{-1}, respectively [63, 64].

To produce heterotrophic 1-butanol, the Clostridia route, a naturally occurring 1-butanol-producing pathway from the genus *Clostridium*, was introduced into *E. coli* [60]. The 2-keto acid route, a synthetic biosynthetic pathway, was constructed into *E. coli* to produce isobutanol [64]. Following the development of this successful butanol-producing pathway, cyanobacteria were used to build and evaluate these pathways. Two unicellular model strains, *Synechococcus elongatus* PCC7942 and *Synechocystis* PCC6803, have been the focus of most engineering research into cyanobacteria for butanol production. Several routes of enzymes involved in butanol formation were over-expressed, introduced and assessed to improve the metabolic ways for butanol [65]. Two base plasmids in the model strain *Synechocystis* have been genetically modified to produce butanol:

(a) pRH-ECT7 - knock-out of *phaEC*, inserts ORF's under control the *phaEC* promoter/RBS, uses T7 terminator (kanamycin resistance plasmid).
(b) pRH-BT7b – knock-in extra ORF's onto the end of the *phaAB* mRNA (after *phaB*), has RBS from *psbA2* gene, uses T7 terminator (chloramphenicol resistance plasmid) [63].

3.2 Production of Ethanol by Cyanobacteria

Renewable energy sources can produce ethanol, which can be used in existing diesel engines without requiring any adjustments [66]. Early in the new millennium, the fermentation of crops like sugarcane, corn, sorghum, etc. was the predominant method for producing ethanol [67]. Cyanobacteria were considered a more desirable than crops for ethanol generation because they undergo natural fermentation. Thirty-seven cyanobacterial strains were examined by Heyer et al. [68] for their capacity to ferment and generate ethanol. Among thirty-seven strains, sixteen strains were able to produce ethanol, while two species of *Oscillatoria* produced a significant amount of ethanol. *Synechococcus* sp. PCC 7942 was the first cyanobacterial strain to undergo genetic

modification for increased ethanol production. Pyruvate decarboxylase and alcohol dehydrogenase II, two genes from the required fermentative prokaryote *Zymomonas mobilis*, were transformed under the control of the cyanobacterial operon promoter rbcLS alone and with the *E. coli* lac promoter [12].

Dexter and Fu [13] also investigated the increased ethanol production when the same gene was expressed with PSB. The light-driven psbA2 promoter system can be used to produce the pyruvate decarboxylase (*pdc*)/alcohol dehydrogenase II (*adh*) gene cassette in *Synechocystis* strains, making it possible to produce and describe ethanol-producing mutants. Approximately 6 days after inoculation, ethanol will accumulate under specific conditions in the liquid media at a concentration of 10 mM or more. Ethanol yields were reported to be 5.2 mmol OD_{730} $unit^{-1}$ $litre^{-1}$ day^{-1} during the log phase of growth [13]. Cellulose is an alternative method for producing ethanol. Cellulose synthase genes from *Gluconobacter xylinus* were employed to genetically alter *Synechococcus* sp. PCC 7942 by which extracellular non-crystalline cellulose was produced making it the perfect feedstock for ethanol synthesis [69].

