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Article

Utilization of Lipid-Extracted Algae Cell Residue in Cultivation of *Chlorella vulgaris* for Biodiesel Production

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Abstract

Lipid-extracted algae (LEA) cell residue is generated as an organic solid waste in the process of biodiesel production from microalgae, and its recycling or reuse is important in the aspect of waste minimization. In this study, the influence of the addition of LEA hydrolysate to *Chlorella vulgaris* (*C. vulgaris*) cultivation on cell growth and biodiesel production was investigated to seek a possible use of LEA as a carbon source. LEA was hydrolyzed by three different methods: acid hydrolysis, autoclave, and ultra-sonication. The resulting hydrolysates were supplemented with three background media: formulated defined medium (BG-11), organic liquid fertilizer (PAL1), and distilled water. Both cell growth and lipid production of *C. vulgaris* were improved under mixotrophic cultivation. By supplementing hydrolysates, biomass productivity was increased several folds because the LEA hydrolysates contained monosaccharides such as glucose and galactose. Lipid contents and biodiesel productivity were bestly increased in PAL1 medium supplemented with ultrasonication (UL) hydrolysate, from 11 to 25% after 14 days, while nitrate concentration was quickly reduced from 55 to below 10 mg/L. The suggested recycling option of the LEA to microalgae cultivation was helpful to improve biodiesel productivity as well as to reduce organic waste generation.

Keywords: *Microalgae, Chlorella vulgaris, Biodiesel production, Lipid-extracted algae cell residues, Hydrolysis*

1. Introduction

Biodiesel is an alternative renewable energy source to replace fossil fuels which cause the increase of atmospheric greenhouse gases and climate change. Because most of other renewable energy are focused to produce electric power, biodiesel is considered unique in that it can directly substitute liquid form of fossil fuel especially in transportation sector. One of the popular routines to produce biodiesel is utilizing vegetable oils from crops. However, crop-originated biodiesel demands a competition between consumption for fuel and food, and thus is not sustainable way of replacing fossil fuels since it disturbs worldwide prices of food, feed and meat markets[1]. Among other feedstock for biodiesel production, microalgae are a good candidate because they are mostly non-edible and require short cultivation time and no arable land compared to terrestrial energy crops[2].

Microalgae are photosynthetic microorganisms capable of converting CO_2 into organic macromolecules such as lipids, polysaccharides and proteins, under light conditions. Although carbon fixation occurs inherently under autotrophic condition, many microalgal species can also grow in mixotrophic or heterotrophic condition too. Some of them show faster growth than in autotrophic condition when the CO_2 and organic carbon sources such as glucose are simultaneously provided under light[3,4]. However, supplying refined organic substrates is not economically feasible for the production of a commodity fuel like biodiesel even though the biomass and lipid productivities of mixotrophic cultivation are higher than those of autotrophic one.

Triacylglycerol as the source of biodiesel is usually recovered from neutral lipids which is accumulated in microalgae cells, and the biodiesel is obtained after extraction of lipids and transesterification using primary alcohols[5]. Cell disruption is usually accompanied depending upon the methods of extraction and transesterification. If methanol is used in the reaction, fatty acid methyl esters (FAMEs) are produced as biodiesel[6]. After the solvent extraction, lipid-extracted algae (LEA) cell residue remains in the extraction unit and is considered as a solid organic waste which requires an appropriate disposal or recycling[7,8]. It is known that the mass of the resulting LEA after lipid extraction is about 80~85% of original algal biomass[7].

In this study, we focused the fact that the major constituents in LEA are from cell wall, membrane-based carbohydrates and proteins and that some microalgae can grow mixotrophically utilizing organic carbons as well as inorganic carbon like CO_2 under light[9,10]. We estimated the possibility of utilizing LEA as a potential carbon source for microalgae growth and biodiesel production. LEA cell residues were recovered after lipid extraction and then hydrolysis was carried out to degrade the polymeric carbohydrates into low molecular weight

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monomers. Utilizing the hydrolysates in the culture of *Chlorella vulgaris* (*C. vulgaris*), its influence on cell growth and lipid accumulation was investigated to find the feasibility of LEA in enhancing biodiesel productivity and in reducing the burden of organic waste treatment and disposal.

