Biochemical Characterization of Lipid-Extracted Microalgal Biomass Residues

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ABSTRACT

Massive interests to microalgal biodiesel are obvious to date, due to the promising prospect of microalgae as biodiesel feedstock. Nevertheless, based on the result of energy analyses and life-cycle assessments, microalgal biodiesel production has consumed large amount of energy and it has not been cost effective yet. Therefore, utilization of lipid-extracted microalgal biomass residues (LMBRs), one kind of residues produced after lipid extraction in biodiesel production should be effectively investigated. This work emphasizes on the overview of researches related to the biochemical characterization of LMBRs. The LMBRs of *Dunaliella tertiolecta* (UTEX LB 999) consisted of total carbohydrates of 82.0%, proteins of 13.4%, and ash of 4.5% (w/w in dry mass). Meanwhile, *D. tertiolecta* LB 999 LMBRs contained carbohydrate of 60%, and its saccharification yield was 42% based on LMBRs mass. Moreover, the biochemical composition of *Haematococcus pluvialis* LMBRs were crude fiber (9.6%), crude protein (40.3%), and crude lipid (0.9%) while *Scenedesmus* sp. LMBRs contained carbohydrate (24.7%), protein (32.4%), lipid (6.5%) and ash (10.0%).

Keywords: microalgal, biodiesel, LCA, LMBRs, characterization, EPS, saccharification

INTRODUCTION

Several problems such as energy security, increase of oil price, resources depletion and climate change are the global issues which have been facing by our society since decades ago. Biofuels and bio-refineries are expected that can mitigate these problems at least to some extent. Nowadays three generations of biofuels have been developed. Biofuel which is produced from sugar/starch crop is the first generation, that produced from lignocellulosic biomass is the second generation, and the third generation biofuel feedstocks are microalgae and macroalgae. Microalgae and macroalgae are more promising than the previous two generations of energy sources, due to the prospect of these organisms which show high biomass yields and lipid productivity without requiring any arable land (Chiaramonti, 2007; John *et al*., 2011; Trent, 2012; Cheirsilp and Torpee, 2012). Moreover, some algal species can grow well in saline, brackish and waste-water environment that makes them more promising as feedstock than terrestrial crops which rely utterly on fresh water. So far, most research on algal biofuels has been conducted in two areas i.e. biodiesel synthesis from algal lipids and fermentative ethanol production from algal feedstock (Daroch *et al*., 2013).

Oleaginous microalgae can utilize both inorganic carbon (CO_2) and organic carbon sources (glucose, acetate, etc.) to synthesize and accumulate lipids more than 20 % of dry weight inside the cells. The components and contents of lipids in microalgal biomass vary from species to species. The classes of lipid

basically are classified into neutral lipids (e.g., triglycerides, cholesterol) and polar lipids (e.g., phospholipids, galactolipids). Triglycerides (neutral lipids) are the main materials in the production of biodiesel. Moreover, it was known that microalgal lipid has several advantages such as: 1) it has fatty acid constituents similar to common vegetable oils; 2) under certain condition it may be as high as 85% of the dry cell weight; 3) it has short-time growth cycle (Huang *et al*., 2010). However, fertilizers consumption, harvesting, and lipid extraction from microalgal biomass consume large amount of energy. It jeopardizes the massive interests of algal biomass from the result of energy analyses and the life-cycle assessment. Therefore, to be more sustainable in developing microalgal biodiesel industry, and in utilizing renewable energy, the effective utilization of lipid-extracted microalgal biomass residues (LMBRs) should be investigated (Zheng *et al*., 2012). Moreover, in line with an increasing of microalgal biodiesel industries which are inevitably would extract lipids from microalgal biomass, the lipid-extracted microalgal biomass residues (LMBRs) would also be abundantly produced. Utilization of these residues (LMBRs) to valuable products not only could reduce the production cost of biodiesel but also could lower the treatment or disposal cost.

An Overview of Microalgal Biodiesel Production

Biodiesel

Biodiesel is commonly known as methyl or ethyl esters of fatty acids which are produced by transesterification of triglycerides under an acid or alkaline condition. Alkaline transesterification was more widely used than acid transesterification in biodiesel production. Figure 1 illustrates the alkaline transesterification of acylglycerol with methanol to produce biodiesel as a main product and glycerol as a by-product. However, under alkaline condition, the undesireable reactions between free fatty acid and alkaline commonly occur and undesirable soap was formed.