In the ethanol production pathway, the enzymes that play important roles are pyruvate decarboxylase (*Pdc*) and alcohol dehydrogenase (*Adh*). The preceding enzyme converts pyruvate to acetaldehyde and CO_2 through nonoxidative decarboxylation and then the acetaldehyde is subsequently converted to ethanol. In the first investigation, *Synechococcus* 7942 was used as the platform for ethanol production by expressing the *pdc* and *adhII* genes from the bound ethanol producer *Zymomonas mobilis* under the direction of the *rbcLS* promoter of the cyanobacteria. Several studies have been done on ethanol production using different different cyanobacteria strains to determine the maximum yield of ethanol. The photoautotrophic conversion of CO_2 to bioethanol using the *Synechocystis* 6803 strain was reported previously [13]. Deng and Coleman obtained an ethanol yield of about 5 mM (0.23 g L^{-1}) after four weeks of culture [12]. In the process of the photobioreactor condition, the engineered strain generated approximately 10 mM of 0.46 g L^{-1} ethanol after 6 days, with an average yield of 0.0766 g L^{-1} day^{-1} [13]. In the oxidative pentose phosphate pathway, glucose-6-phosphate dehydrogenase is encoded by an endogenous gene (*Zwf*). This *Zwf* gene was overexpressed to enhance the yield of NADPH in *Synechocystis* 6803. Moreover, *pdc* from *Zymomonas mobilis* and *yqhD* (encoding NADPH-dependent alcohol dehydrogenase) from *E. coli* were also introduced into the NADPH overproducing platform to enable ethanol synthesis. Ethanol production extended to 0.59 g L^{-1} following 14 days in the strain overexpressing *zwf*, *pdc* and *yqhD*, which was 33% higher than the strain over-expressing only *pdc* and *yqhD* [70].

3.3 Production of Hydrogen by Cyanobacteria

Biofuels may be gaseous, like biogas, syngas and biomethane, or solid like fuel wood, charcoal and wood pellets. They could also be liquid like ethanol, biodiesel and pyrolysis oils [71]. Hydrogen is anticipated to play a significant role in the world's energy future by displacing fossil fuels. A promising new energy source that is gaining prominence is hydrogen [72]. Since it only emits water when burned, it is one of the most promising clean fuels. Oil, natural gas and coal are the main non-renewable energy sources used to produce the bulk of the hydrogen on the planet [73]. Pyrolysis is a commercially well-established thermochemical technique, carried out without oxygen and at temperatures ranging from 300 to 600°C, to convert organic biomass into biofuels or bio-oils [74, 75].

Pyrolysis and gasification of biomass have significant potential for producing renewable hydrogen, which is advantageous for exploiting biomass resources, developing a highly efficient clean way for large-scale hydrogen production and reducing reliance on insecure fossil energy sources. In oxygenic phototrophs, only green microalgae and cyanobacteria have been shown to produce hydrogen. Several types of cyanobacteria can produce hydrogen due to the reversible action of hydrogenase. Nitrogenase, a reversible bidirectional hydrogenase (Hox) and an uptake hydrogenase (Hup) are three different forms of hydrogen metabolism enzymes that have been found in cyanobacteria [76]. Even in a 100% nitrogen gas in the atmosphere, hydrogen is produced as a side reaction at 1/3 to 1/4 the rate of catalyzed nitrogen fixation. In the most efficient H_2-producing species, nitrogenase enzymes generate H_2 as a byproduct of N_2 fixation. An uptake hydrogenase (Figure 4) catalyzes hydrogen consumption produced by nitrogenase and a bidirectional hydrogenase, which can both take up and produce hydrogen [77].

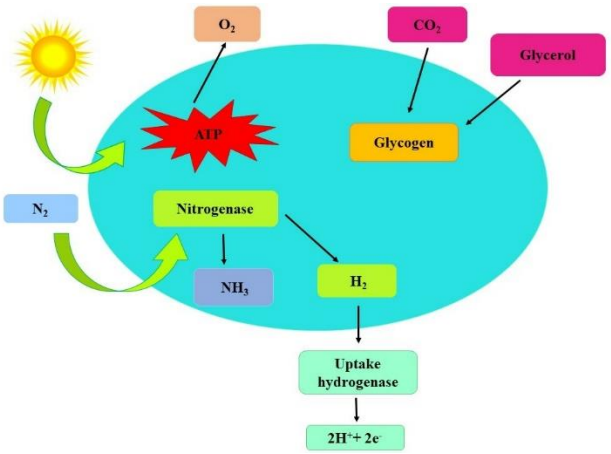

Figure 4 Schematic presentation of hydrogen production from cyanobacteria.

