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Identification of Carbohydrates Metabolic Related Enzymes for Lipid Production in *Botryococcus* sp., a Microalgae Isolated from Taman Negara Endau Rompin

ZUBAINATU ABBA[1,2], SITI FATIMAH ZAHARAH MOHAMAD FUZI*[1] & HAZEL MONICA MATIAS-PERALTA[1,3]

[1]Department of Technology and Natural Resources, Faculty of Applied Science and Technology, Universiti Tun Hussein Onn Malaysia, High Education Hub Pagoh, KM 1, 84600 Panchor, Johor, Malaysia; [2] Federal College of Education Yola, Adamawa, State of Nigeria; [3]College of Fisheries-Freshwater Aquaculture Center, Central Luzon State University, 3120 Science City of Munoz, Nueva Ecija, Philippines
*Corresponding author: fatimahz@uthm.edu.my
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ABSTRACT

Botryococcus is a microalgal genus known for its ability to generate and accumulate substantial amounts of lipids via carbohydrate metabolism. This work determined the metabolic pathways and enzymes involved in carbohydrate metabolism leading to increased synthesis of fat in *Botryococcus* sp. Relevant intracellular and extracellular metabolites were extracted and quantified using chromatographic analysis. Enzymes involved in carbohydrate metabolism leading to lipid formation in *Botryococcus* sp. under natural conditions were also discovered by one-dimensional gel electrophoresis followed by proteomic mass spectrometry (LC-MS/MS) and database searching. Proximate analysis demonstrated 23.0% total carbohydrate, 16.0% protein and 61.0% lipid per milligram biomass dry weight of *Botryococcus* sp. The extracellular metabolites constitute mostly of cyclohydrocarbons, nitrogenated hydrocarbons, siloxanes, phenols, and phenol derivatives. A glycolytic enzyme "enolase," which can create phosphoenolpyruvate and subsequently convert it into pyruvate, was found in this study. This study revealed that enolase provided an alternate pathway to export fixed carbon to the cytoplasm, hence providing a shorter route to lipid production than the normal process via the plastid leading to the manufacture of more lipids in the cells of *Botryococcus* sp. than other microalgae of the same group.

Keywords: *Botryococcus* sp., carbohydrate metabolism, enolase, lipid, phosphoenolpyruvate

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INTRODUCTION

The search for medium to long term source of renewable and environmentally friendly alternatives for fossil fuels have attracted serious attention of sciences worldwide (Ghaffour *et al.*, 2015). One of the utmost interests and is likely to show a significant role in global energy substructure is biofuel, which comprise of energy from geologically current carbon fixation by living organisms such as plants, macro and microalgae. The main components of microalgae for biofuel production are carbohydrates and lipids (Ho *et al.*, 2011). In general, autotrophs produce more of carbohydrates than the lipids due to photosynthesis. However, some microalgae have a strategic feature of rapid alteration of the intracellular energy storage form from starch to lipids, thereby generating more lipids when necessary. Among these microalgal species, the *Botryococcus* sp. known to have a remarkable capability to produce significant amount of cellular lipids up to 80% (Powell & Hill, 2009), and also to synthesize industrially interesting chemicals intracellularly and excrete them into the extracellular matrix. They can also produce a large number of hydrocarbons as compared with their biomass (Chisti, 2007; Ruangsomboon *et al.*, 2020).

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). 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). In fact, according to Zhou *et al.* (2013), metabolic

pathways of energy-rich molecules for carbohydrate and lipid are closely interconnected. Understanding their metabolic process and its relationship with their energy requirements, particularly those for carbohydrates biosynthesis, is essential for increasing microalgal biofuel production.

In related studies by Grima *et al.* (2003), there is a competition between lipid and starch synthesis for triacylglycerol (TAG). Triacylglycerol is the principal precursor in the synthesis of glycerol-3-phosphate (G3P) produced through the catabolism of glucose (glycolysis). Understanding the metabolic pathways and processes involved in the generation of these organic macromolecules is essential to improve biofuels production. However, there are few research on the carbohydrate metabolic route and enzyme discovery that led to the formation of lipids in *Botryococcus braunii*, such as studies by Molnár *et al.* (2012), Kurinjimalar *et al.* (2017), Cheng *et al.* (2018) and Blifernez-Klassen *et al.* (2018). This research work therefore aimed to determine the alternate metabolic pathway and the enzymes involved in the shift from carbohydrate metabolism to biosynthesis of lipid storage in an effort to boost its biofuel production.