There are several reasons make biodiesel as an important energy resource. First, as a renewable energy resource, biodiesel could be sustainably produced (Sheehan, *et al*., 1998). Second, biodiesel has several properties which are environmentally friendly (Antolin *et al*., 2002; Vicente *et al*., 2004). Third, economically, biodiesel has big potential because fossil fuel prices intend to increase predictably in the future (Cadenas and Cabezndo, 1998). Furthermore, biodiesel has several characteristics which are compatible with the petroleum diesel (fossil fuel). For instance, the higher heating value (HHV) of biodiesel (39–41 MJ/kg) is comparable with that of petrodiesel (43 MJ/kg). Other characteristics such as cetane number, kinematic viscosity, and flash point are also similar (Fuls *et al*., 1984).

Nowadays, biomass oils are mostly used as raw materials in biodiesel production. Biodiesel is mainly produced from vegetable oils of plants such as soybeans, corn, and palm. However, an increasing of biofuel consumption has led to a higher production capacity of biofuel. Consequently, it also increases the pressure on biofuel industries to find more biomass. Biodiesel production has begun to face limitations such as availability of vital food sources and the massive area of land required to grow several biomass crops (Chisti, 2007).

Algae

As a word algae is commonly used to describe a large diversity of prokaryotic (Cyanobacteria) and eukaryotic organisms which have a range of phylogenies and morphologies. Those organisms are included in a wide range of species, which can be found from deserts to the Arctic Ocean environments (salt and fresh water). The algae vary in shape, colour, and size (picoplankton is 0.2 - 2 µm, while giant kelp fronds reachs 60 m in length (Barsanti and Gualtieri, 2006). Traditionally, algae were understood having both prokaryotic and eukaryotic domains (Bold and Wynne, 1978; South and Whittick, 1987). Nevertheless, recent research reported that the prokaryotic algae are more similar to bacteria. Therefore, newer definitions exclude prokaryotes from algae (Bhattacharya and Medlin, 1998). The most common classification of the eukaryotes includes four divisions such as *Chromophycota*, *Rhodophycota*,

Euglenophycota, and *Chlorophycota*. *Chromophycota* include all algae which have chlorophylls *a* and *c* and lacking chlorophyll *b*, *Rhodophycota* are red algae, *Euglenophycota* are similar to *Chromophycota* yet plate or disc shaped, and *Chlorophycota* are green algae (South and Whittick, 1987).

Algae can widely be found as diverse groups of chlorophyll containing. They are mainly aquatic, eukaryotic organisms. Moreover, algae are different from higher plants by lacking leaves, roots, stems, and reproductive structures (Lee, 1999). Eukaryotic algae cells have cell wall which is mainly composed of polysaccharides. In addition, there is the plasma membrane surrounding the cell. Figure 2 shows the detailed structure of algae cell with organelles such as a nucleus, golgi apparatus, starch vacuole, mitochondria, chloroplasts, ribosome, etc.

Microalgae

Microalgae are obviously heterogenous group of organisms. The organism has several characteristics such as, small size (commonly microscopic size), colourful (it is because the accessory and photosynthetic pigments), unicellular (but also can live in colonies with little or without cell differentiation), can be found mainly in water, and most microalgae are photoautotrophic (but not necessarily every time). Based on phylogeny analysis, microalgae can be classified as prokaryotic or eukaryotic. Moreover, in evolutionary terms, they can be classified as recent or very ancient organism. The biodiversity of microalgae is very high. Estimately, there are around 200,000 – 800,000 species of microalgae on earth, whereas only around 35,000 species had already been indentified, such as *Chlorella* sp., *Spirulina*, *Nannochloropsis* sp., *Botryococcus braunii*, *Nitzschia* sp., *Tetraselmis suecia*, *Dunaliella primolecta*, etc. Due to a very high diversity microalgae known as, a group, a very rich source of many chemical products which are applied in several industries such as, the nutritional, pharmaceutical, food, cosmetic, and fuel industries (Olaizola, 2003).

Microalgae are a photosynthetic microorganism which can utilize sunlight and carbon dioxide to produce biomass, and oxygen. The oxygen produced is approximately 50% of total atmospheric oxygen. Microalgal cells develop and grow in water suspension, so in utilizing water, carbon dioxide, and other nutrition, microalgae have higher level of efficiency than plants (Deng *et al*., 2009; Widjaja, 2009). Most species of microalgae produce specific products inside and outside the cells such as, fatty acids, sterol, peptides, polysaccharides, enzyme, carotenoid, antioxidants, and toxins (Hossain *et al*., 2008). These products of microalgae are not limited by proper factors. It depends on species and condition of cultivation. So, there is an opportunity to harvest microalgae and their products with certain amount by manipulating the environmental factors such as, temperature, light, pH, carbon dioxide availability, salts and other nutrition (Basmal, 2008).