2. Materials and methods

2.1. Microalgae and Chemicals

Chlorella vulgaris AG-30007 was obtained from Korea Biological Resource Center (Daejeon, Korea), was routinely maintained in BG-11 medium at 25 ± 1 °C and 100 μmol/m^2s light intensity under 12:12 h L/D cycle for preparing inoculum. BG-11 is a popularly-used medium designed for the autotrophic culture of microalgae and cyanobacteria, which includes high levels of nitrates and phosphates, six trace metals and EDTA, but negligible amount of inorganic carbon (20~22 mg/L as TOC) and almost no organic carbon[11]. *Chlorella species* is known to have rigid wall components embedded within a more plastic polymeric matrix[12].

The organic liquid fertilizer PAL1, which is used to a background medium for mixotrophic culture, was supplied by Syaron Co., Korea. The characteristics of this fertilizer are described previously[13]: pH in the range of 8.1~8.5, 630~635 mg/L of COD, 228~232 mg/L of C as TOC, 55~60 mg/L of NO_3-N and 27~30 mg/L of PO_4-P.

Sulfuric acid (H_2SO_4, 95%, Samchun) was used in one of cell hydrolysis methods and sodium hydroxide (97%, Daejung) was used to neutralize the solutions after hydrolysis. n-Hexane (97%, Sigma-Aldrich), chloroform (99%, Showa), methanol (99.8%, Showa) and hydrochloric acid (35~37%, Junsei) were used as the solvents for transesterification process to produce biodiesel from extracted triacylglycerol.

2.2. Lipid extraction

Lipid was extracted from algal biomass using a ternary system with chloroform/methanol/water, based on the method of Bligh and Dyer [14] with some modification. 30 g of dry sample was mixed with a 7.6 mL of chloroform/methanol/water (1/2/0.8, v/v/v), and the mixture was sonicated for 1 min at 100 W and 20 kHz. Then 2 mL of chloroform and 2 mL of water were added, and the mixture was sonicated further for 1 min and centrifuged at 3000 rpm for 5 min to form a two phase system. The chloroform phase was transferred into a pre-weighed vial and was evaporated in a drying oven at 80 °C for 30 min. Then, the amount of lipid obtained from each sample was measured gravimetrically.

After the lipid extraction and transferring the organic solvent phase to lipid analysis, the remaining aqueous phase was recovered to collect LEA cell residue through centrifugation and drying. LEA fractions were used to supplement to the culture and to hydrolyze for the analysis of consisting monosaccharides.

2.3. Hydrolysis

The recovered LEA cell residue was hydrolyzed by three different methods: (i) acid hydrolysis, (ii) autoclaving, and (iii) ultra-sonication. Acid hydrolysis process was carried out based upon Cheshire and Mundie[15] with some modification. Dried LEA sample was treated first with 10 M H_2SO_4 at ambient temperature to solubilize carbohydrate fraction and then subsequently with 0.5 M H_2SO_4 under boiling to convert the solubilized fraction to monomeric forms. Autoclaving hydrolysis was performed at 150 °C for 40 min. For ultrasonic hydrolysis, the Power Sonic 520 Ultrasound Bath (Hwashin, Korea) was used at a resonance of 40 kHz for 40 min at ambient temperature. All hydrolysis procedures were carried out with 3 g of dried LEA in 30 mL of sulfuric acid or distilled water.

After hydrolysis, the resulting hydrolysate liquor was recovered by centrifugation followed by filtration, and then was used in sugar analysis or in microalgae cultivation. The composition of monosaccharides in hydrolyzed LEA liquor were analyzed by HPLC (Agilent 1260 Infinity) with Aminex HPX-87H (Bio-Rad) ion exclusion column, employing 5 N H_2SO_4 mobile phase and 30 °C column temperature.

