Volume IV, No. 1: 18–30 (2020)
ISSN 2507-9638

THE CLSU INTERNATIONAL
JOURNAL OF SCIENCE & TECHNOLOGY
www.clsu-ijst.org

Significance of Carbohydrate Pathway in the Maximization of Biofuel Production in *Botryococcus sp*: A Brief Review

Zubainatu Abba[1, 2*], Siti Fatimah Zahrah Mohammad Fuzi[1], Hazel Monica Matias-Peralta[3], and Muhammad Muhammad Nimaya[4]

[1]Department of Technology and Heritage, Faculty of Applied Science and Technology, Universiti Tun Hussein Onn Malaysia, 86400 Parit Raja, Batu Pahat, Malaysia
[*2]Federal College of Education Yola, Adamawa State, Nigeria
[3]Freshwater Aquaculture Center-College of Fisheries, Central Luzon State University, 3120 Science City of Munoz, Nueva Ecija, Philippines
[4]SPS Agaie, IBB University, Nigeria

Email for correspondence: zubainatuabba@gmail.com

Submitted January 31, 2020. Accepted June 6, 2020. Published online July 31, 2020.

Abstract

Botryococcus spp. are green microalgae (Chlorophyta) known to contain an abundant amount of hydrocarbons, lipids, and other bioactive constituents such as ether-based lipids, fatty acids, polysaccharides, and carotenoids which are used for commercial applications. In comparison with higher plants, after microalgae fix carbon dioxide (CO_2), they have a greater ability to convert it into biomass and subsequently, into products of interest. Some significant studies on the optimization of lipid biosynthetic pathways in microalgae for biofuel production are available since *Botryococcus* spp. have been known to contain a high amount of lipid in their cells. However, carbohydrates pathways, which are also precursors for biofuel production have been overlooked. This brief review aims to provide insights into the need to consider carbohydrates pathway in *Botryococcus* spp. to maximize biofuel production in microalgae.

Key Words: biofuel, *Botryococcus* spp., carbohydrate pathway

Introduction

The significant advancement of industrialization, modernization in lifestyle, and vehicular population of the world has led to a considerable increase in petroleum-based fuel demand (Asadullah, 2014). The consumption of liquid fossil fuel as an energy source has long been considered unsustainable throughout the world, and is due to the rapid lessening of the fossil fuel reserves and the release of significant quantities of greenhouse gases associated with their production and combustion (Alam et al., 2015; Raheem et al., 2015). The environmental impact raised from the burning of fuels has an enormous effect on the carbon cycle, which is related to the combustion of fossil fuels. Besides, exhaustion of different existing biomass without appropriate compensation has resulted in colossal

DOI: https://doi.org/10.22137/ijst.2020.v4n1.02

biomass scarcity, developing environmental problems like deforestation and the destruction of biodiversity. These problems have forced governments, policymakers, scientists, and researchers worldwide to find alternative renewable energy sources like biofuels (Alam et al., 2015; Dragone et al., 2010)

