Third Generation Green Energy: Cyanobacteria, Key to Production of Sustainable Energy Through Metabolic Engineering

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Abstract

Biofuels are important as dependency on fossil fuels has resulted in economic instability in the world and heavy environmental damage. Burning of fossil fuel releases heavy amounts of carbon dioxide in the atmosphere, raising the concern of global warming. Development of alternative energy forms, sustainable and renewable in nature, is thus the need of the hour. In this context, agricultural production of biofuels has gained utmost importance, and more recently industrial biofuel production through cyanobacteria at large scale has almost stabilized the current scenario of global warming and current fuel demands. Modulation in the cyanobacterial biochemical and metabolic pathways at the genetic level for attractive biofuel yields is a challenge for upcoming scientists to offset petroleum and mineral oil usage and dependency.

12.1 Introduction

Fossil fuel has lost its significance as an energy resource. Global warming, depletion of oil and petroleum reserves in the near future (Nashawi et al. 2010), economic and energy crisis due to heavy oil demand at commercial scale, etc. are some of the serious issues that call for a change and replacement in the technology. Bioenergy production, in this regard, has emerged as an innovative substitute for fossil fuels in the past few years to generate heat, power and chemicals (Hall et al. 1993; Goldemberg 2000). Sustainable development of energy using biomass constitutes 75 % of renewable energy sources (Hall and Moss 1983), and this has stabilized environmental, social and economic issues. First- and second-generation biofuel productions using agricultural crops and lignocellulosic wastes, respectively, present a promising solution for heavy yields of clean energy. Agricultural crops such as sugarcane, cereal grains, oilseeds etc. can be processed to produce biofuels through various biochemical or thermochemical routes such as fermentation (sugar to alcohol), gasification, chemical synthesis etc. (Elam 1996). Use of lignocellulosic biomass to produce high amounts of bioenergy can play an important role in addressing both greenhouse gas emissions and dependency on mineral oil. Lignin content interferes with the biofuel

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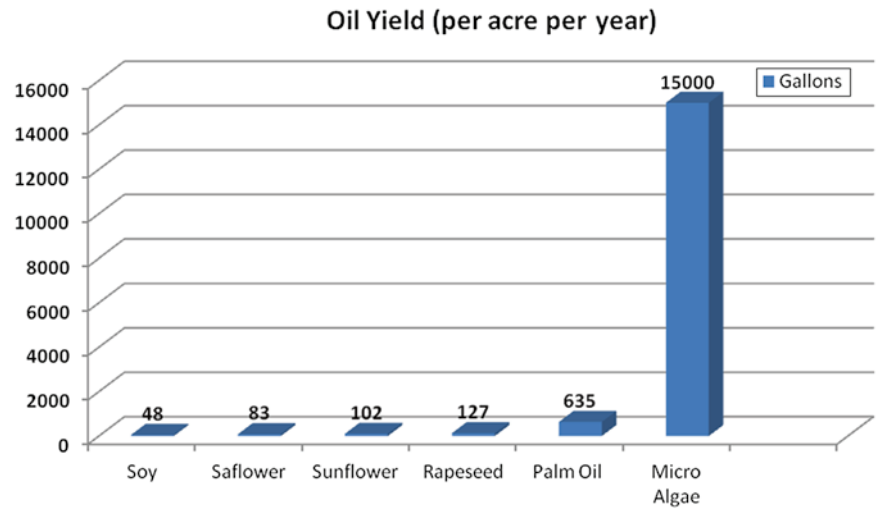

Fig. 12.1 Biodiesel yield of some feedstock (Source: The Green Chip Stocks 2008. http://www.greenchipstocks.com/articles/investing-algae-biofuel/253)