When cyanobacteria are grown in an N_2-limited environment, nitrogenase fixation results in the production of H_2. It was also discovered that heterocystous cyanobacteria produce more hydrogen than non-heterocystous cyanobacteria [58]. Several sources claim that at least 14 genera of cyanobacterial species that can produce H_2 have been grown under different conditions [78]. It was discovered that among these genera, *Anabaena* sp. produced the highest H_2. When employing cyanobacteria to produce hydrogen, the major problem is the simultaneous generation of oxygen and hydrogen by the two photosystems. The evolved O_2 inhibits hydrogenase activity and reduces its synthesis. The availability of reducing substances like ferredoxin and NADPH, which are also essential for other routes like respiration, is another problem. To boost H_2 synthesis, it will be required to redirect some of the electron flow toward H_2-producing enzymes and to produce hydrogenases that can withstand oxygen [46, 79]. The overall H_2 production was five times higher in mutants of *Synechococcus* 7002 lacking lactate dehydrogenase compared to the wild type [80]. A mutant *ldhA* strain of the cyanobacterium *Synechococcus* sp. PCC 7002, was developed by inactivating the d-lactate dehydrogenase gene. The dark anaerobic metabolism of *Synechococcus* 7002 is distinct from other cyanobacteria that have been discovered so far and can produce up to five different fermentation products, including lactate, acetate, succinate, alanine and hydrogen. In comparison to wild-type cells, the *ldhA* mutant exhibits significantly altered fermentative fluxes, a higher NAD(P)H/NAD(P)$^+$ ratio and up to five times as much hydrogen production [80]. The

subsequent deletion of two uptake hydrogenases (1: *hya* and 2: *hyb*) increased the H_2 production yield from 1.2 mol to 1.48 mol mol^{-1} of glucose, demonstrating that the activity of uptake hydrogenases is crucial under anaerobic, glucose fermentation conditions [81]. To produce O_2 and H_2, it takes advantage of the geographic separation in which heterocysts are specialized spaces where cyanobacteria that fix nitrogen produce hydrogen. O_2 is formed in the light and H_2 is produced in the dark when there is a temporal separation [79]. The main purposes of genetic engineering are to increase the O_2 tolerance and effectiveness of hydrogenases [82]. Cyanobacterial cells that have been immobilized; increase the amount of light available and direct absorbed light energy toward H_2 production rather than biomass accumulation [83]. Improvement of growth conditions, including the development of automated photobioreactors, to increase the yield and efficiency of hydrogen photoproduction [84]. Artificial water-splitting technologies are being developed to enhance H_2 output [85]. Cyanobacteria form a large and diverse group [86, 87] of oxygenic photoautotrophic prokaryotes [88], many of which can produce hydrogen. Cyanobacteria have attracted special attention in recent years due to their significant applications in the field of biotechnology [89, 90]. Hydrogen production (Figure 4) has been studied in various cyanobacterial species and strains [91]. Unicellular non-diazotrophic cyanobacterium *Gloeocapsa alpicola* under sulfur starvation showed increased hydrogen production [92]. *Arthrospira* (*Spirulina platensis*) can produce hydrogen (1 μmole H_2/12 hr/mg cell dry weight) in completely anaerobic and dark conditions [93]. Another nitrogen-fixing cyanobacterium, *Anabaena cylindrica*, produces hydrogen and oxygen gas simultaneously in an argon atmosphere for 30 days in light-limited conditions [94]. Symbiotic cyanobacteria within the *Cycas revoluta* or Sago palm and *Zamia furfuracea* showed a significant *in vivo* hydrogen uptake [95]. *Anabaena* sp. can produce a significant amount of hydrogen. Among them, nitrogen-starved cells of *Anabaena cylindrica* produce the highest amount of hydrogen such as 30 mL of H_2 L^{-1} culture hr^{-1}. Hydrogenase-deficient cyanobacterium *Nostoc punctiforme* NHM5 when incubated under high light for a long time, until the culture was depleted of CO_2 showed increased hydrogen production [96]. Several intrinsic factors such as genetic components or sensitive proteins in cyanobacteria may also be addressed by engineering the native hydrogenase. Engineering oxygen-tolerant hydrogenase genes, for example, *hydS* and *hydL* from *Thiocapsa roseopersicina* into sensitive organisms may help reduce oxygen sensitivity [97]. An expression vector pEX-Tran used for cyanobacterium *Synechococcus* sp. PCC7942 transformation is readily available and with minimal modification should be suitable for other cyanobacterial systems as well [98].