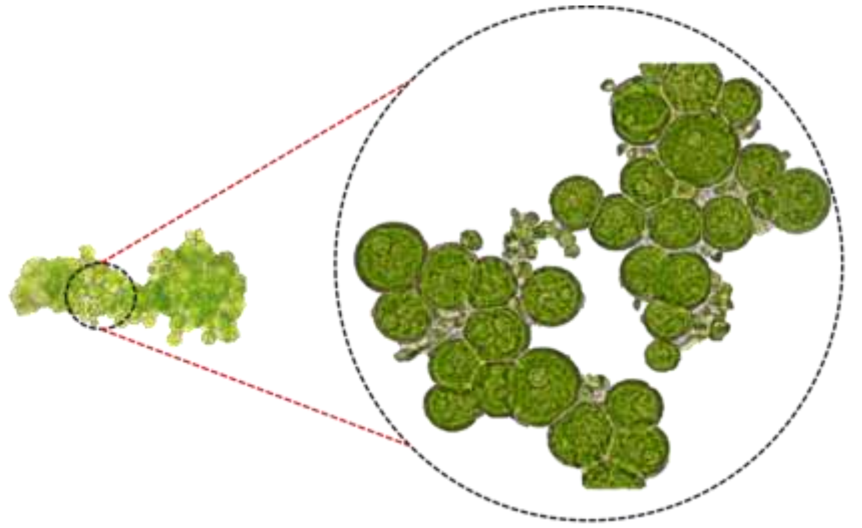
Biofuel production through microalgae is a promising and viable alternative to substitute the usual fossil fuel that has been the main contributor to global warming (Hossain 2019). However, the advancement of microalgal biofuels is faced with challenges since the production cost of microalgae as a biofuel feedstock is higher compared to fossil fuels (Zhu et al. 2014).To improve the economic feasibility of mass production of microalgal biofuels, several efforts have been made to increase strain performance (Xin et al., 2010), cultivation methods (Ketheesan & Nirmalakhandan 2012; Zhang & Hu, 2012) and improving technologies involved in biomass harvesting and extraction (Halim et al., 2012; Hanotu et al., 2012).

Botryococcus spp

Botryococcus, a green colonial microalga (Chlorophyceae), is remarkably rich in renewable source of hydrocarbons and other bioactive compounds. *Botryococous* exists in different ecosystems, such as freshwater and brackish waters of the tropical environment (Ashokkumar & Rengasamy, 2012; Hegedűs et al., 2015). They grow best at an optimal temperature of 23ºC, the light intensity of about 30–60 W/M^2 for a maximum of 12 hours per day, and 0.15 molar NaCl of salt (Qin & Li, 2006). In the laboratory, *Botryococcus* usually grows in cultures of Chu 13 Medium and Bold Basal Medium (Guiry, 2012; Prescott, 1978). *Botryococcu*s colonies are free moving and of uneven shape, without a visible gelatinous enclosure but entirely enclosed by a tough, hyaline, orange-coloured or dark membrane that produce in rough wrinkles (Arulmurugan et al., 2010). Colonies often unite in the compound net like groups using lengthy delicate mucilaginous projection from the colonial envelope. Cells are shaped in an ovoid or ellipsoid and arranged radially at the periphery of the colony (Komárek & Marvan 1992). The inner layer of each cell of the colony is made up of fatty substances and an outer layer of pectin (Recht, Zarka, et al., 2012). Single colonies can reach up to the length of 80 µm, while a compound colony can reach up to 1.5 mm in diameter.

Figure 1

Botryococcus sp. *Colony*



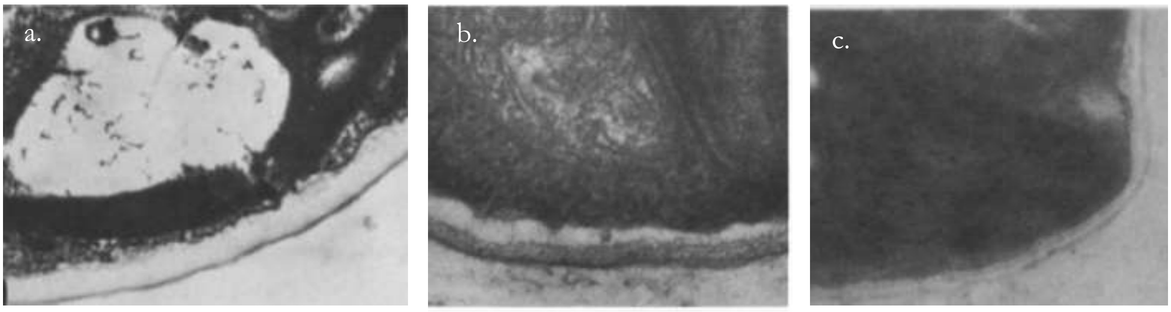
Carbohydrate Composition of Microalgae

Note: Adapted from Gani (2017)