MATERIALS AND METHODS

Microalgae

Locally isolated indigenous freshwater microalga, *Botryococcus* sp. was used in this study. The sample was collected from Upeh Guling, Taman Negara Johor Endau Rompin, located in the Mersing district of Johor, Malaysia, as previously mentioned by Gani *et al.* (2016).

Equipment and Chemical Preparation

Freeze dryer (Martin Christ Alpha LD2, Germany) was used in drying the microalgae. High-performance liquid chromatography (HPLC) Agilent and Shodex sugar SP0810 column equipped Showa Denko America, Inc. were used in carbohydrate identification. Mini-PROTEAN-System from Bio-Rad Laboratories Inc. and LC-MS from Thermo Fisher Scientific were used in enzymes identification. Perkin Elmer Clarus 680SQ8 gas chromatography system (USA) was used in the fatty acid analysis. Bovine serum albumin was used as a standard for calibration curves in a spectrophotometer from Fisher Scientific Sdn. Bhd., sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from Bio-Rad Laboratories Inc., and Folin's Phenol reagent from Sigma.

Microalgal Medium Preparation

Bold basal medium (BBM), a highly enriched medium, was used for the cultivation of many green algae (Nichols, 1973). The BBM was prepared, as previously mentioned by Gani *et al.* (2016). Table 1 shows the physical parameters for cultivating *Botryococcus* sp. The cultures were incubated at room temperature (23 – 30 °C) under light illumination from sunlight during the day with an intensity of 30-60 W/m^2 for 20 days with constant agitation.

Table 1. Physical parameters of cultivation of *Botryococcus* sp. under natural conditions

No	Parameters	Values
1	Temperature	23 – 30 °C
2	Light intensity	30 – 60 W/m^2
3	Salinity	0.15 – 0.3 M
4	pH	7.5

Harvesting

After 20 days of microalgae cultivation, the cells were ready for harvesting due to the change in color of the cell suspension in the flasks, from a light green color to a dark green color. At the end of the cultivation period, the microalgae biomass was harvested at 6,000 rpm for 15 min (Chen *et al.*, 2013).

Freeze-drying

The harvested microalgae were frozen using liquid nitrogen, the frozen paste of crude microalgae was directly introduced to a freeze dryer (Martin Christ Alpha LD2, Germany) at -40 °C and 0.12 Mbar for 24 h. The dry biomass was stored under anhydrous conditions for further analysis. Before any disruption treatment, the cells were vigorously rehydrated in distilled water to ensure proper homogeneity of the sample (Chatsungnoen & Chisti, 2016).

Hydrolysis of *Botryococcus* sp. Biomass

Prior to HPLC and total protein analysis for monomeric sugars and protein content, the microalgal biomass was first subjected to analytical acid hydrolysis. A 25 mg of freeze-dried microalgal biomass was subjected to two-stage sulfuric acid hydrolysis for 1 h in 72 wt.% sulfuric acid at 30 °C in a water bath and then autoclaved for 1 h at 121 °C in 4 wt.% sulfuric acids. The procedure used to detect the content of the monomer was as reported in the National Renewable Energy, Laboratory analytical procedure. After hydrolysis, the insoluble acid residue was separated from the hydrolysate using glass fiber filters (<0.2 μm pore size). The aliquot was neutralised to a pH between 6 and 8 using calcium carbonate.

HPLC Analysis for Total Sugar Content

The neutralised sample was filtered through a 0.2 μm nylon filter (disposable syringes and compatible filters were used). HPLC analysis was carried out using a Shodex sugar SP0810 column equipped with an appropriate guard column and a refractive index detector. Sample volume was 50 μl with a mobile phase of 18.2 MΩ water, 0.2 μm filtered at a flow rate of 0.6 mL/min, the column temperature was 85 °C and detector temperature was 55 °C for 50 min. A series of calibration standards comprising the compounds to be quantified was carried out, as described by Van Wychen and Laurens (2013).

Total Protein Analysis

The sample was analysed using the method described by Lowry *et al.* (1951) and measured with a spectrophotometer at 750 nm and SDS-PAGE as previously mentioned by Hershey *et al.* (1995) and Wang *et al.* (2006). Protein samples were separated using 1-dimension SDS-PAGE, then lanes were cut into bands for parallel processing. Before the enzymatic digestion of proteins, reduction and alkylation were carried out. Peptides were isolated for LC-MS/MS analysis, which was followed by searches of protein sequence databases (Dzieciatkowska *et al.*, 2014).

