Bioresource Technology 302 (2020) 122809

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Biocomponent-based microalgal transformations into biofuels during the pretreatment and fermentation process

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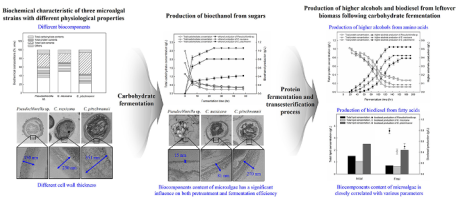
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Microalgae
Cell wall disruption
Biochemical constituents
Fermentation
Biofuels
Energy recovery

ABSTRACT

Microalgal cell wall integrity and composition have a significant impact on the fermentation process and biofuel recovery. In this study, various biofuels (bioethanol, higher alcohols (C3-C5), and biodiesel) were produced by the fermentation of carbohydrates and proteins, and transesterification of lipids from three different microalgal strains (*Pseudochlorella* sp., *Chlamydomonas mexicana*, and *Chlamydomonas pitschmannii*), each possessing different proportions of bioconstituents (carbohydrates, proteins, and lipids). Changes in the cell wall structure and thickness were observed before and after fermentation using transmission electron microscopy. *Pseudochlorella* sp. showed the highest yields of bioethanol (0.45 g-ethanol/g-carbohydrates), higher alcohols (0.44 g-higher alcohols/g-proteins), and biodiesel (0.55 g-biodiesel/g-lipids), which consequently revealed a maximum energy recovery (42%) from whole constituents. This study suggests that different physiological properties, including cell wall thickness and the proportion of bioconstituents in microalgae, could have a significant impact on the pretreatment and fermentation efficiencies for biofuels production.

1. Introduction

The use of fossil-based fuels and increasing energy demands have resulted in the increasing emission of carbon dioxide (CO_2), which has consequences for global climate change, sea-levels rise. These factors are driving the search for alternative sustainable energy solutions (Blifernez-Klassen et al., 2012; Georgianna and Mayfield, 2012; Salama et al., 2017). Microalgae have been considered as one of the most promising feedstock for the production of biofuels (bioethanol, higher alcohols (C3-C5), and biodiesel) that can replace fossil fuels due to their high growth rate and high biochemical compositions (Goh et al., 2019; Huo et al., 2011). Microalgal biomass serve as potential substrate for the production of biofuels because it possesses high biochemical composition (approximately 90% organic matter) rich in carbohydrates,

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https://doi.org/10.1016/j.biortech.2020.122809
Received 11 November 2019; Received in revised form 10 January 2020; Accepted 11 January 2020
Available online 15 January 2020

proteins and lipids (El-Dalatony et al., 2017; Phwan et al., 2018). Carbohydrates from microalgae can be used to produce bioethanol, an environmentally-friendly, commercially viable liquid fuel through yeast fermentation (Sanchez Rizza et al., 2017; Zabed et al., 2017). The protein and lipid components from microalgae can be converted to higher alcohols containing more than two carbons, and biodiesel via the Ehrlich pathway of fermentative microbes and transesterification, respectively (El-Dalatony et al., 2019a; El-Dalatony et al., 2019b; Goh et al., 2019).

Pretreatment is a crucial step to extract the major components (carbohydrate, protein, and lipid) from microalgae which can be converted into economically commercialize biofuels (Abbassi et al., 2014; Chng et al., 2017). However, the bioaccessibility of the fermentative microorganisms utilizing these intercellular compounds in biomass is a major challenge due to the tough interlinking biopolymers structure of microalgae cell walls with high tensile strength (Gunerken et al., 2015; Velazquez-Lucio et al., 2018). Biological pretreatment, including the application of microbial fermentation among various pretreatment methods, is an eco-friendly approach to accelerate substrate hydrolysis, reduce energy consumption and downstream costs (El-Dalatony et al., 2017; Lai et al., 2016a; Zabed et al., 2019). A previous study has investigated the production of biofuels through successive fermentations as a cost-effective bio-pretreatment capable of co-extracting protein components from *Chlamydomonas mexicana* (El-Dalatony et al., 2019b).

Physiological properties of microalgae including cell constituents, cell wall thickness, cell size and density, and cell morphology all have an important effect on disruption efficiency during biofuels production (Baroni et al., 2019; Yap et al., 2016). The ability of microalgae cells to resist biological rupture has been linked to the constituents of the cell and the thickness of the cell wall (Aligata et al., 2018; Middelberg, 1995). The presence of large quantities of carbohydrates or lipids in microalgae cells has been reported to reduce extraction efficiency, which subsequently affect biofuels yield (Wijesinghe and Jeon, 2012; Yap et al., 2016). In another detailed study, they reported reduced digestibility of *Daphnia* that swallowed microalgae with thick and rigid cell walls (Donk and Lurling, 1997). However, most of the studies have been limited for observing the physiological properties of cultured microalgae. The effects of different biocomponents on biofuels production in relation to cell disruption, such as changes in cell wall thickness, have not been well-investigated. Although major effort has been made to optimize the key parameters of both the pretreatment and fermentation processes (El-Dalatony et al., 2016; Eldalatony et al., 2016), the influence of the microalgae biocomponents content on the production of biofuels during fermentation is still needs further investigation.

This study is the first report to explain how internal cell structure and cell wall thickness with different biocompositions in microalgal strains effect on biofuels (bioethanol, higher alcohols, and biodiesel) production. The type of pretreatment and its feasibility are important factors to decrease overall energy demands and reduce biomass processing costs through better investigation and understanding in biofuels production studies using microalgae strains with different physiological properties for improving bioaccessibility and bioavailability of microorganisms during fermentation. Furthermore, the release of soluble proteins as a nitrogen source during carbohydrate fermentation was measured. In addition, the use of these proteins to produced higher alcohols production was also evaluated during protein fermentation. The mass balance was calculated during the overall biofuels production process, for all microalgal strains.