production. It has been observed that plant form with high cellulose content and low or modified lignin content is easily digestible in biofuel reactors by the respective enzymes which lead to enhanced production of biofuels (Gressel 2008; Barriere et al. 2004; Gressel and Zilberstein 2003). Lignin-degrading peroxidases are known, but they cannot be used in bioreactors as they have high energy requirements and these enzymes work in living microbes only in direct association with lignocelluloses. Transgenic modification of the lignin content and up-regulation of cellulose biosynthesis can help in clean production of biofuel in large amounts, and this can be achieved through partial silencing of the phenyl-propanoid pathway enzymes leading to reduced lignin synthesis. Many new molecular techniques such as antisensing and RNAi strategies can be helpful. Biofuel production using annual crops such as rapeseed, cereals, beet, etc. in comparison to perennial crops presents certain limitations—high production costs, low net energy yield (100–200 GJ/ha year), requirement of valuable agricultural land, high fertilizer requirement, etc. (Berndes et al. 2003). Other controversial issues like (a) competition between edible and energy crops for available land and water, (b) decline in the ecological value of the area with the plantation of energy crops and (c) influence on ecosystem diversity also limit the use of annual crops for biofuel production. In this respect, lignocellulosic biomass (e.g. wood and grasses) offers a better promising future with the use of biofuels than agricultural crops. Second-generation biofuel production using lignocellulosic wastes (e.g. ethanol production from lignocellulosic biomass) leads to higher overall energy conversion efficiencies and offers lower overall costs for longer term (Arthur 1999). Moreover, better-projected economics and less energy requirement by their feedstocks for growth and harvest are additional benefits of second-generation biofuels.

Green Chip Stocks 2008 special report states that the biofuel production from microalgae accounts for 20 % higher production efficiency than from palm oil without any competition with other feedstocks (Fig. 12.1). Microalgae can be grown in enclosed systems, and all the input requirements can be monitored and regulated accordingly for higher production of biofuel stock.

Cyanobacteria as a microalgae can be a good source of third-generation biofuel resource in contrast to energy crops and lignocellulosic wastes. Cyanobacteria have emerged as an innovative and promising platform for sustainable biofuel production. This viable alternative energy resource is devoid of the major setbacks associated with the first- and second-generation biofuels

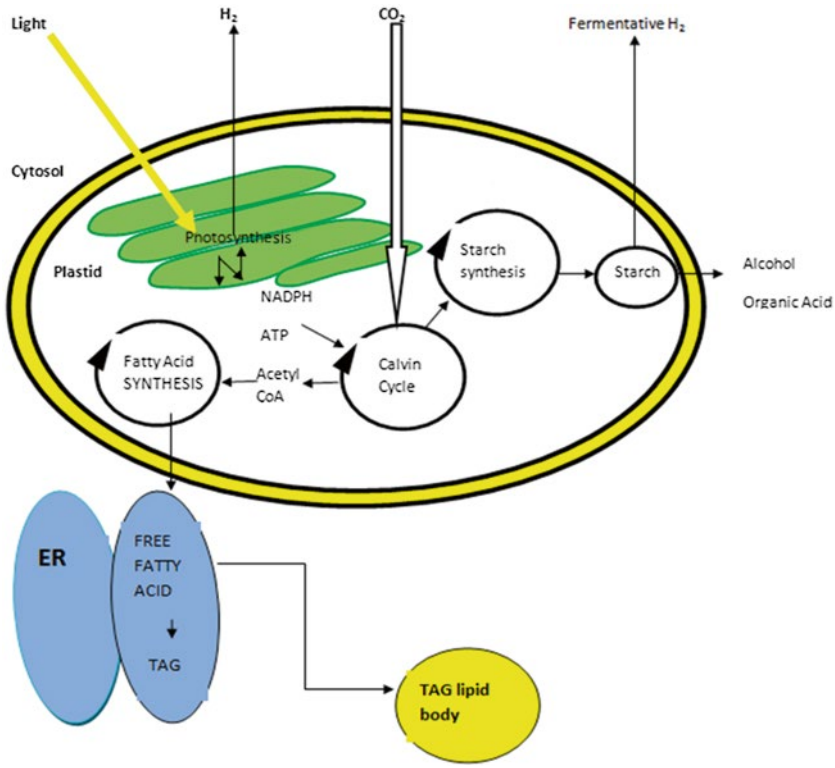

Fig. 12.2 Microalgal metabolic pathways that can be leveraged for biofuel production. *ER* endoplasmic reticulum, *TAG* triacylglycerol