LC-MS/MS Analysis

According to Gundry *et al.* (2010), prior to protein identification for LC-MS/MS analysis, the following processes were performed. After destaining the gel, visible protein bands were excised from the gel. The gel was placed on a glass plate well-lit by a lightbox. Razor blade was used to excise all individual band/spot from the freshly prepared polyacrylamide gel and placed into a 50 ml microcentrifuge tube. The destaining of the excised bands was continued in the tube until the bands were completely colourless. The gel pieces were dehydrated with 400 μl of 100% acetonitrile for 10 min, after which the supernatant was poured out, and the gel pieces were dried in a vacuum centrifuge (Gundry *et al.*, 2010).

Reduction of Disulfide Bonds and Alkylate Free Cysteine

About 100 μl of 10 mM Dithiothreitol (DTT) was added to the gel pieces, and they were incubated for 45 min at 55 °C. The solution was removed, and 100 μl 55 mM iodoacetamide (IAA) was added, and it was incubated at 37 °C for 30 min in the dark. The IAA solution was removed, and 400 μl gel wash solution was added, and it was incubated at room temperature and shaken for 15 min. This step was repeated twice. The gel pieces were again dehydrated with 400 μl of 100% acetonitrile for 10 min, after which the supernatant was removed, and the gel pieces were dried using a vacuum centrifuge.

Enzymatic Digestion

Gel enzyme stock solution was diluted approximately 1:1000 ratio with 25 mM ammonium bicarbonate to obtain a 10 to 20 μg/ml working solution. An adequate amount of the gel enzyme working solution was poured to cover the gel pieces, and it was incubated on ice for 1 h. The excess gel enzyme solution was removed, and a sufficient amount of 25 mM NH_4HCO_3 was poured over the gel pieces, and it was incubated at 37 °C overnight.

Extraction of Peptides

The procedure was carried out as mentioned by Gundry *et al.* (2010). The supernatant, which contains peptides, was removed and poured into a new clean microcentrifuge tube. A 100 μl gel extraction solution was added to the gel pieces and incubated while shaking for 20 min at room temperature. The solution was removed and combined with solution in a microcentrifuge

tube. This was done twice. The combined supernatants were dried using a vacuum centrifuge, and the peptides were desalted before LC-MS/MS analysis using Mass Spectrometer (Orbitrap Fusion – Thermo Fisher Scientific). The mass spectra were analysed and matched to the enzymes database of the National Center for Biotechnology Information (NCBI) using MS-Fit.

Lipid Extraction

For the extraction of lipids, n-Hexane an extractable material for sludge, sediment and solid samples were used in this study. The technique was adopted from the United States Environmental Protection Agency (EPA Method 9071B). Briefly, a sample of *Botryococcus* sp. biomass was mixed with anhydrous sodium sulfate and placed into an extraction thimble (or between two plugs of glass wool). The extraction was done using an *n*-Hexane solvent in a Soxhlet extractor. Further clarification of the protocol was mentioned in previous research by Gani *et al*. (2017).

Total Fatty Acids Analysis

The fatty acids were first converted into methyl esters by direct trans-methylation of the lipid extracts. The fatty acid methyl esters obtained were analyzed by the Perkin Elmer Clarus 680SQ8 Gas Chromatography system (USA) equipped with a flame ionization detector as described by Ruangsomboon *et al.* (2013).

RESULTS AND DISCUSSION

Intracellular Metabolites of *Botryococcus* sp.

Total sugars content

According to Cheng *et al.* (2015), most microalgae contain hemicellulose, a polysaccharide made up of different types of monosaccharides among galactose, mannose, and arabinose. The monomeric composition of the total carbohydrates shows galactose and glucose as the main monomers of this *Botryococcus* sp. Galactose has the most significant portion of the total carbohydrate produced in the *Botryococcus* sp. (Table 2). Gouveia *et al.* (2017) reported that galactose and glucose to be the main monomers across 16 strains of *Botryococcus*. High galactose content is an indication of high viscosity which is a desirable trait in biofuel production (Bayona & Garces, 2014). The monosaccharide galactose produced during photosynthesis by microalgae is used as an energy and carbon source in the production of proteins, lipids, and other carbohydrates. In a similar study Mori *et al.* (2019) noted that UDP-galactose and glycerol 3-phosphate (G3P) are synthesized from photosynthesis within the plastid in the unicellular red alga, *Cyanidioschyzon merolae*.

Table 2. Composition of monomeric sugars in *Botryococcus* sp.