3.4 Production of Isoprene from Cyanobacteria

2-Methyl-1,3-Butadiene is another name for isoprene. It is an indistinct liquid hydrocarbon with the chemical formula C_5H_8. Since many isoprenoids have compact cyclic structures, they offer much potential as high-density fuels [98]. Several species that use photosynthetic energy also produce and emit isoprene. The two major pathways for isoprene production are the mevalonic acid (MVA) pathway and the 2-C-methyl- D-erythritol 4-phosphate (MEP) pathway. The MEP pathway produces two final products: isopentenyl diphosphate and dimethylallyl diphosphate (DMADP). DMADP serves as a precursor for carotenoids, the phytol of chlorophyll and quinones, which act as essential cofactors for photosynthesis [99, 100]. Dimethyallyl pyrophosphate (DMAPP) is cleaved by the enzyme isoprene synthase to produce isoprene and diphosphate. As a replacement for dwindling

fossil fuels, renewable energy fuels can be made from isoprene as a feedstock. Isoprene is biosynthesized more sustainably and environmentally favorable in photosynthetic cells. As a biofuel, isoprene has various advantageous properties over other biofuels, including greater energy density, higher octane ratings, lower water miscibility and better low-temperature fluidity [101]. In cyanobacteria, glyceraldehydes-3-phosphate (G3P) and pyruvate (*Pyr*) serve as feeder molecules for the formation of DMAPP and IPP via the methylerythritol phosphate (MEP) route. Cyanobacteria have the MEP route, but they lack the enzyme isoprene synthase (*IspS*) needed to produce isoprene. As a result, transgenic organisms bearing the transgene for isoprene production were developed. By expressing the *isp's* gene for the isoprene synthase from the isoprene-emitting kudzu vine, *Pueraria montana*, *Synechocystis* sp. PCC 6803 was engineered to produce isoprene (50 lg g^{-1} dry cell weight daily) [102]. The heterologous expression of the mevalonic acid pathway in *Synechocystis* increased the photosynthetic carbon partitioning toward the formation of isoprene (120 lg g^{-1}), enriching the pool of precursors to isoprene, isopentenyl-diphosphate and dimethylallyl-diphosphate [103]. Recently, the genes encoding enzymes of the MEP pathway have been identified and functionally characterized, mainly in *E. coli* [104, 105]. This knowledge allowed genome searches and revealed that genes for the MEP pathway (Figure 5) enzymes are present in all cyanobacteria, which are mainly involved in synthesizing photosynthetic pigments. The initial step of isoprene synthesis in the cyanobacterium *Synechocystis* sp. PCC 6803 *via* the MEP pathway is catalyzed by 1-deoxy-d-xylulose 5-phosphate synthase (DXS), which uses pyruvate and d-glyceraldehyde 3-phosphate as precursors. It has been shown that DXS activity controls the emission of isoprene in plants [106-108]. All kingdoms of life contain terpenoids, commonly known as isoprenoids [109, 110]. Terpenoids can be categorized into hemi- (C_5), mono- (C_{10}), sesqui- (C_{15}), di- (C_{20}), tri- (C_{30}), and tetra- (C_{40}) terpenoids because they essentially consist of one to eight isoprene units (C_5) [111]. In recent years, the cyanobacteria *Synechococcus* sp. PCC 7002, *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 has all shown great promise as hosts for the production of terpenoids [112]. As part of their photosynthetic process, these unicellular cyanobacteria naturally produce terpenoids, such as carotenoids, hopanoids and the phytol tail of chlorophyll [113]. Cyclic monoterpenes like limonene have a higher energy density and their freezing point and boiling point are comparable to Jet A^{-1} aviation fuel [114]. Due to its characteristics, including a similar cetane number, carbon length and ring structure to diesel (C^{16}), bisabolene, a derivative of sesquiterpenes bisabolene (C^{15}), can be used as a substitute for diesel [115, 116]. By heterologously expressing limonene synthase (*Lms*) from *Schizonepeta tenuifolia*, *Synechocystis* PCC 6803 synthesized the volatile monoterpene limonene. A codon-optimized *lms* was co-expressed with the native *dxs, crtE* and *ipi* MEP pathway genes under the control of the Ptrc promoter. Limonene was produced by strains expressing only *lms* at a rate of 41 g L^{-1} d^{-1}, while strains expressing three additional MEP pathway genes produced 56 µg L^{-1} d^{-1} [117]. Under the direction of *cpcBA*, heterologous expression of the *Mentha spicata lms* in *Synechococcus* 7002 led to the production of 4 mg L^{-1} of limonene at a rate of 50 µg L^{-1} d^{-1} [118].