(Mata et al. 2010). High carbon components in the atmosphere released from the automobiles and industries are sunk down by these plant forms through oxygenic photosynthesis in the aquatic environments to usable energy products. Cyanobacteria trap down the sun's energy through phycobilisomes present in the thylakoid membranes of their chloroplast systems converting light energy into chemical (renewable) form of energy (Griffiths and Harrison 2009). There are enormous advantageous features of cyanobacteria that make these species best suited for biofuel development. Non-requirement of bioproductive lands and freshwater for their growth, zero competition with agriculture, nonfood-based feedstock for the generation of biofuel, very short harvesting cycle, significant high growth rates and photosynthetic levels, basic nutritional requirements, inexpensive cultivation, high yields, considerable amounts of lipid content in their membrane systems, potential to thrive in elevated CO_2 conditions, ability to mitigate the progression of climatic change and production of other eco-friendly coproducts are some of the unique attributes of cyanobacterial forms that attract scientists to use this living biomass for the development of bioenergy (Parmar et al. 2011; Rittmann 2008) (Fig. 12.2). CO_2 emissions from automobiles and industrial exhaust gases get fixed up by these living forms photosynthetically in a carbon-neutral way to produce metabolites which act as potential feedstocks from which biofuel can be extracted. The burning of the biofuel in turn generates equal amounts of CO_2 maintaining the overall environmental integrity and nature's balance.

Eukaryotic algae- and cyanobacteria-based fuels are leading candidates for the replacement of fossil fuels. However the cost of harvesting the algae and extracting intracellular lipid from them offers certain limitations at the large scale which demand fuel production from engineered

cyanobacteria (Pienkos and Darzins 2009; Yang et al. 2011). Engineering of cyanobacteria at the gene level is important as it results in successful bulk amount of biofuel yields at large scale. This introduces a new dimension in the current scenario of biofuel production—*metabolic engineering*. Genetic engineering of metabolic pathways at some important steps in cyanobacteria for improved and efficient fuel productivities is possible. With the advent of many new technologies and most importantly with the available full sequence data of most of the cyanobacterial species, it is easy to modify the enzymatic pathways at the gene level for higher production of free fatty acid and other lipid/sugar compounds for improved yields of biofuel. *Genetic engineering* in cyanobacteria can be focused on the following metabolic pathways for substantial yield of biofuel at commercial scale:

1. Carbon metabolism, genetic strategies to increase:
   (a) Glucan storage
   (b) Starch biosynthesis
   (c) Starch biodegradation
2. Lipid metabolism:
   (a) Lipid biosynthesis
   (b) Lipid catabolism
3. Modification of lipid characteristics

12.2 Carbon Metabolism

Carbon dioxide fixation to usable chemical products (sugar/lipid) and their extraction to several distinct energy forms is the key for sustainable biofuel development in cyanobacteria. Full-length genome sequences of most of the cyanobacterial species are known, knowledge of which helps to manipulate the rate-limiting steps in the carbohydrate metabolic pathways in cyanobacteria for maximum bioenergy yield. Also relatively small genome of cyanobacteria makes it possible to readily genetically engineer the genome to enhance biofuel production.

Rubisco plays an important role in central carbon metabolism in higher plants. Rubisco fixes carbon dioxide during photosynthesis. Cyanobacteria are also photosynthetic forms that fix atmospheric carbon dioxide with cyanobacterial Rubisco, which has a lower affinity for carbon dioxide. In view of the above problem, a carbon dioxide concentrating mechanism (CCM) is developed (Badger et al. 2006) in which two carbon-fixing enzymes Rubisco and carbonic anhydrase are stored in organized micro-compartments—carboxysomes—through the cell away from competing oxygen (Savage et al. 2010). This leads to enhanced carbon dioxide fixation and hence improved biofuel yield. Also, certain manipulations at the gene level in the carbon dioxide concentrating mechanism are possible. Genetic modification can be introduced by:

1. By over-expressing carbonic anhydrase enzyme
2. By inserting more efficient Rubisco
3. By inserting multiple copies of inorganic carbon transporters (responsible for intracellular delivery of carbon dioxide and bicarbonates)

12.2.1 Glucan Storage

Sugars represent the main source of energy. Genetic modulations to increase glucan storage in cyanobacteria can help lead to heavy accumulation of storage compounds. Biosynthetic pathways of starch and other storage compounds can be genetically modulated, and also certain desirable traits can be added to the metabolic pathways for the same. Resulting bulk amount of storage compounds can be extracted readily to distinct forms of biofuels.