2.4. Cultivation

The microalgae cultivation was performed in 1 L bubble-column type photobioreactors including 500 mL working volume of medium. The cells in photobioreactors were suspended with fresh air supplying from the bottom. Photobioreactors were maintained at 20~25 °C and illuminated continuously with white Kumho FL20SD/18 20 W fluorescent (light density of 100 μmol/m^2s). The background culture medium was either one of BG-11, liquid organic fertilizer (PAL1), or distilled water (DW) as a reference. All culture media were autoclaved at 121 °C for a 15~20 min prior to inoculation. Right after start-up of cultivation, the 30 mL of hydrolysate liquor (obtained from 3 g of dried LEA) was added to the culture. Cell suspension and mixing was maintained by bubbling fresh air without pure CO_2 supply and therefore a small amount of atmospheric CO_2 was introduced to the culture, which made it mixotrophic environment.

As mentioned earlier, PAL1 contains about 228~232 mg/L of non-glucose organics as COD and BG-11 contains 20~22 mg/L of inorganic carbon as TOC[13]. The composition of dried LEA from *C. vulgaris* is in the range of 34~46% proteins and 13~18% carbohydrates [16]. Therefore, the amount of carbohydrates added to the reactor through hydrolysate corresponded to 930 mg/L as a median value of the carbohydrate range.

2.5. Transesterification and fatty acid composition

Dried biomass was mixed with 0.2 mL of chloroform and methanol (2:1, v/v) and 0.3 mL of 0.6 M HCl in methanol, in a glass vial which was sealed using PTFE screw cap. The microalgal lipid was transesterified at 85 °C for 1 h in a drying oven. After transesterification, samples were cooled for 15 min at room temperature and 1 mL of n-hexane was added and vortexed. After vials were left undisturbed for 1 h at room temperature to allow phase separation, the hexane layer was transferred to GC vials to analyze the composition of the resulting fatty acid methyl esters (FAME). GC-FID (YL6500 GC, Younglin Instrument Co., Korea) was used with HP-INNOWAX capillary column (Agilent 19091N-213), in which helium was used as a carrier gas.

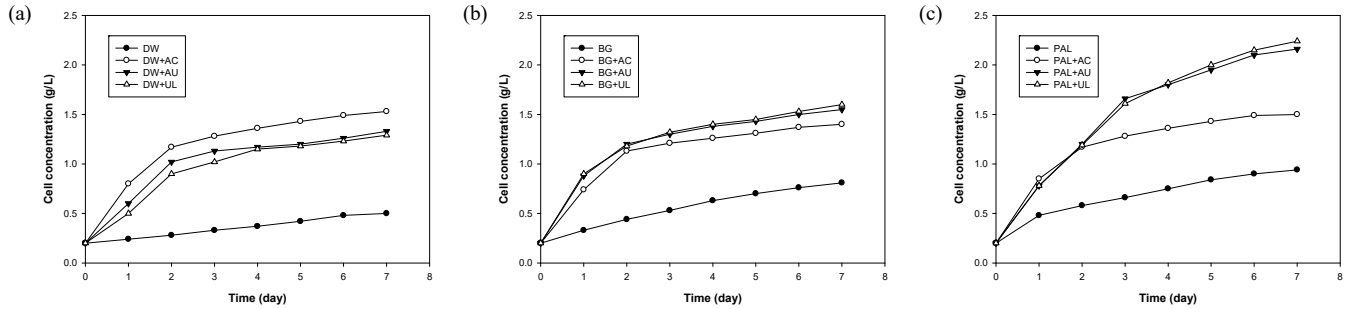

Figure 1. Effects of hydrolyzed LEA addition on cell concentration of *C. vulgaris* growing in (a) DW, (b) BG-11 and (c) PAL1. AC, acid hydrolysate; AU, autoclave hydrolysate; UL, ultrasonic hydrolysate.