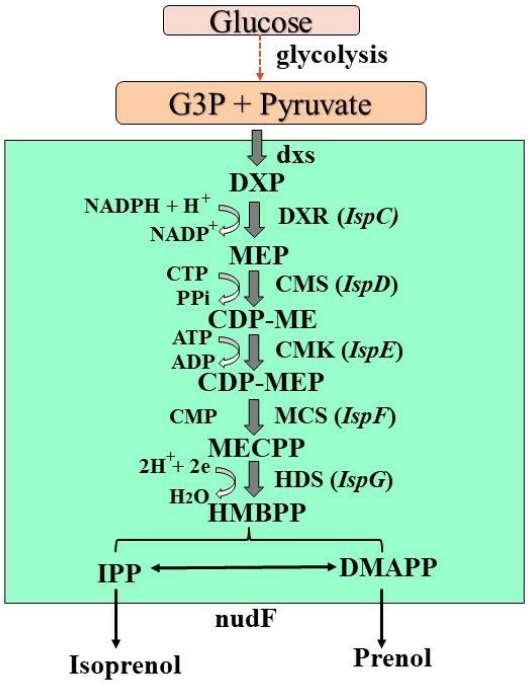

Figure 5 MEP pathway for the production of isoprene. (MEP: 2-C-methyl-D-erythritol 4-phosphate; G3P: Glyceraldehyde 3-Phosphate; DXP: 1-deoxy-D-xylulose 5-phosphate; CDP-ME: methylerythritol cytidyl diphosphate; CDP-MEP: methylerythritol 4-phosphate cytidyl diphosphate; cMEPP: 2-C-methyl-D-erythritol-2, 4-cyclopyrophosphate; MCS: cMEPP synthase; HMBPP: (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; IPP: Isopentenyl diphosphate; DMADP: Dimethylallyl diphosphate; GGPP: Geranylgeranyl pyrophosphate; DXP: Deoxyxylulose 5-phosphate; DXS: DXP synthase; DXR: 1-Deoxy-d-xylulose 5-phosphate reductoisomerase; CMS: 4-Diphosphocytidyl-2C-methyl-D-erythritol synthase; CMK: 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; HDS: 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; HDR: 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase).

After 30 days of cultivation, *Synechocystis* 6803 produced 1 mg L^{-1} of limonene with the help of the *Ptrc* promotor and *dxs, crtE, ipi*, and *lms* genes [117]. *Anabaena* 7120 produced 0.52 mg L^{-1} of limonene after 12 days of cultivation with the aid of the *Pnir/PpsbA1* promotor and the *lms, dxs, idi* and *gpps* genes [119]. With the aid of the *PpsbA* promotor and the *lms* gene, *Synechococcus* 7942 generated 2.5 mg L^{-1} of limonene after 4 days of cultivation [120]. *Synechococcus elongatus* UTEX 2973 produced 16.4 mg L^{-1} of limonene after 2 days of cultivation with the aid of the *Ptrc10* promotor and the *dxs, crtE, idi, and lms* genes [121].