Microalgae are known as microorganism with capability as biofuel factory. It is because, there are several biofuels produced from microalgae such as, biodiesel (produced via transesterification reaction), bioethanol (produced via fermentation), hydrogen, and biogas (Basmal, 2008). Comparing to food based plants/crops, the utilization of microalgae as raw material in biofuel production gives several advantages such as, higher growth rate, higher productivity, utilizing freshwater or sea water, lower water consumption, not compete with food, and relatively low production cost. Microalgae grow faster than higher plants. It is because the simple structure of their cells, and the water suspension environment. Therefore, wider cell surface can capture more light and increase the transfer of mass, resulting faster substrate consumed and more efficient photosynthesis (Miao and Wu, 2006; Sheehan *et al*., 1998).

Potential of microalgae as biofuel feedstock

Microalgae are microorganism which can provide energy by conducting photosynthesis. They also grow so quickly. Some species can adapt and grow very well in various environments. It is obviously an advantage for further development of microalgae as biofuel's raw materials because they can be grown in various areas. There are differences with other biofuel's raw material such as jatropha

oil, soy bean, and sun flower which can only be developed in certain area. Moreover, microalgae have higher growth rate and higher productivity than other raw materials. Furthermore, to produce the same amount of biofuel produced by using other raw materials, microalgae only needs 49-132 times of smaller land. In addition, the utilization of microalgae as biofuel's raw materials does not compete with human consumptions. Microalgae are promising organisms which have potentials becoming biofuel's raw materials (Deng *et* al., 2009; Li *et al.*, 2008; Gouveia and Oliveira, 2009

It averagely needs 10 days of cultivation before harvesting the microalgae. The productivity of microalgae as biodiesel's raw materials is very high. The productivity reach 120,000kg biodiesel/Ha year, it is more than 20 times of palm oil productivity (5,800kg biodiesel/Ha year) and 80 times of jatropha oil productivity (1,500kg biodiesel/Ha year) (Teresa *et al*, 2010).

Composition of microalgae

Microalgal cell contains some lipids, carbohydrates, and proteins. Lipids content of certain microalgal found are more than 50%, those microalgae also have very high growth rate (Hossain *et al*., 2008; Hu *et al.*, 2008; Massinggil, 2009). Meanwhile, the carbohydrate content of microalgae (*Chlorella* species) is around 29-31% of dry biomass. It is higher than in cassava which is 23% of dry biomass. One of the major constituents of the algae biomass is proteins (up to 50% w/w). Proteins are expected to play an important role in algae biorefinery (Williams and Laurens, 2010). Based on their abundance and their amino acid profile, microalgal proteins have been considered as an alternative protein source in foods (Spolaore *et al*., 2006).

Lipid

Definition of lipids, "Lipids are hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters (fatty acids, polyketides, etc.) and/or by carbocation-based condensations of isoprene units (prenols, sterols, etc.)" (Fahy *et al*., 2005). Or "Lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds" (Christie, 2003).

Most of the important oils and fats from plant and animal almost wholly consist of triacylglycerols (the simple lipid class) or in older literature known as triglycerides. Triacylglycerols consist of a glycerol-liked structure with each hydroxyl group esterified to a fatty acid. Naturally, triacylglycerols are synthesised by system of enzymes, which make carbon-2 of the glycerol structure is a centre of asymmetry, so they exist in enantiomeric forms. The main biological function of those triacylglycerols is as energy storage molecules (Christie, 2003).

Lipid production by microalgae

High oil content of some algal species is potentially attractive as a raw material of biodiesel production. Research for the production of oil utilizing algae has focused on the microalgae not macroalgae. It is because microalgae have higher lipid content than macroalgae. Green algae (*Chlorophyta*) and diatoms (*Bacillariophyta*) are the algal species which are mostly considered for biodiesel production (Sheehan *et al*., 1998).

Microalgae can utilize both inorganic carbon (CO_2) and organic carbon sources (glucose, acetate, etc.) for lipids production. The constituent and contents of lipids in microalgal cells differ from species to species. The type of lipids basically are classified into neutral lipids (e.g., triglycerides, cholesterol) and polar lipids (e.g., phospholipids, galactolipids). Triglycerides (neutral lipids) are the most important materials in the biodiesel production. The biosynthesis routes of triglycerides in microalgae may consist of the three steps: (a) the formation of acetyl coenzyme A (acetyl-coA) in the cytoplasm; (b) the elongation and desaturation of carbon chain of fatty acids; and (c) the biosynthesis of triglycerides in microalgae.