Table 1. The Mean Values of Biomass Productivity (g/L/d) and Specific Growth Rate (d^{-1}) for 7 Days

Medium	Without hydrolysate		+ Acid-hydrolysate		+ Autoclave-hydrolysate		+ Ultrasonic-hydrolysate	
	Biomass productivity	Specific growth rate	Biomass productivity	Specific growth rate	Biomass productivity	Specific growth rate	Biomass productivity	Specific growth rate
DW	0.04	0.13	0.17	0.28	0.16	0.27	0.17	0.28
BG-11	0.09	0.20	0.18	0.29	0.20	0.29	0.21	0.30
PAL1	0.11	0.22	0.19	0.29	0.28	0.34	0.30	0.36

Productivity (Px) = $\Delta X/\Delta t$; Specific growth rate (μ) = $\frac{1}{X}\frac{dX}{dt}$, where X is cell concentration (g/L).

2.6. Analysis methods

The dry cell weight (g/L) was measured gravimetrically. Microalgae cells were filtered by a sterile membrane filter (0.45 μm pore size, 47 mm in diameter, Whatman, UK), which was pre-dried and pre-weighed. After filtration, the cells on the membrane surface were dried at 80 °C for 12 h and were kept in the desiccator until the weight was invariant. Active growth of microalgae cells accompanies nitrate consumption which was determined by the APHA standard method[17]. using UV spectrophotometer (Hach DR4000/U).

3. Results and discussion

3.1. Effects of LEA hydrolysate on cell growth

Figure 1 shows the effects of the addition of hydrolysates on the cell growth in three different background media (DW, BG-11 and PAL1). The current *Chlorella* strain grew faster in PAL1 than in BG-11 and DW, and this trend was also maintained when the LEA hydrolysate was added. It is known according to previous study[18] that the current *C. vulgaris* strain can grow mixotrophically or sometimes heterotrophically depending upon culture condition. The highest cell growth in PAL1 experiments (Figure 1b) can be explained by the fact that the amount of organic carbons is larger than that in BG-11 and DW (Figure 1a and 1c) because organic carbons are present in both LEA hydrolysates and organic fertilizer. BG-11 is a chemically-defined medium for the culture of microalgae and cyanobacteria including nitrogen, phosphate and some minerals without any carbon source[11].

It can be seen clearly that the addition of hydrolysates affects cell growth positively regardless of the employed background media and hydrolysis methods. The cell concentrations in DW, BG-11 and PAL1 after 7 days without hydrolysate were 0.49, 0.81, and 0.94 mg/L, respectively, while they were increased up to 1.53, 1.60, and 2.74 mg/L in the presence of hydrolysate. Growth enhancement was highest in PAL1 compared to BG11 and DW overall.

When autoclave (AU) hydrolysate was added, cell concentration of *C. vulgaris* increased steadily and reached 1.33, 1.55 and 2.16 g/L after 7 days in DW, BG-11 and PAL1, respectively. Next, the addition of ultrasonic (UL) hydrolysate into DW, BG-11 and PAL1 resulted in reaching 1.29, 1.60 and 2.74 g/L, respectively. The biomass concentration went up to 1.52, 1.42 and 1.50 g/L, respectively, when the acid hydrolysis (AC) hydrolysate was added.

Table 1 compares the mean values of biomass productivity (g/L/d) and specific growth rate (d^{-1}) of *C. vulgaris* in three types of culture medium, with and without the supplement of LEA hydrolysate. The cultures with hydrolysate supplement showed a significantly improved performance in growth, compared to the culture in original background medium alone without hydrolysate. The organic fertilizer (PAL1) was superior to the chemically-defined BG-11 as a background medium in the presence of LEA hydrolysates, and the extent of growth enhancement was best in the addition of UL hydrolysate compared to AC and AU.