Carbohydrates are the first products of photosynthesis and carbon fixation metabolism. They also include various types of molecules, such as starch, which is the reserve material, and cellulose, as the main component of cell walls (Chen et al., 2013). Carbohydrates are the main end products derived from the process of carbon fixation metabolism, otherwise known as the Calvin cycle (Ho et al., 2011). These carbohydrates are stored in the plastids as reserve materials, for example, starch, or they become the main constituent of cell walls such as cellulose, pectin, and sulfated polysaccharides. However, the composition and metabolism of carbohydrates, primarily starch, and cellulose in microalgae may vary considerably from species to species (de Oliveira Rangel-Yagui et al., 2004; Rismani-Yazdi et al., 2011). The carbohydrate content in microalgae can be as high as 70% (Becker, 1994; Nahak et al., 2011), and starch content can reach 60% of the dry weight of algal biomass (Brányiková et al., 2011). The microalgal cell wall consists of an inner and an outer cell wall layer. The outer cell wall layer can further be grouped into three types, namely those: (1) with a trilaminar outer layer, (2) with a thin outer monolayer, and (3) without an outer layer (Yamada & Sakaguchi, 1982). Figure 2 shows the different outer cell wall layer types: The cell wall composed of two layers, the inner bulky electron-transparent or microfibrillar layer and the outer trilaminar layer (Figure 2a); the cell wall composed of also two major layers but the outer layer is not trilaminar (Figure 2b); and the cell wall composed of only one microfibrillar layer (Figure 2c).

Figure 2

Electron Micrographs Showing Different Thin Sections of a Microalga Cell Wall



Note: Adapted from Yamada & Sakaguchi (1982)

The composition of the outer cell wall differs from species to species, but it is generally comprised of polysaccharides, such as pectin, agar, and alginate. However, the inner cell wall layer of microalgae is mostly made up of cellulose and other materials such as hemicellulose and glycoprotein (Yamada & Sakaguchi, 1982). The compositions of the cell walls and their storage products in different microalgal species are shown in Table 1. In certain microalgae, the significant components in the cell walls and their stored products are glucose polymers produced through cellulose/starch metabolism (Metting, 1996). Microalgae are said to be a promising feedstock in the production of bioethanol because they possess cellulose-based cell walls, with accumulated starch as its key carbohydrate source (Wang et al., 2011). Currently, the most widely used biofuel is bioethanol, which is produced from sugar-based (sugarcane) and starch-based (maize) feedstock (Bai et al., 2008; Chen et al., 2013). Brazil with a limited sugar-based bioethanol production is the only exporter of sugar-based bioethanol (Goldemberg, 2007). Developing starch-based bioethanol suffers from the food vs. fuel dilemma. The lignocellulosic biomass cannot be efficiently and economically converted to biofuels due to its costly pretreatment process, even though they exist abundantly in nature (Himmel et al., 2007). In contrast, carbohydrates from microalgae are suitable for bioethanol production since microalgae-based carbohydrates live in the form of starch and cellulose, but without lignin, and are thus more easily converted to monosaccharides, to eventually produce bioethanol (Alam et al., 2015; Harun et al., 2010a).

Alternatively, once saccharified, microalgae-based carbohydrates could serve as a feedstock for most industrially relevant microorganisms.

Carbohydrates, such as glucose, can also be converted into alkanes (C7 to C15) in an aqueous phase catalysis process (Huber et al., 2005). Researchers have found that certain microalgal species can accumulate a lot of carbohydrates, including *Chlorella, Dunaliella, Tetraselmis,* and *Chlamydomonas* (John et al., 2011). Recently, algal biomass residues enriched with carbohydrates coming after biodiesel production have been studied for producing biogas and bioethanol (Georgianna & Mayfield, 2012; Lee et al., 2013).

Carbohydrate Metabolism Pathway of Microalgae

The main components of microalgae for biofuel production are carbohydrates and lipids. Carbonates are the primary products derived from the carbon fixation metabolism (i.e. the Calvin cycle) (Ho et al., 2011).