3.5 Production of Lipids from Cyanobacteria

Biologically sourced lipids are trans-esterified in biodiesel to produce fatty acid methyl ester (FAME) [122]. Thus, lipid content significantly impacts the processes and quality of biodiesel production. Because cyanobacteria provide lipids, they are crucial components in biotechnology and biofuels. The best fatty acid profile and high lipid output are important factors to consider when choosing cyanobacteria for biodiesel production. An important factor determining biodiesel's

quality is fatty acid esters' structure, which affects vital biofuel characteristics like cetane number, ignition temperature, viscosity, oxidative stability and fluidity at low temperatures [123]. As a result, biofuels containing molecules with many saturated bonds have a stronger resilience to oxidation and glycerol polymerization does not occur during combustion, considerably increasing engine reliability [124].

Engine reliability is considerably enhanced when numerous saturated bonds are present in biofuels because they exhibit better oxidation resistance and avoid glycerol polymerization during combustion. Regarding lipid accumulation, *Synechocystis*, *Anabaena*, *Synechococcus*, *Oscillatoria* and *Spirulina* are currently well-studied. They can retain significant amounts of diacylglyceride as a reserve lipid (20 to 50% of the cell's dry mass) under photooxidative stress or other adverse environmental conditions [125]. The findings on a sufficient quantity of lipids in the cells of cyanobacteria *Croococcidiopsis* sp. (22.7%), *Synechocystis* PCC6803 (12.5%), *Limnothrix* sp. (20.73%), *Leptolyngbya* sp. (21.15%), *Synechococcus* sp. (30.6%) and *Oscillatoria* sp. (31.9%), *Microcystis panniformis* (35.8%), *Microcystis proteolysis* (41.5%), *Anabaena variabilis* (46.9%), *Synechococcus* sp. MK568070 (21.4%), *Limicolariam artensiana* (5%) are reported [126-130].

According to our study, cyanobacteria that secrete fatty acids, lipids, isoprene, ethanol, etc. hold great promise for developing renewable biofuels.

4. Global Status of Biofuel Production and Utilization

Globally, biofuel production has been rising consistently, reaching a peak in 2017. With bioethanol making up more than 60% of global biofuel production, the United States is the world's top producer of these fuels [131, 132]. Biodiesel hardly meets 25% of the requirement, but bioethanol makes up 75% [133, 134]. On a global scale, bioethanol and biodiesel can produce up to 1.91×10^6 and 0.82×10^6 TJ year^{-1} of energy, respectively [134]. The top two consumers of bioethanol and biodiesel are the United States and Brazil, with France, Germany, China, Canada and Italy following closely after. The United States set a record for renewable energy production in 2020, demonstrating its position as a global leader in producing renewable energy despite the global calamity of COVID-19 [135]. In contrast to the global scenario, India's biofuel production has experienced a brief uptake and has successfully sustained a long-term gain, showing a peak in 2016. The Indian government has started several programs, including the National Biodiesel Mission, the Biodiesel Blending Program and the Ethanol Blended Petrol (EBP) Program, to encourage the sale of blended fuels in India. According to the National Biofuel Policy (NBP) 2018, gasoline and diesel containing 20% ethanol and 5% biodiesel will be sold by 2030.