Microalgal lipid composition

Lipid accumulation of four strains of microalgae (fresh water *Chlorella* sp., marine *Chlorella* sp., *Nannochloropsis* sp. and *Cheatoceros* sp.) had been studied by Cheirsilp and Torpee (2012). They found that under

photoautotrophic cultivation, microalgae produced the highest lipid content compared to those under mixotrophic and heterotrophic cultivation. Marine *Chlorella* sp., *Nannochloropsis* sp., and *Cheatoceros* sp. had the lipid content of about 30% based on dry weight. In contrast, fresh water *Chlorella* sp. had lower lipid content of about 10%. Those results were in line with the studies of Chojnacka and Noworyta (2004) and Liang *et al*. (2009), they reported that the lipid content of *Chlorella vulgaris* which were cultivated under photoautotrophic condition was higher than those cultivated under mixotrophic and heterotrophic conditions.

Lipid content of microalgae varies from species to species in range of dry biomass 5 to 77 wt.% (Brown *et al*., 1997; Chisti, 2007). Lv *et al*. (2010) investigated lipid compositions of some microalgal species. They found that some microalgal species are rich in neutral lipids content. The composition and fatty acid profile of microalgal lipids are affected by the microalgal life cycle and the cultivation conditions, such as medium composition, temperature, illumination intensity, ratio of light/dark cycle, and rate of aeration (Guzman *et al*., 2010; Ota *et al*., 2009; Ramadan *et al*., 2008).

Microalgal cells which were harvested during stationary phase have lower polar lipid contents than the same species harvested during the logarithmic phase. Some microalgal species could also increase their lipid contents from ~10 wt.% to almost 20 wt.% during deprivation of oxygen (Dunstan *et al.*, 1993). Microalgae commonly respond a nutrient starvation by intensifying their metabolism for biosynthesizing of neutral lipids. Nevertheless, the increasing of cellular lipid production commonly does not in line with an overall increasing of oil productivity per unit mass. Due to the inter and intraspecific variations during cells growth. Suitability of microalgal lipids for biodiesel production is also difficult to be assessed. It often needs to be examined on a case-by-case basis (Halim *et al.,* 2012).

Microalgal fatty acids vary from 12 to 22 carbons in length and could be found either as saturated or unsaturated type. The number of double bonds in the fatty acid chains never exceeds 6 and almost all of the unsaturated fatty acids are cis isomers (Medina *et al*., 1998). The fatty acid profile of lipid extracted from *Tetraselmis suecica* during early stationary phase could be seen in Figure 7 (Halim *et al.,* 2012). *T. suecica* is a common green microalga and its fatty acid profile could be used to illustrate the suitability of microalgal lipids for biodiesel production. Their principal fatty acids such as C16:0, C18:1, and C18:2 show that *Tetraselmis* lipid has the required fatty acid profile for conversion to high-quality biodiesel. Moreover, in terms of lipid classes, it was known that acylglycerols commonly have a lower degree of unsaturation than polar lipids. Therefore, acylglycerols are more suitable for biodiesel conversion.

Harvesting of microalgae

Microalgae which were cultivated in open ponds or tubular photobiorector commonly have low final biomass concentrations (0.5–5 g/L dry weight) (Lam and Lee, 2012). The low final concentration of microalgal biomass and small size of microalgae make harvesting of the biomass is challenging. There are several methods which have been used for microalgal harvesting, such as centrifugation, foam fractionation, filtration, flocculation, and gravity sedimentation (Chen *et al*., 2011).

Most of commercial systems choose centrifugation to harvest microalgae, but it consumes a large amount of electric power (Divakaran and Sivasankara Pillai, 2002). Filtration could also be used in harvesting process, but membranes will be rapidly fouled by the extracellular organic matter if the medium was filtered directly (Babel and Takizawa, 2010). Based on life cycle analysis reported by Sander and Murthy (2010), the microalgal biomass which were harvested by centrifugation or filtration (bulk harvesting) need large amount of energy and intensive maintenance. Therefore, in the view of economic and technological feasibility, flocculation can be a convenient and an effective method for harvesting microalgae from large scale of microalgae cultures (Wu *et al*., 2012).

Flocculation of microalgal cultures by addition of inorganic compounds was known as the most promising method for harvesting

microalgal biomass. It was because the flocculation could be easily scale-up and could be applied for various species of microalgae (Uduman *et al*., 2010). Based on several studies of microalgal flocculation, the addition of inorganic salts demonstrated that the flocculation could be induced both under acidic or alkaline conditions. Inorganic flocculants such as iron chloride and aluminum sulfate needed acidic pH for optimal microalgal flocculation (Uduman *et al*., 2010). Meanwhile, microalgal flocculation in alkaline conditions (pH > 10) was induced by precipitation of magnesium (Mg) and/or calcium (Ca) salts which present in the media (Sukenik and Shelef, 1984).