12.2.2 Starch Biosynthesis

The rate-limiting step of starch synthesis (storage product) is the ADP-glucose pyrophosphorylase (AGPase) catalyzed reaction of glucose-1-P with ATP (Stark et al. 1992).

$$\text{Glc-1-P} + \text{ATP} \xrightarrow{\text{AGPase}} \text{ADP-glc} + \text{PPi}$$

Manipulations at the genetic level controlling the rate-limiting step of starch synthesis can improve starch accumulation. AGPases, the

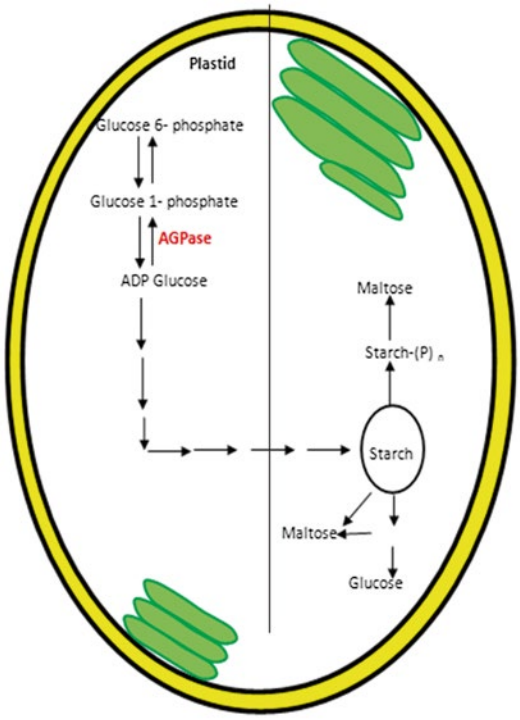

Fig. 12.3 Rate limiting step of starch synthesis catalyzed by ADP-glucose phosphorylase. *AGPase* ADP-glucose pyrophosphorylase

important enzymes, have been studied for their biocatalytic properties (Fig. 12.3). Allosteric structure and interactions of this enzyme with the respective substrate have also been studied (Smith 2008). Designer AGPases have been studied to increase starch content in higher plants (Giroux et al. 1996). These enzymes can be introduced and expressed in cyanobacteria also with no native AGPase activity. Another approach to increase glucan storage is to introduce genes responsible for starch synthesis into the protoplast through genetic engineering.

12.2.3 Starch Degradation

Starch content in the protoplast can also be increased by checking the starch degradation processes. Hydrolytic and phosphorolytic mechanisms are known that control starch degradation processes in cyanobacteria (Smith et al. 2005). By controlling the starch catabolic pathways at the gene level, heavy level of starch content can be achieved in the protoplast which later can be recovered and extracted to different biofuel forms. Gene knockout technologies are found useful for controlling starch degradation processes. Phosphorylation and hydrolytic steps demonstrate important gene knockout targets which help to display a starch accumulation phenotype in cyanobacteria. It is important here to refer that oxidative pentose phosphate (OPP) pathway is the major route of sugar catabolism in cyanobacteria. One of the key enzymes of OPP pathway is glucose-6-phosphate dehydrogenase. Its regulation and control at the level of gene expression can increase carbon dioxide fixation and hence can improve biofuel production (Kaplan et al. 2008; Stanier and Cohenbazire 1977; Vanderoost et al. 1989).

Most of the cyanobacteria also produce certain polymeric substances in their extracellular environment, for example, cellulose (Pereira et al. 2009). These species can be genetically modified for significant yields of extra-polymeric substances which can be extracted to produce biofuels.

12.3 Lipid Metabolism

Facile genetic manipulation in the lipid metabolic pathway for potential fuel excretion in cyanobacteria is possible. Tailor designing of biocatalyst for large-scale fuel production through genetic engineering in cyanobacteria also plays an important role. Understanding the lipid synthetic and catabolic pathways in cyanobacteria is of great interest for the ultimate production of fuel surrogates. There are really important pathways that control the saturation and length of fatty acids that also have been studied in cyanobacteria. Cyanobacteria have this unique ability to excrete fuel precursors such as free fatty acids extracellularly. This makes the recovery process and the further downstream processing steps relatively easy and simple (Kaczmarzyk and Fulda 2010).