Table 1

Composition of Microalgal Cell Wall and Storage Products

Division	Cell wall made up	Storage products
Cyanophyta	Lipopolysaccharides, peptidoglycan	Cyanophycean starch
Chlorophyta	Cellulose, hemicellulose	Starch/lipid
Dinophyta	Absence or contain few celluloses	Starch
Cryptophyta	Periplast	Starch
Euglenophyta	Absent	Paramylum/lipid
Rhodophyta	Agar, carrageenan, cellulose, calcium carbonate	Floridean starch
Heterokontophyta	Naked or covered by scales or with large quantities of silica	Leucos/lipid

Microalgae phyla such as *Chlorophyta*, *Dinophyta*, *Glaucophyta*, and *Rhodophyta* store glucans in the form of linear-1,4 and branched-1,6 glycosidic linkages (Stern, 2009). Similarly, in green microalgae, starch is manufactured and stored in the chloroplast, while in other species such as *Dinophyta*, *Glaucophyta*, and *Rhodophyta,* starch is stored in the cytoplasm and the peri plastidial space in *Cryptophyceae* (Deschamps et al., 2006).

Sugars accumulate in microalgae through CO_2 fixation during the photosynthetic process. Photosynthesis is a biological process which utilizes adenosine triphosphate/nicotinamide adenine dinucleotide phosphate (ATP/NADPH) to fix and convert the CO_2 taken in from the atmosphere to yield glucose and other sugars through a metabolic pathway called the Calvin cycle (Kliphuis et al., 2012). Carbon assimilation in cells take place through various pathways together representing a carbon metabolic network (Baroukh et al., 2015). The CO_2 fixation occurs in the chloroplast localized Calvin-Benson cycle in the presence of light through the enzyme Ribulose-1, 5-bisphosphate (RuBP). Ribulose is a double phosphate ester of the ketopentose, an organic substance which reacts with carbon dioxide to form RuBisCO that is involved in photosynthesis (Baroukh et al., 2015). According to Mohammed (2013), *Botryococcus* sp. uses light energy (provides the biochemical reductant, NADPH2) to dissociate the water molecules by splitting it into protons, electrons, and oxygen, which is used to reduce CO_2 to form the RuBisCO. Apart from the starch present in plastids, the cell wall, particularly, microalgal extracellular coverings, is another carbohydrate-rich part transformable to biofuel (Harun et al., 2010b). Nevertheless, these compositions of extracellular covers in microalgae are diverse by species

(Domozych et al., 2012). Among the compositions is cellulose, one of the main fermentable carbohydrates found in most green algae (Radakovits et al., 2010).

Cellulose synthesis is a complicated process that involves many enzymatic reactions. UDP-glucose formed from the result of uridine triphosphate (UDP) and fructose and catalyzed by the enzyme sucrose synthase (Figure 1) is the starting substrate for cellulose synthesis (Kimura & Kondo 2002).

Proposals and Attempts Made at Improving Biofuel Production in Microalgae

Radakovits et al. (2010) stated that biofuels production from microalgae-based carbohydrates is essential to understand and manipulate the related metabolisms to achieve higher microalgal lipid accumulation via strategies like decreasing glucan storage and increasing starch degradation. Metabolic pathways of energy-rich molecules for carbohydrate and lipid are closely interconnected (Figure 3).