Flocculation of three freshwater and two marine species of microalgae were studied by Wu *et al*. (2012) based on the presence of magnesium salts in the media. They set the pH of marine algal culture to range of 8-12, Mg^{2+} dosage of around 1.5-4.5 mg/L and initial biomass concentration of around 1.7 g/L. They found that percentages of flocculation efficiency were more than 90% after 10 minutes of flocculation. They also reported that Mg^{2+} in the growth media produced $Mg(OH)_2$ which precipitated at high pH. The agglomerates then induced flocculation of microalgal cells by sweep flocculation and charge neutralization. Vandamme *et al*. (2010) also reported that the presence of Mg^{2+} in the growth medium was essential for flocculation and a condition of pH more than 10 could induce flocculation of microalgae (*C. vulgaris*).

Processes Resulting LMBRs in Microalgae-based Biodiesel production

There are two kinds of processes that usually used in biodiesel production then produce LMBRs as residues. They are including 1) the lipid extraction process which are separately conducted prior to transesterification process, and 2) the simultaneous lipid extraction and transesterification process which are simultaneously conducted in the reactor.

1) Lipid extraction

One important factor to optimize advantages from lipid-producing microalgae is the ability to efficiently extract lipids from the microalgal biomass (Mercer and Armenta, 2011). Some extraction methods and their performance to recover lipids (mainly, valuable fatty acids) can be seen on Table 2. There are several important procedures for extracting lipids from microalgae, such as mechanical pressing, milling, homogenization, solvent extraction, ultrasonic-assisted extraction, supercritical fluid extraction, enzymatic extractions, and osmotic shock. Every method has its individual advantages and disadvantages. Pressures from pressing and homogenization processes are aimed to rupture cell walls, so lipids inside the cell can be recovered. Meanwhile, milling uses grinding media (with small beads) and agitation is aimed to disrupt cells.

Those above methods are commonly combined with several solvent extractions. Solvent extraction is used for extracting microalgal lipids by washing microalgal cells repeatedly with an organic solvent. Figure 8 demonstrate schematic diagram of the proposed organic solvent extraction mechanism. Supercritical fluid extraction is conducted utilizing chemical compounds which have properties of both liquids and gases (i.e. CO_2) in increased temperatures and pressures conditions. They act as an extracting solvent. No residues are left behind when the system is set back to atmospheric pressure and room temperature. Enzymes can also be used to hydrolyze cell walls in order to release lipids into an extracting solvent. The use of a combination method with sonication method potentially shows faster extractions and higher yields. The sonication enhances extraction process via cavitation process. The cavitation mechanism can be described as, when ultrasonic waves produce bubbles within the solvent, the bubbles then burst close to the microalgal cell walls. That results shocking waves which trigger the releasing of cells' contents (i.e. lipid) into the solvent (Cravotto *et al*., 2008; Wei *et al*., 2008). In the study of Neto *et al*. (2013), they used microalgal dry biomass (approx. 100mg) to evaluate the lipid extraction methodology regarding a sonication bath as pretreatment cell disruption technique (20 min sonication at room temperature) followed by vortex mixing and n-hexane as solvent (20 mL). The results showed that the

proposed method could obtain a lipid content of 15.5% (% dry weight) for *C. minutissima*, 40.3% for *T. fluviatilis* and 39.5% for *T. pseudonana*.

2) Simultaneous lipid extraction and transesterification

Simultaneous lipid extraction and transesterification is a one-step method. In this method both extraction and transesterification of the algae lipid conducted simultaneously in the reactor. This method not only could decrease the numbers of procedures but also could reduce biodiesel production cost. In contrary, the two-step method of traditional solvent extraction and transesterification of lipid to biodiesel often requires the consumption of a large amount of energy because of algae dewatering and grinding into powder. For biodiesel production from dry algae, algae drying accounts for the majority of the total energy consumption (84%) (Patil *et al.*, 2012). Furthermore, the complex process of the traditional two-step method often requires a total of 0.5–1.5 h for extraction and transesterification.

To address the large energy consumption of microalgae dewatering and to simplify the conventional two-step method for biodiesel production, wet microalgal biomass should be used to produce biodiesel directly. Cheng *et al*. (2013) studied the simultaneous microwave-assisted lipid extraction and transesterification (one step method). Approximatly 1 g wet microalgal biomass (water content, 80 wt.%) was placed in the digestion reactor and mixed with 4 mL of chloroform, 4 mL of methanol, and 0.2 mL of sulfuric acid at temperature of 60 ^{o}C, prior to 20 minutes of reaction time the reaction mixtures were heated via microwave irradiation for 40 s. The biodiesel yield calculated was 10.51%.