Monosaccharide	ppm	mg/100 ml	Sugars (%)
Glucose	0.2985	23.88	29.99
Galactose	0.5774	46.20	58.01
Arabinose	0.0762	6.10	7.66
Mannose	0.0432	3.46	4.34
Total		79.63	100

Total Protein Estimation

However, it is also important to highlight the protein content of this microalgae as part of the total nutrient that this species provides. The complete protein estimated for the *Botryococcus* sp. was 16% which is within the range of 11% – 46% DW for freshwater microalgae (Boyd, 1973; Dayananda *et al.*, 2007; Tsarenko, 2011). High protein content has been found and reported in some microalgae species such as *Spirulina maxima* (60% – 71%), *Synechococcus* sp. (63%), *Anabaena cylindrical* (43% – 56%), and *Chlorella vulgaris* (41% – 58%) as previously mentioned by Wang *et al.* (2021). These microalgal species with high protein content have been used and are the sources of nutrients for functional foods, nutraceuticals, and food additives. According to Bi and He (2013), a high protein level in microalgae was indicative of a high nitrogen content, rendering it unsuitable for biofuel production. Intriguingly, *Botryococcus* sp. has a low protein

concentration; hence, it may be a suitable source for biofuel production.

Most proteins in the extract of microalgae are enzymes involved in photosynthesis and other essential activities for its survival and growth as mentioned by Contreras *et al.* (2008) and Schwenzfeier *et al.* (2011). These enzymes can consist of multiple polypeptide chains. In this study, the migration distance of four distinct bands isolated in the *Botryococcus* sp. was measured to be 55, 45, 31, and 24 kDa (Figure 1). In addition, the enzymes identified by the LC-MS/MS fall within the band range of the SDS-PAGE. These were enzymes phosphokinase, enolase, GADPH, and activator acetyl dehydrogenase with molecular weights 50.08, 46.7, 40.3, and 25.6 kDa, respectively (Table 3).

Total Lipid Estimation and Fatty Acid Analysis

The gas chromatography separation identified 19 individual fatty acids in *Botryococcus* sp. as shown in Figure S1. Total percentages of saturated and unsaturated fatty acids in this species were 38.628% and 61.372%, respectively. A total of nine (C4:0, C6:0, C11:0, C12:0, C14:0 C15:0, C16:0, C17:0, C18:0) saturated fatty acids (SFA), five (C14:1, C15:1, C16:1, C17:1, C18:1n9c) monounsaturated fatty acids (MUFA), and four (C18:2n6cis, C18:3n6G, C18:3n3A, C22:2) polyunsaturated fatty acid (PUFA) were identified and quantified as the percentages of total lipid (Table 4).

The major SFAs found were C16:0 (palmitic acid) and C18:0 (stearic acid) at 27.95% and 8.635%, respectively. PUFAs made up most of the unsaturated fats, with C18:2n6cis (linoleic acid) making up 22.758% and C18:3n (linolenic acid) making up 17.046%, whereas, a MUFA, C18:1n9c (oleic acid) contributed 13.057%. In total, the grouping of carbon 16 to carbon 18 alone of *Botryococcus* sp. contributed 95.384% of the total fatty acids, which was similar to the results obtained from the studies described by Mata *et al.* (2010), Dayan *et al.* (2010), Ashokkumar & Rengasamy (2012) and Ruangsomboon (2015). The ratios of SFA, MUFA, and PUFA contents have a major effect on the biodiesel quality. This is because biodiesels with good flow performance at low temperatures can be achieved through short-chain fatty acids and with a high degree of unsaturation (Knothe (2005), Nascimento *et al.*, (2013), and Stansell *et al.*, (2012)). The results from this study showed that the combined percentage of PUFAs (linoleic acid and linolenic acid) amounted to 39.804%, which have higher degree of unsaturation than the SFAs. Biodiesels with more SFA content have high melting points and could crystallize at normal engine temperatures. This parameter is defined as poor flow performance property in terms of biodiesel (Knothe, 2008). Another important consideration for a good biodiesel quality is the presence of oleic acid which was present in *Botryococcus* sp. at 13.27%. Oleic acid helps in balancing ignition quality and cold flow properties, hence, biodiesel with high oleic acid content does not normally cause polymerization during combustion as mentioned by Knothe (2005), Nascimento *et al.* (2013) and Stansell *et al.* (2012).