3.2. Monosaccharide composition in hydrolysates

The reason for the enhancement of growth rate and biomass productivity in the presence of LEA hydrolysates can be explained by the results of sugar analysis in hydrolysates. Figure 2 shows the monosaccharide compositions in LEA after three different hydrolysis methods. According to the HPLC analysis for the consisting monosaccharides in hydrolysates, the major monosaccharides from the current *C. vulgaris* LEA were glucose and galactose, which are two most easily bio-digestible monosaccharides for the culture of many kinds of

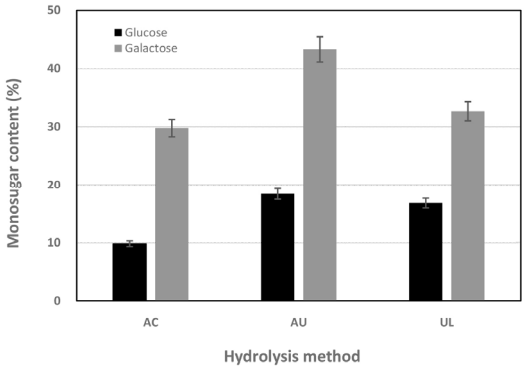

Figure 2. Monosaccharide content in LEA after three hydrolysis methods. AC, acid hydrolysis; AU, autoclave hydrolysis; UL, ultra-sonicaiton hydrolysis.

microorganism. The content of galactose was 29~44%, which was always higher than that of glucose in the range of 9~18%. The rest of the contents excluding glucose and galactose in the HPLC analysis is the mixture of several disaccharides and organic acids produced from hydrolysis procedure. The resulting amount of monosaccharides (sum of glucose and galactose) was in the order of AU > UL > AC. Glucose content was also highest in AU hydrolysate as 18.7%, while 16.8% in UL and 9.8% in AC hydrolysate.

Since the LEA from *C. vulgaris* is composed of 34~46% proteins and 13~18% carbohydrates[16], the amount of carbohydrates added to the reactor through hydrolysate corresponded to 930 mg/L as a median value of the carbohydrate range. According to Figure 2, the glucose contents in hydrolysates were 9.8, 18.7, and 16.8% in AC, AU, and UL, respectively, and thus the actual concentrations of glucose supplied by the hydrolysate addition in Figure 1 were estimated approximately as 91.1, 173.9, and 156.2 mg/L in the cases of AC, AU, and UL, respectively. In the other hands, galactose concentrations added were 227, 401.8, and 304.1 mg/L in the case of AC, AU, and UL (Figure 2), because the galactose contents were 29.8, 43.2 and 32.7%, respectively.

Although acid hydrolysis using sulfuric acid is usually known as a stronger hydrolysis method than physical hydrolysis methods such as autoclave or sonication due to its reaction nature as oxidative degradation[19], Figure 2 shows that the contents of both glucose and galactose after AU and UL were higher than those after AC. The possible reason for the lowered monosugar contents in AC than in AU or UL is because some monosugars can be converted to ring-opened by-products such as levulinic acid during the hydrolysis by strong acid like sulfuric acid[20]. Physical hydrolysis methods such as autoclave or ultra-sonication are more effective than acid hydrolysis in that they generate higher amount of intact monosaccharides (sum of glucose and galactose) as general organic carbon sources for microorganisms cultivation.

However, it is also known that galactose is difficult for most Chlorophyta to directly utilize in heterotrophic or mixotrophic culture [21]. The rate of galactose utilization of many microalgae species including *Chlorella* is quite low compared to the utilization of glucose, and the existence of galactose is sometimes inhibitory to glucose uptake and resulting cell growth[22]. Although AU resulted in higher amount of monosugar overall than UL, we chose UL as a subsequent hydrolysis method for biodiesel production experiments because glucose contents are not significantly different between AU and UL (Figure 2) while UL contained less amount of galactose and showed higher biomass productivity and growth rate than AU (Table 1), especially in the PAL1 medium.