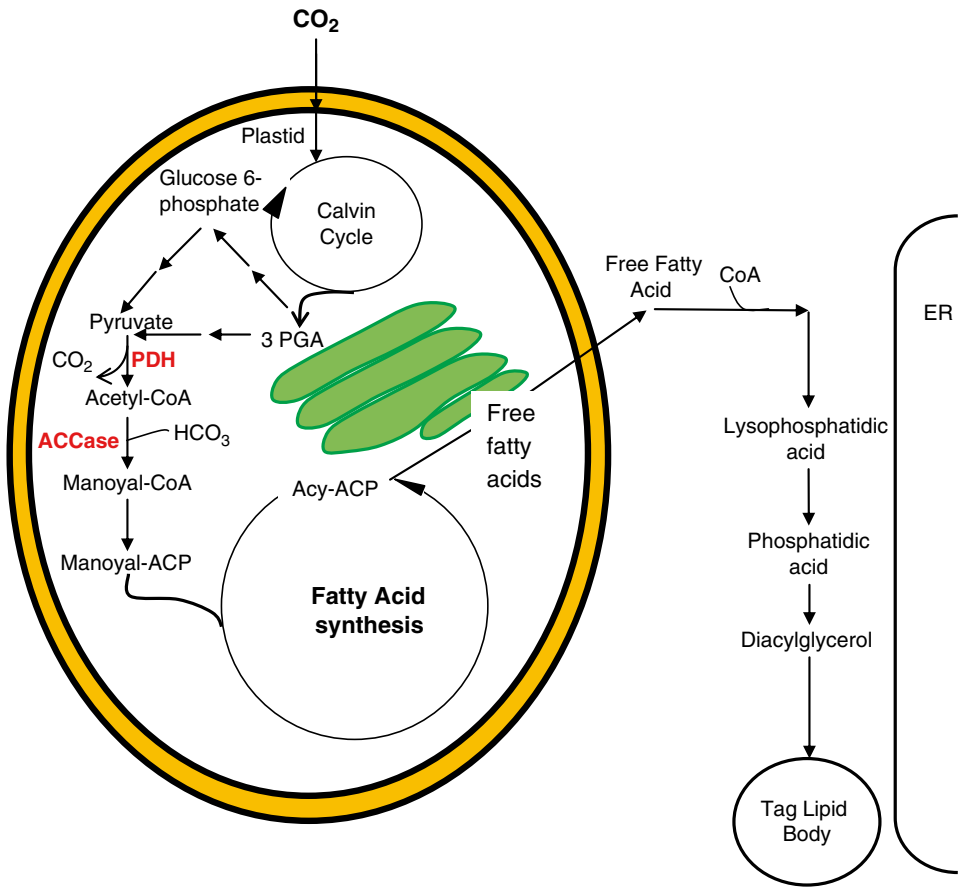

Fig. 12.4 Schematic overview of the metabolites and representative pathways in microalgal lipid biosynthesis shown in *black* and enzymes shown in *red*. *ACCase* acetyl CoA carboxylase, *ACP* acyl carrier protein, *CoA* coenzyme A, *G3PDH* gycerol-3-phosphate dehydrogenase

12.3.1 Lipid Biosynthesis

In recent years, much of the work has been done on cyanobacteria to understand the biochemical pathways and regulation of the enzymatic pathways at the gene level. Strategies have been established to increase the lipid content in the cell. Transgenic over-expression strategies have successfully resulted in heavy lipid production and accumulation in cyanobacteria. One early rate-limiting step in fatty acid synthesis is the conversion of acetyl-coenzyme A (CoA) to malonyl-CoA, catalyzed by acetyl CoA carboxylase (ACCase). Genetic modification of this committed enzyme can increase the fatty acid production and can result in quantitative yield of biofuels (Dunahay et al. 1995) (Fig. 12.4). Over-expression of the lipid biosynthesis genes encoding acetyl CoA carboxylase controlled by an inducible promoter can be activated once the microalgal cells have grown to a high density and have entered stationary phase. Similarly, over-expression of genes involved in triacylglycerol (TAG) assembly has shown some meaningful results. Glycerol-3-phosphate dehydrogenase (G3PDH) catalyzes the formation of glycerol-3-phosphate, which is needed for TAG formation. Over-expression of G3PDH can result in improved fuel production (Vigeolas et al. 2007). Another possible approach to increase cellular lipid content is to block starch synthetic pathways. For example, starchless mutant of *Chlorella*

pyrenoidosa has been shown to have elevated polyunsaturated fatty acid content (Ramazanov and Ramazanov 2006).