Table 3. Identified enzymes related to carbohydrates metabolism

Enzyme	EC numbers	5′ to 3′ Score Sequence from LC-MS results	Function of enzyme
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.59	VPTPTVSVVDLVVQVEK	To capture CO_2
Phosphoglycerate kinase 1	2.7.1.59	ELDYLDGAVSAPK.	To regenerate RUb
Phosphoglycerate kinase 2	2.7.2.3	ELDYLDGAVSAPK.	To produce 1,3 Bp
Enolase 2	4.2.1.11	SGETEDTFIADLVVGLR	To generate PEP
Fructose bisphosphate aldolase	4.1.2.13	ATPEqVASYTLK	To convert FBP to F6P
Fructose bisphosphate aldolase	4.1.2.13	GILAMDESNATCGK.	To convert F6P to glucose
Triose phosphate dehydrogenase	1.2.1.9	VPTVDVSVVDLTVK	In glycogen-esis
Triose phosphate isomerase	5.3.1.1	RVPTPTVSVVDLVVQVEKK	In glycolysis II

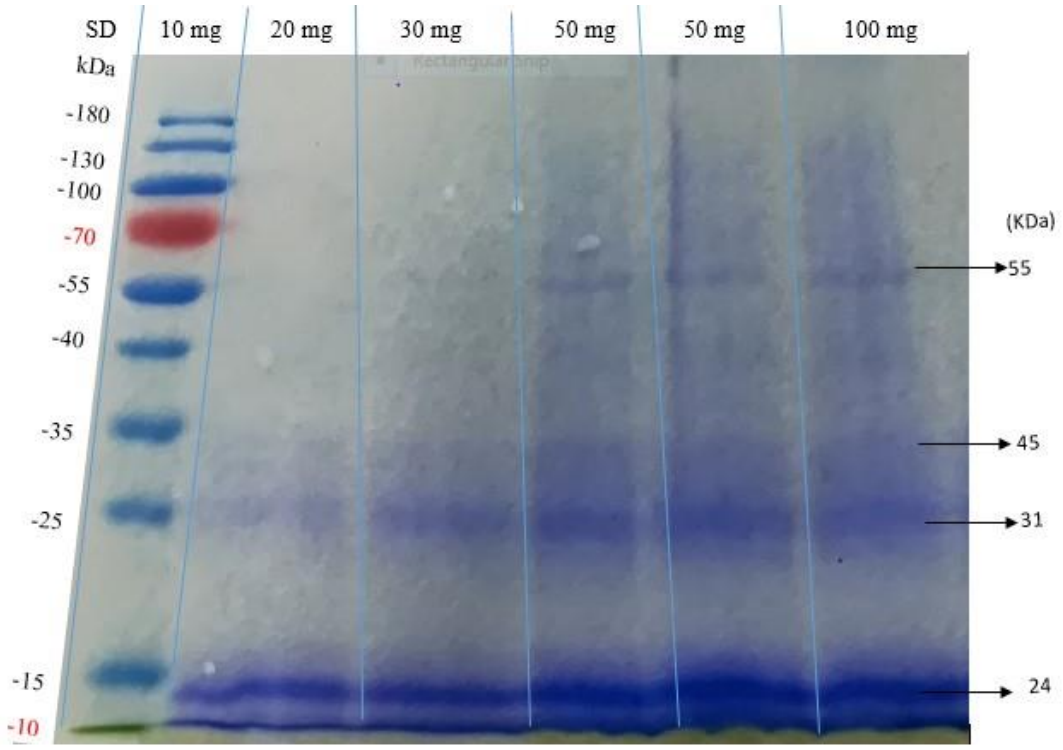

Figure 1. SDS-PAGE analysis of the major protein fractions from *Botryococcus* sp. (arrows showing apparent bands)

Table 4. Fatty acids composition of *Botryococcus* sp.

Sample	Lipid Numbers	Common Name	%
Saturated fatty acids			
1	C4	Butyric acid	0.255
2	C6	Caproic acid	0.110
3	C11	Undecylic acid	0.404
4	C12	Lauric acid	0.089
5	C14	Myristic acid	0.436
6	C15	Pentadecylic acid	0.129
7	C16	Palmitic	27.950
8	C17	Margaric acid	0.618
9	C18	Stearic acid	8.635
			38.626
Unsaturated fatty acids			
1	C14:1	Myristoleic	0.083
2	C15:1	C15:1 (*cis*-10) Fatty acid	0.051
3	C16:1	Palmitoleic	1.453
4	C17:1	C17:1(*cis*-10) Fatty acid	1.532
5	C18:1n9c	Oleic acid	13.270
6	C18:2n6c	Linoleic	17.046
7	C18:3n6G	Linolenic (GLA) acid	2.122
8	C18:3n3A	Linolenic (ALA) acid	22.758
9	C22:2	Docosadienoic acid	3.057
			61.372
Total percentage of fatty acids			100

Carbohydrate Metabolism Pathway of *Botryococcus* sp.