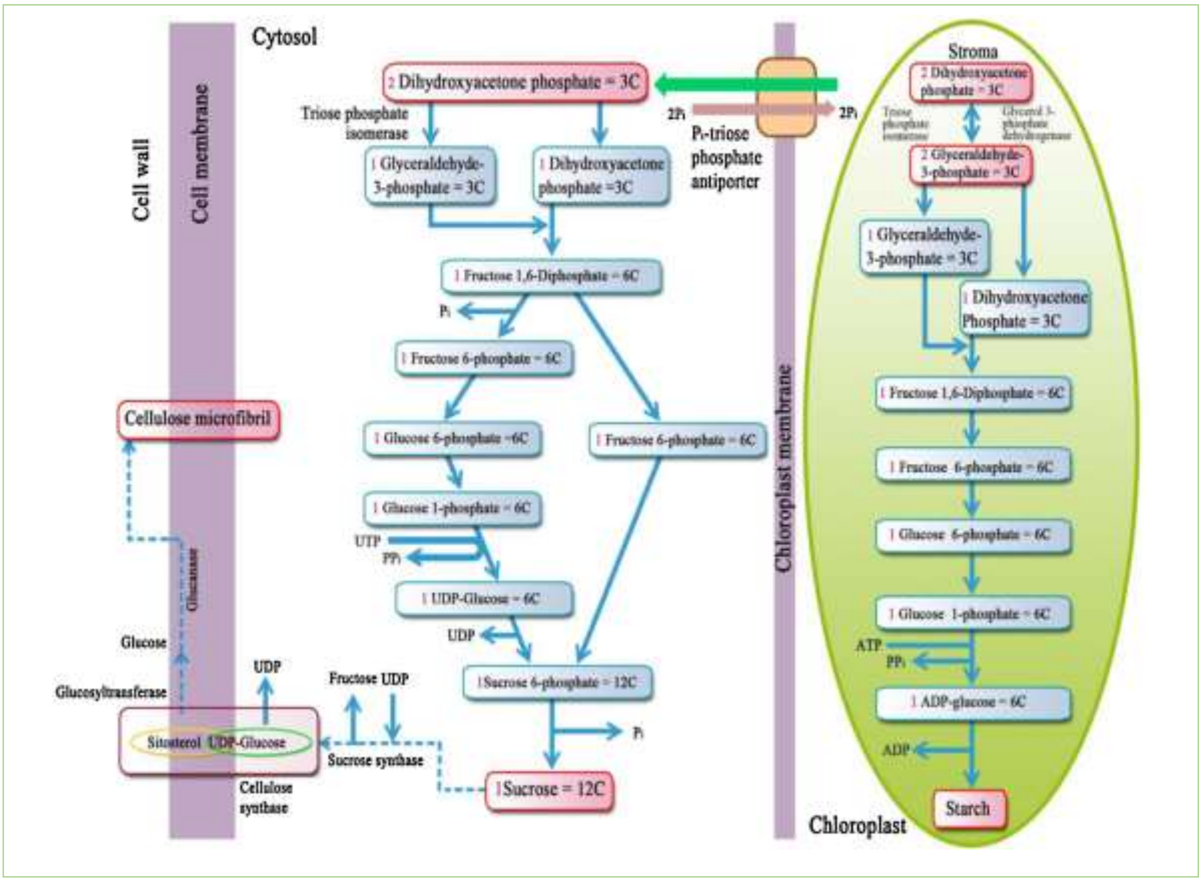
Metabolic pathways of energy-rich molecules for carbohydrate and lipid are closely interconnected. To improve biofuel production from microalgae carbohydrates, it is essential to understand and manipulate the linked metabolisms to attain higher microalgal biofuel, through the modification or manipulation of their metabolic pathway, especially that of carbohydrates. These manipulations are made possible by the identification and isolation of some enzymes that participate in the biosynthesis.

Since starch and lipid shares common precursors in their synthesis, it is possible that carbohydrate and lipid could be inter-convertible (Zhou et al., 2013). According to Grima et al. (2003), Ho et al. (2012), and Rismani-Yazdi et al (2011) there is a competition between lipid and starch synthesis because triacylglycerol (TAG) is the primary precursor in the synthesis of glycerol-3-phosphate (G3P), which is produced through catabolism of glucose (glycolysis).