12.3.2 Lipid Catabolism

Lipid catabolic pathways can be silenced to increase cellular lipid accumulation. This is one strategy wherein the genes involved directly in β-oxidation of fatty acids can be inactivated through gene knockout technology to achieve quantitative yields of biofuel (De Riso et al. 2009; Molnar et al. 2009; Zhao et al. 2009). Quoting one example—in *S. elongatus* 7942–any free fatty acid (FFA) released by membrane degradation is recycled for membrane synthesis via acyl-ACP synthetase (aas) (Fulda 2010). Cellular FFA accumulation can reach high peaks if this acyl-ACP synthetase enzyme is targeted for gene knockout. Silencing of this respective enzyme can lead to heavy accumulation of acyl-ACP in the cytosol followed by effective production of biofuel.

12.3.3 Modification of Lipid Characteristics

Not only the quantity but the quality of biofuel also matters. Quality of the lipids is checked by few properties such as carbon chain length and degree of unsaturation of the fatty acids. These affect the oxidative, cold flow and stability characteristics of the biofuel/biodiesel. The chain length of fatty acids is determined by acyl-ACP thioesterases, which release the fatty acid chain from the fatty acid synthase enzyme. Suitable expression of thioesterase can change the lipid profiles in cyanobacteria. Ideal fatty acid chain length for biofuel production should be 12:0 and 14:0. Appropriate thioesterase expression can improve the suitability of microalga-derived diesel feedstock (Voelker and Davies 1994).

12.3.4 Biosynthesis of Ethanol

Bioethanol is one of the renewable energy resources which is produced from fermentation of agricultural crops (Goldemberg 2007). In contrast to edible crops, cyanobacteria can be used for bioethanol production without the addition of yeast starter culture (for fermentation) (Heyer and Krumbein 1991). To maximize bioethanol generation in cyanobacteria, genetic manipulations are also possible, for example, genes encoding pyruvate decarboxylase and alcohol dehydrogenase (enzymes controlling ethanol biosynthesis) from bacterium *Z. mobilis* have been introduced into the cyanobacterial genome for high bioethanol production during photoautotrophic growth (Deng and Coleman 1999). Cyanobacteria have been studied to produce cellulose extracellularly at a yield of up to 25 % of the cell dry volume (Dewinder et al. 1990). Modification of *Synechococcus* (cyanobacteria) with cellulose synthesis gene from *Gluconobacter* can yield maximum amount of extracellular non-crystalline cellulose, an ideal feedstock for ethanol production (Nobles and Brown 2008).

12.3.5 Biosynthesis of Hydrogen

Renewable energy is produced from natural sources and cyanobacterial biomass stands out as one such natural resource in today's generation for bioenergy development. Biohydrogen has emerged as one of the promising renewable green energy sources, and nitrogenase-based photobiological hydrogen production from cyanobacteria offers a major platform for its high rate production under aerobic conditions (Tamagnini et al. 2002, 2007). Optimization of cyanobacterial strains is central to improved biofuel/biohydrogen yields. For commercial viability and economic feasibility, efficient biohydrogen production directly using the sun's energy has been studied in a number of photosynthetic species possessing nitrogenase and hydrogenase enzymes. *Cyanothece* strains maintain marine nitrogen cycle and grow in basic nutritional environment limited in dissolved inorganic nitrogen compounds (Zehr et al. 2001). In one of the works on *Cyanothece* sp. ATCC 51142 strain, it has been described that its nitrogenase enzyme can be modulated to convert

solar energy into bioenergy at unexpected high rates under aerobic conditions. *Cyanothece* strains derive their nutritional requirements by performing photosynthesis during daytime and storing carbon in the form of glycogen. During dark period suboxic intracellular environment created as a result of respiration process drives oxygen-sensitive nitrogenase and hydrogenase enzymes to carry out nitrogen fixation and hydrogen production energy-rich processes at the expense of glycogen accumulated during light period (Schneegurt et al. 1997). Diurnal cycling patterns and other unique metabolic traits of this strain have made this strain an ideal organism for photobiological biohydrogen production (Stockel et al. 2008). *Cyanothece* 51142, a unicellular diazotrophic cyanobacterium, possesses a two-phase intracellular biohydrogen production pathway (Toepel et al. 2008): (a) growth phase, cells are grown aerobically under 12 h light/12 h dark cycles, followed by (b) incubation phase, cells are collected at the end of 12 h light period and are incubated at a specific temperature under continuous white light source for 12 h. The physiological activities of the cells get in sync with the dark condition during this latter phase, enhancing nitrogen fixation and hydrogen production processes. This particular experiment has led to a significant conclusion that it is possible to achieve a high-fold increase in biohydrogen production in *Cyanothece* 51142 strain during sub-aerobic/anoxic dark period by oxygen-sensitive hydrogenase and nitrogenase enzymes under photoactive conditions. Determination of hydrogen levels at the end of the experiment provided strikingly new results as most unicellular microbial strains till now utilize anaerobic environment for hydrogen production, but this case was different as more than 150 um of hydrogen per mg of chlorophyll per hour was produced.