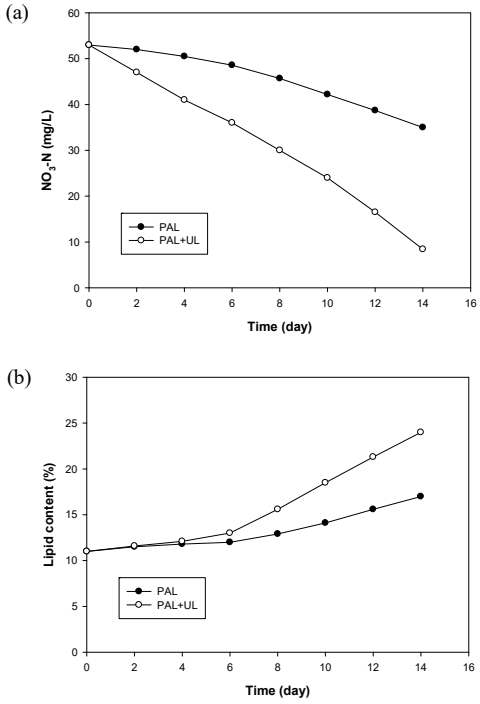

Figure 3. Nitrate consumption (a) and lipid accumulation (b) of *C. vulgaris* in original PAL1 and in PAL1 supplemented with ultra-sonication hydrolysate (PAL+UL).

3.3. Effects of hydrolysates on lipid accumulation

In order to investigate the effect of hydrolysate addition on lipid production, the cultivation of *C. vulgaris* was carried out in organic fertilizer (PAL1) with and without the addition of UL hydrolysate. The concentration of glucose to be added to the cuture is estimated approximately as 156.2 mg/L because the glucose content in UL hydrolysate was 16.8% in Figure 2.

Figure 3 shows nitrate consumption and lipid accumulation of *C. vulgaris* in original PAL1 medium and new PAL1 medium in which ultrasonic (UL) hydrolysate was added (PAL+UL). Nitrate level is important in biodiesel production since the synthesis of neutral lipid is triggered when the nitrate level drops below a certain level in many microalgae species[6,23]. It is clearly observed in Figure 3a that the NO_3-N was consumed more quickly (3.2 mg/L/d of N consumption rate) in PAL+UL than in original PAL1 (1.3 mg/L/d of N consumption rate). Nitrate concentration in PAL+UL was lowered to below 30 mg/L after 6 days and 10 mg/L after 13 days. Nitrogen is the most essential

nutrient for algal growth, because it is a component of all structural and functional proteins, chlorophylls, energy transfer molecules and genetic materials in algal cells[24,25]. Microalgae are able to utilize various forms of nitrogen, including nitrate, ammonium and organic nitrogen such as urea. Fast growing and actively metabolizing microalgae cells consume nitrogen quickly, and therefore cell growth rate and nitrogen consumption rate are approximately proportional[18,26].

For the purpose of biodiesel production using cellular neutral lipids (triacylglycerides), the changes in total neutral lipid content were monitored as shown in Figure 3b. By comparing Figures 3a and 3b, lipid synthesis was indeed induced by nitrate starvation from about 11% at the beginning. The lipid content in PAL+UL started to increase after 6 days when nitrate concentration became below 30 mg/L and reached up to 25% after 14 days at which nitrate concentration was below 8 mg/L. The lipid content of *C. vulgaris* in original PAL1 was not changed much for a loner time, and it went up slowly to 16% after 12 days. Here the % of lipid content is based upon the dried cell mass.

It is known that some microalgae species including *C. vulgaris* can grow even in nitrogen deficient conditions by utilizing their intracellular nitrogen reserves such as pigment protein molecules[23,27]. Microalgae cells firstly synthesize protein and carbohydrates to store energy in nutrient-rich condition, and then initiate accumulating lipids to prepare for possible environment stress such as nutrient (N and/or P) starvation[10,26]. Under N limitation, metabolic pathway of carbon assimilation diverts from protein synthesis to lipid or carbohydrate production as carbon and energy storage[28,29]. The results in Figure 3 demonstrated that the addition of LEA hydrolysate enhanced lipid accumulation through the quick consumption of nitrate. It is concluded that the additional supply of assimilable carbohydrate (glucose in UL hydrolysate) to organic fertilized medium (PAL1) was advantageous to improve biodiesel production performance.