Some metabolic engineering investigations to optimize biofuel lipid metabolic engineering have been recently carried out in the model green microalga *Chlamydomonas reinhardtii* (Miller et al. 2010; Msanne et al. 2012). Alteration of metabolic pathways involves different approaches such as overexpressing a rate-limiting enzyme involved in the synthesis of the desired product, down-regulating or blocking the competing channels, and over-expressing transcription factors controlling the critical pathway enzymes (Jeong et al., 2002). Wang et al. (2012) showed that transient accumulation of starch is linked with the slow accumulation of lipid in response to nitrogen deficiency in *C. zofingiensis* cells. Msanne et al. (2012) revealed that nitrogen depletion triggered a similar form of the new synthesis of starch followed by significant lipid increase in two reasonably different green microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169 (Recht, Re et al., 2012). Recht, Zarka et al. (2012) also suggested that the central instant response to stress in *Haematococcus pluvialis* was the intensive making of carbohydrates, which later changed to fatty acids.

Figure 3

Carbohydrate Metabolism of Green Algae



Note: Adapted from Chen et al. (2013)

It is well-known that starch reduction provides metabolites for the production of fatty acids (Li et al., 2011; Rismani-Yazdi et al., 2011). Li et al. (2011) suggested that *Pseudochlorococcum* used starch as a central carbon and energy storage product, and cells moved the carbon splitting into neutral lipid as a minor storage product after N-depletion. It is worthy of believing that algae tend to yield lipid as a long-term storage mechanism in response to extended environmental stress. Therefore, if biodiesel is desired, it is necessary to maintain the culture under pressure for a long time to gain enough lipid. Microalgae are rich in energy stock mixtures, such as diacylglycerol, triacylglycerol, and starch (Chang et al., 2011; Chisti, 2007; Radakovits et al., 2010; Stern, 2009; Van De Meene et al., 2006).

Attempts have been made to improve the oil contents in the cells, by blocking the metabolic pathways as well as to limit the steps of genes that overexpress itself. Another possible approach to aggregate the cellular lipid content is by blocking metabolic paths that lead to the increase of energy-rich storage compounds, that is starch. Under N depletion in the presence of acetate, the wild-type of *Chlamydomonas reinhardtii* lipid content has increased 15-fold. When starch biosynthesis was blocked in the star6 and star7 mutant, the lipid content increased 30-fold, demonstrating that genetic manipulation can enhance the production of lipids.

According to Wang et al. (2009), the levels of TAG in these mutants have increased during nitrogen deficiency. A different starchless mutant of *Chlorella pyrenoidosa* has also been revealed to have raised polyunsaturated fatty acid content under nitrogen starvation (Ramazanov & Ramazanov, 2006). Likewise, Moellering and Benning (2010) used N-depletion and RNAi suppression, to increase the total fatty acid content by 2.4-fold per cell of which 65% of the total fatty acids esterified to TAG in oil bodies of *Chlamydomonas reinhardtii*.

According to Rismani-Yazdi et al. (2011), starch degradation provides metabolites for the making of fatty acids in *Dunaliella tertioletcta*. The complete changes in enzymatic activity and metabolic changes of carbohydrate biosynthesis in microalgae are poorly understood. A better understanding of the biochemistry of microalgae carbohydrate metabolisms will help in the development of genetic engineering of microalgae (Chen et al., 2013).

Microalgae are also known to synthesize cell wall carbohydrates, proteins, and membrane lipids for cell structure under favorable growth conditions. A process called photosynthetic carbon partitioning can be employed in which there is a switch towards the production of energy-rich storage compounds like starch and lipid, which in turn is converted into biofuels.

Based on this information, the carbohydrate production in the cells can be converted to lipids by manipulating the carbohydrate pathway to increase the lipid content, thereby maximizing the overall biofuel production from *Botryococcus* spp. Therefore, understanding the metabolic pathways and processes involved in the generation of these organic macromolecules is essential to improve biofuels production.

Metabolic Pathway Modification in Microalgae

The metabolic pathways produce molecules such as the carbohydrate and lipid, which are energy-rich and closely linked molecules. To improve biofuel production from microalgae-based carbohydrates, it is essential to understand and manipulate the linked metabolisms to attain higher microalgal biofuel through the modification or manipulation of their metabolic pathway, especially that of carbohydrates. These manipulations are made possible by the identification and isolation of some genes and enzymes that participate in the biosynthesis. Since starch and lipid shares common precursors in their synthesis, it is possible that carbohydrate and lipid could be inter-convertible (Zhu et al., 2013).