Biochemical Characterization of LMBRs

Recent researches in characterizing of LMBRs

Nowadays, researches of microalgae composition after lipid extraction were scarcely reported. Based on our knowledge after studying literatures, it was found that research of LMBRs characterization has been reporting since 2010, and to date totally only 4 reports were found. Here we presented the overview of those recent researches:

1. Goo *et al*. 2013
   - Algal strain:
     - *Dunaliella tertiolecta* (UTEX LB 999), a green halophilic algae that accumulated β-carotene.
   - Algal cultivation:
     - 70 L plate type PBR, 28 days, artificial sea water plus f/2 medium, at 25 ^{o}C, with 0.1 vvm aeration with compressed-air containing 2% CO_2, under 150 μE(m^2s) with fluorescent light irradiation, turn on/off to simulate circadian cycle, without controlling the pH.
   - Harvesting method:
     - Cells were collected from the culture broth by centrifugation at 10,000 rpm for 30 min and washed 10 times volume of distilled water.
   - Pretreatment to cell:
     - Cell was lyophilized
   - Lipid extraction:
     - Lipid were extracted twice with 10 times volume of extracting solvents (chloroform : methanol = 1:2 v/v) from the dried cells. Cells obtained were used as crude defatted biomass (LMBRs).
   - Methods to characterize LMBRs:
     - Extracellular Polysaccharide (EPS) analysis: Dried LMBRs biomass were washed to remove protein from LMBRs by using 0.1 M NaOH solution, to obtain partially pure EPS. Pure EPS then were analyzed for its C, H, N content using Elemental Analyzer
     - Carbohydrate analysis: Total amount of carbohydrate in EPS was determined by a modified phenol-sulfuric acid method.
     - Protein analysis: LMBRs biomass were treated by sonication at 50 ^{o}C for 30 min to disperse all component in distilled water, and concentration of protein was determined by Bradford method

- Ash analysis: EPS was burnt to ash at 700 ^{o}C and is completed when the cool residue was nearly white. The ash quantity was expressed to dry matter.
- Monosaccharide composition analysis: determined by High Performance Anion Exchange Chromatography (HPAEC) of its hydrolysate.
  EPS was resuspended in 1 mL of distilled water and then mixed with equal volume of 4.0 M trifluoroacetic acid (TFA). Samples were stand for 6 h at 100 ^{o}C to allow acid hydrolysis, filtered through 0.45 µm syringe filter, and vacuum dried using a Speed-vac. Residual acid was removed by repeated vacuum drying, and dried sample was analysed on a CarboPacPA-1 column.
- FT-IR Spectroscopy: absorption FT-IR spectra (400 – 4000 cm^{-1}) of EPS and potato amylase were recorded in KBr pellet by FT-IR spectrometer.
- NMR Spectroscopy: the ^{1}H and ^{13}C NMR spectra of purified EPS were recorded on NMR in D_2O solution. Working frequency were 499.8 for ^{1}H and 125.7 for ^{13}C, respectively. Correlation spectroscopy ^{1}H, ^{1}H COSY and ^{1}H, ^{13}C HSQC were applied for signal assignment.
- Dilute acid hydrolysis of EPS:
  For optimization of acidic hydrolysis, various amounts of EPS (0.1-1 g) and 0.5-1.5% sulfuric acid solution (20-100 mL) were mixed at 50-90 ^{o}C up to 24 h. Undigested solids was removed from the solution by centrifugation and kept frozen for analysis and processing. The collected hydrolysates were examined by TLC or HPAEC-PAD as described above. Hydrolysis of EPS was defined as the relative content of glucose in dried hydrolysate.
- Enzymatic hydrolysis of EPS:
  The enzymatic hydrolysis of EPS with an enzyme mixture consisting of a-mylase (EC 3.2.1.1) and a-glucosidase (EC 3.2.1.20) was tested in comparing with artificial substrate pNP-glucoside. The enzyme activity of a-glucosidase towards pNP-glucoside was determined by photometry at 405 nm (absorption of releasing free p-nitrophenol (pNP), molar absorption coefficient 18,400). One unit of a-glucosidase activity was defined as 1 nmol of pNP released per minute. To complete the conversion of EPS to glucose, a-mylase and a-glucosidase (10 U/each) known as an endo- and exo-type enzymes was mixed with defatted dried algal biomass (0.5 g) in 50 mM sodium phosphate buffer (pH 6.8) and incubated at 37 ^{o}C for 0-48 h. The sample was then boiled for 10 min at 90 ^{o}C to quench the reaction. Centrifugation at 9000g for 15 min was performed to separate the solid residue and the supernatant. The release of soluble sugars (basically glucose) formed in preliminary hydrolysis experiments was determined by reducing sugar assay (Somogyi, 1952) and corrected by blank tests on substrate and enzymes. The enzymatic digestibility was defined as a relative amount of EPS digested to glucose.