3.4. Fatty acids composition in biodiesel

Fatty acid methyl ester (FAME) is the principal component of biodiesel and the composition of fatty acids in FAME plays a critical role in the properties of biodiesel as a fuel. Table 2 shows the compositions of fatty acids in produced biodiesel after transesterification of extracted lipids, derived from the *C. vulgaris* biomass which was cultivated in original PAL1 and PAL+UL. The most abundant fatty acid was C18:1 (oleic acid) in common, and the next main fatty acids were C20:0 (arachidic acid) in original PAL1, while C16:0 (palmitic acid) and C18:3 (*a*-linolenic acid) in PAL+UL. Compared with the original PAL1 medium, the fraction of total saturated fatty acids (SFA) was decreased, while the fraction of total unsaturated fatty acids (UFA) was greatly increased from 56.2% to 73.7% in PAL+UL. It is noted that the fraction of polyunsaturated fatty acids (PUFA) is almost tripled in the culture with PAL+UL employing LEA hydrolysate.

Table 2 shows that the produced biodiesel from the original PAL medium contained a high level of MUFA like oleic acid (C18:1) and long-chain SFA like arachidic acid (C20:0). Many microalgal species including *Chlorella* contain a substantial amount of UFAs such as oleic acid, and it is known that the fraction of longer chained fatty acids such as arachidic acid among SFAs tends to increase when inorganic carbon supply is insufficient[30,31]. The results in Table 2 implies that PAL+UL favored the synthesis of fatty acids with shorter chain length (C18 and C16) and with increased unsaturation (C18:1, 18:2, 18:3). The increase in UFA would improve the cold flow property of the biodiesel fuel in a cold weather, while reducing the oxidative stability. Excessively large amount of PUFA is not desirable as a fuel because it sacrifices oxidative stability of the fuel and is vulnerable to free radical attack. Those problems can be solved by blending with petroleum diesel, or by utilizing various additives such as antioxidants or cold flow improver[32,33].

Table 2. Fatty Acid Profile of *C. vulgaris* Cultivated in Original PAL1 and New PAL+UL which is Supplemented with Ultrasonication (UL) Hydrolysate

FAME (%)	Original PAL1	PAL+UL
C10:0	1.5	0.3
C16:0	2.3	13.0
C16:1	10.3	8.0
C18:0	-	6.3
C18:1	38.4	43.7
C18:2	3.5	9.6
C18:3	-	12.4
C20:0	33.1	1.5
C22:0	6.9	2.1
Others	4.0	3.1
SFA	39.8	21.5
UFA	56.2	73.7
MUFA	48.7	51.7
PUFA	7.5	22.0

SFA, saturated fatty acids; UFS, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

The results of Table 2 imply that the use of LEA hydrolysate UL as a carbon source together with organic fertilizer PAL1 greatly influence the produced fatty acid composition which can affect biodiesel quality. Nevertheless it can be concluded that supplementing LEA hydrolysate to the culture of microalgae in organic fertilizer medium enhanced both cell growth rate and lipid content, which lead to an increased biodiesel productivity.

4. Conclusions

LEA is a byproduct which is produced inevitably in the process of biodiesel production from microalgal biomass. If there is no appropriate recycle method, it is considered as an organic solid waste that is supposed to dewater and then dispose through landfill or incineration. Otherwise, it can be added to undergoing anaerobic digestion process to reduce organic volume and possibly to produce methane. None of these options efficiently utilizes the remaining monosaccharides in LEA for the constructive purposes. The results in this

study demonstrated that it is feasible to recycle the hydrolysates of LEA to microalgae cultivation again to improve cell growth and biodiesel production. Such recycling option of LEA into microalgae culture can contribute to the minimization of waste generation in microalgal biodiesel production and will further drive the microalgae cultivation system towards a more sustainable direction.

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