The carbohydrate pathway of the *Botryococcus* sp. was constructed with the aid of protein sequence identification in the Calvin Benson cycle (reductive pentose phosphate pathway) and the glycolysis pathway. The schematic diagram of the carbohydrates pathway made from the experimental results of the research starts from the carbon fixation of CO_2 to the different vital cellular constituents, in an autotrophically grown green microalgal cells (Figure 2). The carbon assimilation in cells took place through various pathways together, representing a carbon metabolic network (Boroukh *et al.,* 2015). The CO_2 fixation occurs in the chloroplast localised Calvin Benson cycle in the presence of light through the enzyme Ribulose-1, 5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco). According to Baroukh *et al.* (2015), it catalyses the addition of CO_2 to enolized ribulose 1,5-bisphosphate (RuBP), resulting in the formation of 3-phosphoglyceric acid (3-PGA), which is then transformed into sugars. Ribulose 1,5-bisphosphate (RuBP) is a double phosphate ester of the ketopentose, while 3-phosphoglyceric acid (3-PGA) is carbon 3 compound. *Botryococcus* sp. uses light energy (provides the biochemical reductant, $NADPH_2$) to dissociate the water molecules by splitting it into protons, electrons, and oxygen, which is used to reduce CO_2 to form two molecules of 3-PGA (Figure 2). The 3-PGA reacts with ATP in the presence of phosphoglycerate kinase to produce 1, 3-biphosphate, and ADP according to Eq. (1).

$$3-\mathrm{PGA}+\mathrm{ATP}\xrightarrow{2.7.2.3}1,3-\text{bisphosphoglycerate}+\mathrm{ADP} \quad \text{Eq. (1)}$$

The reaction of 1, 3-biphosphoglycerate (1,3-BPG) was further catalyzed by glyceraldehyde 3-phosphate dehydrogenase (GADPH) in the presence of $NADPH_2$ to form glyceraldehyde 3-phosphate (G3P) and NADP according to Eq. (2).

$$1,3\text{- BPG} + \mathrm{NADPH_2}\xrightarrow{1.2.1.59}\mathrm{G3P}+\mathrm{NADP} \quad \text{Eq. (2)}$$

Condensation of two triose phosphate molecules resulted in the creation of hexose sugar (glucose and fructose) synthesis when 3-PGA was reduced into triose phosphates. Ten molecules of the triose phosphate used to regenerate ribulose-1,5-biphosphate for the continuation of the Calvin Benson cycle (Khan *et al.,* 2018). The hexose sugar (glucose) breaks down (glycolysis) to produce pyruvate. After which the pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase (Figure 3).

The alternative pathway of glycolysis exists, through which the 3-PGA from the chloroplast was exported to the cytoplasm with the action of triose-phosphate/phosphate translocator. Garibay-Hernández *et al.* (2017) and Melis (2013) stated that the absence of the lower part of glycolysis from the chloroplast and the presence of its enzymes only in the cytoplasm requires that the chloroplast must export fixed carbon into the cytoplasm fraction of the cell. The glycolytic enolase enzyme could generate phosphoenolpyruvate (PEP), which can then be converted into pyruvate. The cytoplasmic PEP could then be imported back into the chloroplast through phosphoenolpyruvate/phosphate translocator. Similarly, a study by Polle *et al.* (2014) reported that cytosolic localisation involves the carrying across of the organic carbon from the plastid to provide a substrate for the enolase and ensuing re-import of organic carbon back into the plastids. Therefore, the enolase signifies an essential adjusting obstacle in carbon partitioning in this *Botryococcus* sp. (Figure 4).

The finding by Troncoso-Ponce *et al.* (2010) reported that a high level of presence of enolase was considered necessary for oil accumulation in sunflower seeds supported the significance of enolase in carbon partitioning. Therefore, a cytosolic enolase activity seems to be able to support plant growth, whereas the disruption of cytosolic enolase activity has detrimental effects on growth (Voll *et al.,* 2009). The internal transfer of a phosphate group in 3PG is being catalysed by the enzyme phosphoglycerate mutase, which resulted in 2-phosphoglycerate (2PG). The enzyme enolase catalysed the conversion of 2PG to PEP. The pyruvate undergoes decarboxylation to get converted to acetyl-CoA. The identified enzymes related to carbohydrates metabolism were selected, and these are listed together with their matched peptide sequences in Table 3.