- <u>The results of LMBRs characterization:</u>
  - The defatted biomass was obtained from the total microalgal biomass with the yield of 69.4%.
  - The significant changes of EPS production were depend on the cell growth rate and amount of biomass.
  - Investigation of cell growth and accumulation of EPS responding to environmental changes is considerable point.
  - The composition of EPS from *Dunaliella tertiolecta.*
  - Single monosaccharide component was observed after dilute acid hydrolysis of EPS (glucose was the major component).
  - The temperature of 80 ^{o}C is more preserving condition for acid hydrolysis. The total glucose of 90% was observed of 80 ^{o}C, 60 min in 1.0 % H_2SO_4.

- Main factors influence hydrolysis: acid concentration, ratio of temperature/acid concentration.
- EPS from *D. salina* hydrolisate were mainly galactose, xylose, and glucose (Mishrav and Jha, 2009)
- *Dunaliella* species and other algae are sensitive to environmental condition supporting photosynthetic assimilation of carbon dioxide and its utilization as energy sources
- Purified EPS was digested by endo-type α-amylase and exo-type α-glucosidase. As the most abundant constituent, EPS can be used as fibers or fermentable sugars (glucose) for bioethanol production
- Removal of impurities such as lipids or proteins is assumed to increase the accessibility and digestibility of EPS and thus lead to greater glucose production.
- EPS was completely converted into glucose by combination treatment with α-amylase and α-glucosidase.
- TLC, HPAEC-PAD analysis of acidic and enzymatic hydrolysates: the EPS isolated from defatted (LMBRs) of *D. Tertiolecta* consist of only glucose units. It should be glucan.
- To obtain more structural information, EPS was analyzed by FT-IR spectroscopy (various functional groups such as amine, amide bonds, aromatics, alkyl, etc.). The EPS spectrum was compared with the spectrumof potato amylase. Higly overlapped IR bands in region of 950-1200 cm^{-1} (CO and CC stretching vibration in carbohydrates) predominated in the both spectra. Analysis of other structurally IR bands of polysaccharide vibration also identified EPS to be amylose-like α-glucan.
- EPS structurally was similar to amylose, but is different from other polysaccharides used in bioethanol production, i.e. heteroxylan from plants (Simas *et al*., 2004), and cyclic (1,2)-β-D-glucan from other microalgae (Suarez *et al*. 2005).
- NMR analysis results to confirm about structure of EPS predicted from FT-IR. It showed that only six signals of 1,4-linked-α-glucopyranose units typical for amylose. Therefore, NMR data confirm that EPS is linear (1,4)-α-D-glucan structurally similar to amylose.
- Conclusively, EPS od *D. tertiolecta* is identified as a homopolysaccharide consisting of glucose, promising candidate for industrial exploitation for the source of biorefinery.

2. Lee *et al*. 2013
   - Algal strain:
     - *Dunaliella tertiolecta* LB999
   - Algal cultivation:
     - *D. tertiolecta* was cultured in a 70L plate type PBR (photoautotrophic culture) with fluorescent lighting 60 $\mu E/m^2s$) at 20-25 ^oC for seven days. The culture was bubbled with air containing 2% (v/v) CO_2 (2 vvm; volume of air added to the liquid volume air per minute). The initial biomass concentration was 0.3 g/L.
   - Harvesting method:
     - The cells were harvested after seven-days cultured by centrifugation. The biomass concentration was 9.1 g fresh cell/L.
   - Pretreatment to the cells:
     - Lyophilized
   - Lipids extraction:
     - The total lipids were extracted twice from the freeze-dried cells. Fifteen volumes of chloroform and methanol (1:2 (v/v)) were added to the freeze-dried biomass, and the lipid was extracted with magnetic stirring and reflux at 65 ^oC for 2 h or at room temperature for overnight. After lipid extraction, the residual biomass was dried at 42 ^oC for 2 h or at room temperature for overnight. The dried LMBRs then was ground into powder using pestle and mortar. Approximately 80% of the residual biomass powder had diameter from 75 to 300 µm.
   - Saccharification:

- For chemical saccharification: 5% (w/v) of the residual biomass was autoclaved at 121 ^{o}C for 15 min in the presence of HCl (0.1 - 1 M) or H_2SO_4 (0.05 - 0.5 M).
- For enzymatic saccharification: Enzymatic saccharification was performed with 5% (w/v) of the residual biomass (LMBRs) at various temperatures (35 – 55 ^{o}C) and pH (3.5–6.5). The reaction mixture was incubated with commercial cellulase, amyloglucosidase, and/or Viscozyme L (Viscozyme L is a multi-enzyme complex containing arabanase, cellulose, β-glucanase, hemicellulase, and xylanase.
- For the chemo-enzymatic saccharifi-cation, acid-generated hydrolysates were adjusted to a pH of 5.5 with 0.1 M sodium acetate buffer. Commercial cellulose, Viscozyme L, or amyloglucosidase were added for enzymatic saccharification. The samples were then centrifuged at 7000 rpm and 4 ^{o}C for 10 min before analysis of the reducing sugars.