Most studies focused on the lipid metabolic pathways and their associated genes in other algae such as *Chlamydomonas, Nannochloropsis,* and *Cyanidioschyzon merolae* (Zhu et al., 2014). However, studies on carbohydrate metabolic pathway and enzymes discovery leading to the production of lipid in *Botryococcus* sp. are minimal. Hence, the need to study the metabolic pathways and identify the enzymes involved in the metabolism of carbohydrates in *Botryococcus* spp. that lead to more lipid production with the hope of increasing its biofuel content.

Plant constituents are generally composed of carbohydrates, starch, proteins, and lipids. Carbohydrate presence is usual in plants, as it constitutes the highest proportion of plant nutrients. Unlike animals that store excess energy in the form of lipids, autotrophs produce more carbohydrates than lipids due to photosynthesis. Conversely, microalgae have a strategic feature of rapid alteration of the intracellular energy storage form from starch to lipids, thereby generating more lipid content. More so, all the carbohydrate produced by *Botryococcus* sp can further be converted into lipid similar to animal fats and cultivation of microalgae do not require an extensive land space but a small water body and few cells. The advantage of lipid production via *Botryococcus* sp. as stated earlier justifies the study on an alternative pathway for lipid production in *Botryococcus* sp.

Although microalgae have so many potentials as alternative feedstock to fossil fuels, the commercial level of microalgae biofuels production is still not feasible due to several factors. These factors include: low biomass concentration, low lipid content in some species, and the high cost of biofuel production. The production of algal biomass using the available technology is not economical and the existing algal species could not be grown sufficiently cheaply and, at the same time, produce oil usable as a source of fatty acids for biodiesel (Ratledge & Cohen, 2008).

However, the viability of their output can be realized by designing advanced photo-bioreactors and developing low-cost technologies for high biomass harvesting, drying, and oil extraction. Production of low-cost microalgal bio-products requires either improvement of algal biology through genetic and metabolic engineering or modifying the culture conditions in some species or combination of both (Chisti, 2007). Commercial-scale manufacture can also be possible by improving genetic engineering strategies and by engineering the carbohydrate to lipid metabolic pathways for high lipid production (Singh et al., 2016).

Keasling (2010) reported that through transferring product-specific enzymes or entire metabolic pathways, microbial production of natural products has also been achieved from rare or genetically intractable organisms to readily engineered ones. Furthermore, production of products such as particular chemicals, bulk chemicals, and fuels, has been made possible by combining enzymes or pathways from different hosts into a single microorganism and by engineering the enzymes to have new functions (Keasling, 2010; Medipally et al., 2015). In the photanol approach proposed by Hellingwerf and De Mattos (2016), conversion of CO^2 and water into biofuel could be achieved by combining the light reactions of photosynthesis and the Calvin cycle with a fermentative pathway from a chemoheterotrophic microorganism.

The above perspectives highlight a range of examples which demonstrate how feasible the metabolic pathways can be successfully manipulated with a variety of metabolic engineering approaches. Alternative microalgae pathways can be constructed in the native producer to develop new properties, such as better and increased biofuel production.

Conclusion

In order to meet the demand of the biofuels market, pathway analyses of microalgae biofuels producing system should also be carried out to assess the commercial feasibility of converting microalgae-based carbohydrates into biofuel. Some of the advantages of microalgae-based carbohydrates have over lignocellulosic materials as the feedstock for biofuels production include easy saccharification and requiring less pretreatment. Hence, understanding the fundamentals of carbohydrate metabolism of microalgae is a prerequisite for manipulating the pathway to increase lipid productivity.

Acknowledgements

The author gratefully acknowledges the Fundamental Research Grant Scheme (Vot 1476) of the Ministry of Higher Education, Malaysia for providing the research funding, and the Research Management Center (RMC) of the University Tun Hussein Onn Malaysia (UTHM) and Federal College of Education Yola Nigeria for their support to study in Malaysia.

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