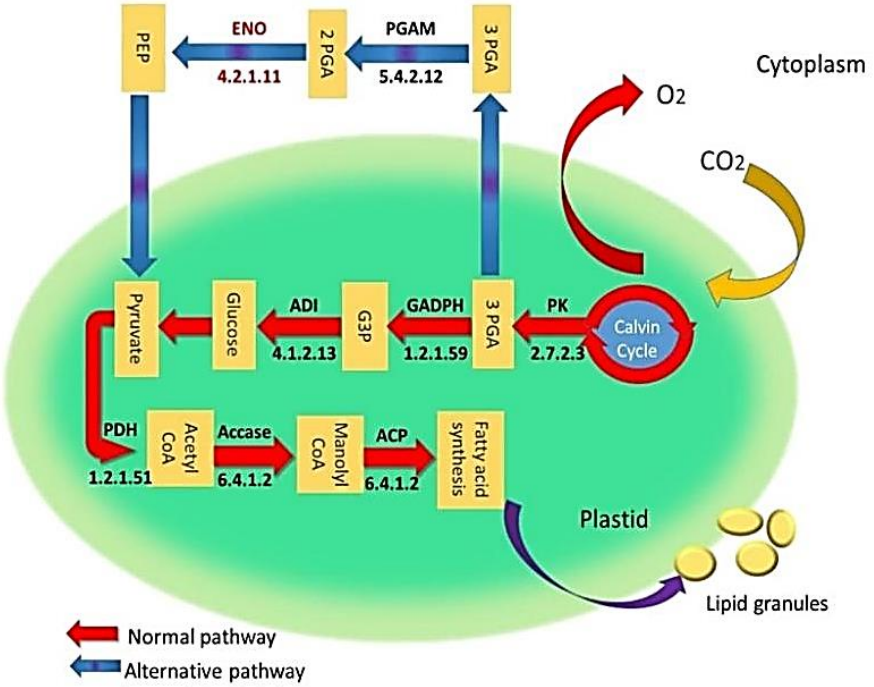

Figure 2. Summary of the carbon metabolism proteins identified by LC-MS/MS in *Botryococcus* sp. Protein abbreviations: Acetyl-CoA Carboxylase Kinase, ACCase (EC 2.7.1.111); Enolase, ENO (EC 4.2.1.11); Fructose biphosphate aldolase, FBP (EC 4.1.2.13); Glyceraldehyde-3-phosphate dehydrogenase, GADP (EC 1.2.1.59); Malate dehydrogenase, MDH (EC 1.1.1.39); Phosphoglycerate kinase, PK (EC 2.7.2.3), Phosphoribolose kinase, PRK (EC 2.7.1.19), Phosphoglycerate mutase, PGM (EC 5.4.2.12) glucose 6 phosphate isomerase GPI (EC 5.3.1.9) Phosphofructokinase, Pyruvate dehydrogenase, PDH (EC 1.2.1.51), Ribulose-1,5-biphosphate, RuBP (EC 4.1.1.39); Triosephosphate isomerase, TPI (EC 5.3.1.1). The proposed diagram is based on research by Baroukh *et al.* (2015) and Shi *et al.* (2021)