- Method to characterize the LMBRs:
  - Total reducing sugar analysis: the total reducing sugar in the saccharification products of the LMBRs was determined by DNS method. Reducing sugar concentration and composition were also determined by HPLC
  - Saccharification efficiency is calculated as follows: saccharification efficiency (%) = (reducing sugars determined by DNS or HPLC/residual biomass (mg)) x 100.
- The results of LMBRs characterization:
  1. Chemical saccharification
     - After harvesting, the composition of algal biomass was determined. Composition of whole biomass of *D. tertiolecta* are carbohydrate (37.8 %), lipid (20.6 %), protein (25.5 % w/w), ash (9.6 % w/w), and moisture (6.5 %).
     - 48 g lipid and 172 g LMBRs were obtained. It indicated that lipids were nearly completely extracted by methanol and chloroform.
     - The carbohydrate content in LMBRs was approximately 51.9% w/w.
     - Effects of sulfuric acid and hydrochloric acid concentration on the saccharification yield.
     - The amount of reducing sugar was approximately 14.8 mg/mL, represented as the D-glucose equivalent, when 5% (w/v) of the residual biomass was hydrolyzed by 0.5 M HCl with autoclaving at 121_C for 15 min. A saccharification yield of 29.5% (w/w, based on the residual biomass) was obtained. The yield based on the total carbohydrate amount of the residual biomass after lipid extraction was 56.7% (w/w).
  2. Enzymatic saccharification
     - The results from enzymatic saccharification of LMBRs was summarized in table 6.
  3. Chemical and enzymatic saccharification
     - The overall efficiency of this saccharification method was nearly the same as enzymatic saccharification method.
- Conclusion:
  - The saccharification of microalgal residual biomass was successfully performed. AMG 300L-catalyzed saccharification produced 1.0 mg/mL of reducing sugar with 42.0% yield based on the residual biomass mass. The saccharification yield based on the total amount of carbohydrates of the residual biomass was 80.9% (w/w).

3. Ju *et al*. (2012)
   - Algal strain:
     - *Haematococcus pluvialis*
   - Algal cultivation:
     - no data
   - Harvesting method:
     - no data
   - Lipid extraction:
     - super-critical CO_2 for astaxanthin

- DMM/LMBRs characterization:
  - Proximate composition was analyzed following the methods by AOAC (2000).
  - Moisturewas determined by drying a 2 g of representative sample in an oven with air circulation at 105 °C for 16–24 h.
  - Crude protein (CP) was determined from total nitrogen by a LECO's Nitrogen/Protein Determinator using a multiplication factor of 6.25.
  - Total crude lipid (CL) was determined by ethyl-ether extraction using an Accelerated Solvent Extractor.
  - Ash was determined by incineration of a representative 0.5 g sample in an oven at 550 °C for 6 h.
  - Mineral content was analyzed by an inductively coupled plasma atomic emission spectroscopy.
  - Gross energy was determined using bomb calorimetry
  - Amino acid (AA) profiles of the sampleswere analyzed using an Agilent 1200 HPLC

- Results:
- Conclusion: the DMM/LMBRs can be utilized as a valuable alternative protein and natural pigment sources for shrimp culture
- More study: DMM effect on shrimp palatability and digestability

4. Yang *et al*. (2010)
   They informed LMBRs composition.
   - Algal strain:
     - *Scenedesmus* sp.
   - LMBRs:
     - from oil extraction process (no data for method of lipid extraction)
   - LMBRs composition:
     - protein (32.4%), carbohydrate (24.7%), lipid (6.5%), and ash (10%).

CONCLUSIONS

It has been being a need to study or to characterize LMBRs for its future utilization. Based on this review, for the future perspective of LMBRs characterization, it would be better to be more focused on pigment characterization as well as pigment's isolation method from LMBRs, while optimizing sugars and amino acids production from LMBRs should be studied too. It was because producing natural pigment needs fewer steps or fewer reactions started from LMBRs than producing fermented products from LMBRs. Moreover, for sugars utilization from LMBRs, one factor that should be consider is the algal cells have already experienced many chemical and/or enzymatic reactions as well as mechanical treatments prior to be the LMBRs. So the LMBRs have already become very tough materials.

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