Continued work on this particular strain has helped scientists to assess the effect of varying environmental conditions on cyanobacterial photobiological activity for improved biohydrogen yields. Cells grown under elevated CO_2 concentrations and under the presence of additional carbon sources have shown higher growth rates.

12.4 Biological Limitations

No doubt, lots of research to increase cellular lipid content and fat accumulation for maximum bioenergy yield has been done, but there are certain limitations also, to use such species at commercial scale for heavy biofuel production.

1. Risk of Contamination: Laboratory organisms when grown under field conditions for large-scale biofuel production can come under the risk of contamination by other indigenous local organisms.
2. Production and Harvesting Costs: High production cost is required for algal growth and for separate lipid producing ponds as most algae either grow or produce fat bodies but not both simultaneously. Harvesting of algae is a cost-prohibitive factor.
3. Carbon Dioxide Enrichment: Most of the higher plants and animals grow well when aerated with 5 % CO_2, but algae and cyanobacteria grow happily when enriched with 100 %CO_2. Still they pose a problem and their response to added CO_2 is not as good as it can be. Conversely, at such high CO_2 concentrations high algal growth can surely occur, but at such CO_2 levels it has been studied that overabundance of RUBISCO enzyme (scavenger of rare CO_2 present in air or water) at the expense of more needed rate-limiting enzymes occurs.
4. Light Penetration: At high light intensities in photo-bioreactors, algal growth can be inhibited due to photoinhibition.
5. Seasonality: Algal growth and high yields in ponds is usually seasonal and temperature dependent. Very low temperatures and extremely high temperatures both pose a problem in algal growth.

12.5 Conclusion and Future Prospects

Biofuel production is important as (a) it contributes in reducing carbon dioxide levels in the atmosphere; (b) it cuts off petroleum dependency; (c) its production is cost effective,

sustainable and renewable in nature; and (d) it stabilizes economical and environmental issues. Fuels from lignocellulosic biomass (second-generation biofuels) and beyond have made a real dent in the petroleum usage and have created a strong impact in this field as a sustainable clean energy resource. First-generation biofuels consist of other food crops which not only have implications for the world's food supply but also impede the amount of land available to grow it on. Second-generation biofuels solve the food problem but not the land problem. As the generation feedstock microalgae (cyanobacteria) solve many of the previous problems, it solves the food vs. fuel issue, the land issue, the footprint issue and the scalability issue. Moreover, with the introduction of new molecular techniques, genetic tools, high-throughput analytical techniques, systematic biological approaches, etc., modification of the genomes of small microorganisms such as cyanobacteria at the genetic level has relatively become easy which has led to cost-effective optimum production of renewable and sustainable form of bioenergy. Lipid and carbohydrate metabolic pathways of these microorganisms have been regulated at the rate-limiting biochemical/enzymatic steps for successful biofuel generation. The problems which have been faced in this field till date can be solved in the near future. The strength of biofuel production can be substantially increased by expansion of cultivation areas for growing feedstock crops. This expansion can be achieved by converting waste uncultivated lands such as forests lands to growing new sources of biofuel feedstocks. Transgenics and the use of other modern technologies are big practical solutions to overcome such limitations. Risk of contamination, algal growth, light penetration, temperature, carbon dioxide enrichment, etc., can be sorted out by genetic modification technology. For example, cyanobacterial growth under high light intensities can cause the problem of photoinhibition, but the use of optical fibres to diffuse the light throughout the depth of the culture can offer a practical solution for the same (Sheehan et al. 1998). Also, metabolic pathways controlling lipid/carbohydrate metabolism can be manipulated by gene technology using novel molecular technologies for enriched bioenergy generation.

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