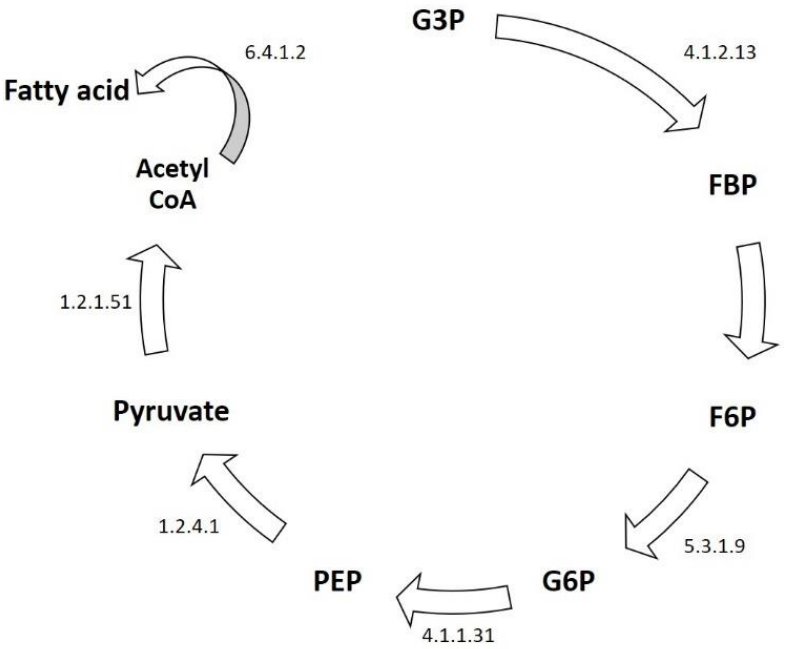

Figure 3. The hexose sugar (glucose) breaks down (glycolysis) to produce pyruvate. After which the pyruvate is converted to acetyl CoA by pyruvate dehydrogenase (Khan *et al*., 2018)

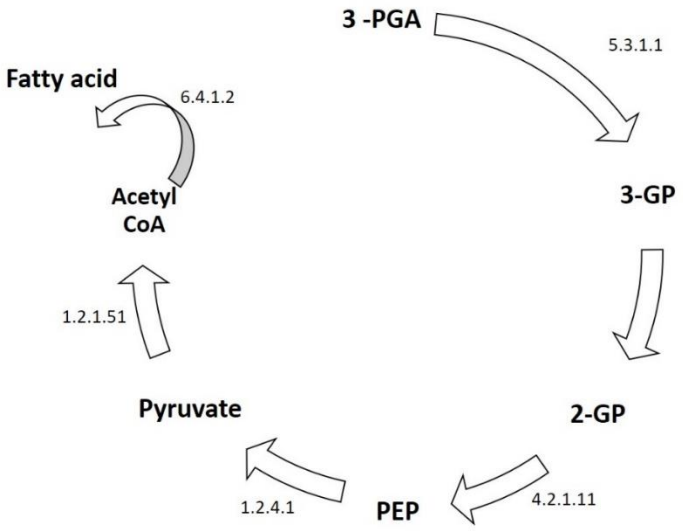

Figure 4. The alternative pathway of glycolysis exists, through which the 3-PGA from the chloroplast was exported to the cytoplasm with the action of triose-phosphate/phosphate translocator (Garibay-Hernández *et al*., 2017)

Alternative Pathway

Blast protein results

Identification of similar sequences in large sequence databanks is typically the first step in the analysis of new protein. Structural and functional annotations of the related sequences can be used to infer the functions and structural features of the new sequences. The enolase enzyme sequences from the *Botryococcus* sp. was identified in this study, and it was discovered to have three types of the enolase enzyme. The first sequence for enolase that is found in *Botryococcus* sp. according to blast result shows that for Query_316718 of 5′-RSGETEDTFIADLVVGLRT-KIEISELNRV-3′ is similar to the enolase found in this study. There are two other sequences for enolases that are found in this *Botryococcus* sp. The blast result shows that Query 313694 5′-KWLSGPQLADLYHSLVKK-3′ and Query_22067 KYPIVSIE 5-RSGETEDTFIADLVVGLRT-3′ are located within the plastids. From the description of the blastp results, it shows that the first enolase C-terminal which is represented as ENOc, is enolase localized to the cytosol (Voll *et al.*, 2009). The role of it is to catalyze the fixed carbon 3-phospho D glycerate that was sent to the cytosol to PEP. These enolase enzymes found in the cytosol formed the basis of constructing the alternative pathway (that is, a pathway of lipid synthesis) in this study. The second and third enolases are plastid-localized enolase are responsible for performing normal functions as reported by Prabhakar *et al.* (2009). Interestingly, it is strongly suggested that the presence of enolase enzymes located in the cytoplasm is responsible for the *Botryococcus* sp. ability to produce higher lipids compare with others microalgae in the same group.

CONCLUSION

Understanding the metabolic pathways and processes involved in the generation of these organic macromolecules was essential in developing high lipid yielding algal strains. This study was able to establish an alternative pathway of lipid synthesis that was not based on genetic modification but through natural process which is the export of the 3-PGA to the cytoplasm, providing a shorter route to lipid production than the normal process via the plastid. This pathway proved a different route to producing more lipids that was not established before. The alternate pathway shows the main reason why the *Botryococcus* sp. was able to accumulate significant amount of lipids than carbohydrates as its main source energy storage. Overall, this is a fundamental study into the basic composition and metabolic biosynthesis of the microalga *Botryococcus* sp. which is an indigenous strain from the Taman Negara Johor Endau Rompin, Malaysia.

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