The Indian government recently presented the expert committee's report on the roadmap for ethanol blending in India by 2025 in honor of World Environment Day, which states that 20% ethanol blending in gasoline will be implemented by that year [136]. This indicates that India would achieve its aim of blending 20% ethanol into gasoline, five years earlier than expected. Some developed countries have set goals and made it mandatory to use biofuel. Examples include the United States' goal of using 25% ethanol by 2020 and Brazil's implementation of B20 by 2020. Due to their lack of environmental awareness, some developing nations export biofuel but do not use it. To minimize worldwide IC engine emissions, the governments of those emerging nations can take the initiative to use biofuel as a fuel in their transportation sector. Thus, the beneficial effects of government actions will soon be seen in increased biofuel production and consumption, a decrease in GHG

emissions, and a decrease in dependency on foreign sources of oil. Encouraging new bioenergy start-up businesses will also improve the nation's economy [137]. Because of this, using biofuel on a larger scale can provide a long-term supply of energy and contribute to improving the environment by lowering Greenhouse gases (GHG) and other pollutants. Carbon dioxide from the atmosphere is sequestered into biomass, which reduces the risk of climate change and the associated natural calamities. Additionally, numerous countries, particularly India, now have in place policy frameworks for blending biofuels with fossil fuels, which has expanded the industry for biofuels. In general, China has increased scientific research next to the United States, which will soon pay off in the form of rising biofuel production [136].

By 2050, the International Energy Agency (IEA) hopes that biofuels will supply more than a quarter of the world's demand for transportation fuels, thereby reducing the reliability of petroleum. To fulfill the IEA's sustainable development scenario, biofuel production and consumption are not on pace. To meet the IEA's target between 2020 and 2030, the world's biofuel production must rise by 10% annually. However, only a 3% increase each year is anticipated for the following five years.

5. Conclusion

Cyanobacteria are considered safe, non-competitive and fast-growing organisms that can withstand adverse environmental conditions with high oil content, providing a promising platform for biofuel production. Because cyanobacterial biofuel contains no dangerous substances, the environment may be kept clean after combustion. Cyanobacterial cells can be employed as biological factories for producing biofuels and value-added products, which will help make biorefineries commercially feasible. Biofuels that are mainly produced by cyanobacteria are butanol, ethanol, hydrogen, isoprene and lipids etc. The advantages and disadvantages of the four different generations of biofuel production to meet the energy requirement have been discussed above. When comparing the benefits of biofuels to those of fossil fuels, it is important to note that biofuels contribute significantly to lowering the atmospheric carbon intensity, lessening the GHG emission and reducing the oil dependence on limited resources for sustainable growth and development. Cyanobacteria have promising potential for their use in the energy sector due to recent advances in genetic engineering to manipulate these photosynthetic organisms to meet our ever-growing energy needs in an eco-friendly way. The ideal growth conditions for cyanobacterial cultures depend on strain and biomass productivity. Light and temperature are the two main abiotic variables that considerably impact the productivity of cyanobacterial biomass. Cyanobacterial metabolic engineering offers excellent prospects for modifying biofuel-related pathways to boost productivity. Moreover, cyanobacteria play a vital role in choosing the suitable genes for overexpression or disruption, enabling more effective and focused production. It would be ideal to conduct a thorough search and analysis of new cyanobacterial species that are naturally occurring and have certain biotechnological features. To satisfy the criteria of today's demands, the technology of cyanobacterial biomass conversion to bio-oil and cyano-diesel needs to be improved and standardized. The global demand for biofuels can be solved on a large scale through genomics research and metabolic engineering of cyanobacteria without affecting the environment. Through genomics, researchers can improve their understanding of using different renewable energy sources, including lignocellulosic biomass, microalgae and cyanobacteria. The development of sustainable biofuels through the genetic engineering of an enzyme may be effective in replacing fossil fuels.

Certain properties of cyanobacteria such as the ability to produce or store energy-rich hydrocarbons, faster growth rate, metabolic capabilities and high photosynthetic conversion efficiencies have allowed for successful genetic manipulations in these species. Cyanobacteria will facilitate the discovery of fifth-generation biofuels, but it still needs additional research and substantial funding from the government to gain commercial appeal in the green energy platform.

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Author Contributions

S. Mishra, N. Kumari and VK Singh wrote the manuscript. RP Sinha conceptualized the topic and edited the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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