2. Materials and methods

2.1. Microalgae biomass preparation and characterization

The three microalgal strains (*Pseudochlorella* sp. GU732422, *Chlamydomonas mexicana* GU732420, and *Chlamydomonas pitschmannii* GU732416) used in this study were isolated from the effluent of a municipal wastewater treatment plant (Wonju Water Supply and Drainage Center, Republic of Korea). The microalgae strains were cultivated in 5 L Erlenmeyer flasks containing 2 L Bold's Basal Medium (BBM) at a concentration of 10% ($V_{inoculum}/V_{media}$) and then placed on a magnetic stir plate at 150 rpm, 27 ℃ under white fluorescent light with an illumination of 60 μmol/m^2/s. Air was sparged at a flow rate of 0.3 vvm using a pump (Toshipump Co., Ltd, Japan) for two weeks (Eldalatony et al., 2016). The dry cell weight and specific growth rates were measured using the method described in a previous study (El-Dalatony et al., 2016). The carbohydrate, protein, and lipid content of the microalgal strains were determined using the phenol–sulfuric acid, Lowry, and Bligh and Dyer method, respectively (Dubois et al., 1956; Lowry et al., 1951; Bligh and Dyer, 1959). The total chlorophyll and carotenoid content was measured using a previously reported method (Porra et al., 1989). Experiments were performed in triplicate and data are expressed as mean ± SD.

2.2. Pretreatment and carbohydrate fermentation processes of microalgal biomass

The microalgal strains were harvested at the end of the exponential phase by centrifugation at 4000 rpm for 15 min (Combi R515, Hanil Scientific Inc., South Korea) and adjusted to 5 g/L of suspension for pretreatment by preparing microalgal cell suspension in water. The pretreatment experiments were performed using a 40 kHz ultrasonicator with a maximum output power of 450 W (SH-2300, Saehan Ultrasonic Cleaner, South Korea) at 50 ℃ for 30 min. After sonication, the pH of the microalgal suspension was adjusted to 5 using 1 N hydrochloric acids, and cellulase enzyme (*Trichoderma reesei* ATCC 26921, Sigma Aldrich, USA) was added to the microalgal suspension to reach a concentration of 1% of the microalgae biomass (w/w) (Eldalatony et al., 2016) for performing simultaneous saccharification and fermentation process. *Saccharomyces cerevisiae* YPH499 purchased from ATCC 204679™ (Meyen ex E.C. Hansen, USA) was used as the fermentative microorganism and cultivated in 100 mL of yeast extract-peptone-dextrose (YPD) medium (yeast extract 10 g/L, peptone 20 g/L, and dextrose 20 g/L) at 30 ℃ and 150 rpm for 24 h (El-Dalatony et al., 2016). The yeast cell suspension (10^8 cell/mL) was immobilized in Ca-alginate (2%) using an electrostatic droplet method (Nikolić et al., 2009). Serum bottles with a capacity of 130 mL and a working volume of 90 mL were used for fermentation experiments. The microalgae suspension (5 g/L) was inoculated with 30 immobilized yeast beads (5×10^7 CFU/g of 4 mm diameter beads). The serum bottles were flushed with N_2 gas (99.99% pure) for 15 min to provide an anaerobic environment and then sealed tightly with a butyl rubber stopper and aluminum crimp. The bottles for carbohydrate fermentation were placed in a shaking water bath (HS-SHWB-30, Hansol Tech, South Korea) at 27 ℃ and 120 rpm for 3 days. The immobilized yeast was separated out after carbohydrate fermentation using a sieve (CISA, USA) and the fermentation broth was distilled at 90 ℃ for 120 min to retrieve ethanol (El-Dalatony et al., 2016). The distilled bioethanol was characterized according to ASTM D97 (Sampling and Analysis of, 2004), and analyzed using gas chromatography.

2.3. Protein fermentation and transesterification of the leftover biomass

The remaining fermentation broth after distillation was further used for protein fermentation process by *S. cerevisiae* S288C immobilized yeast for higher alcohols production. The protein fermentation process for higher alcohols production (butanol, propanol, *iso*-butanol, and 3-methyl-1-butanol) was performed in 130 mL serum bottles following carbohydrate fermentation. Each bottle contained 90 mL working volume of leftover biomass following carbohydrate fermentation. *S. cerevisiae* S288C purchased from ATCC 204508™ (Meyen ex E.C. Hansen, USA) was cultivated in YPD medium and immobilized in Ca-alginate

(2%). A total of 30 immobilized yeast beads (5×10^7 CFU/g of 4 mm diameter beads) were inoculated into the microalgae suspension for protein fermentation. The serum bottles were flushed with N_2 gas for 15 min to provide an anaerobic environment and then placed in a shaking water bath at 27 °C and 120 rpm for 7 days. Higher alcohols produced after protein fermentation was retrieved by distillation and analyzed using gas chromatography (El-Dalatony et al., 2019b).

The immobilized yeast which used for protein fermentation were separated from the fermentation broth using a sieve, and remaining biomass portion rich in lipids were extracted using chloroform solvent by the Bligh and Dyer method (Bligh and Dyer, 1959), and then transesterified and converted to biodiesel using following method. A total of 50 mL of a mixture of chloroform and methanol (1:2, v/v) was added to the leftover biomass in a shaking water bath for 30 min. This was then centrifuged (4000 rpm for 5 min) and 15 mL of distilled water was added to separate the lipid-chloroform and aqueous methanol layers. The chloroform containing lipids were transferred to a Pyrex tube containing methanol (50% of chloroform layer) and sulfuric acid (10% of methanol layer), and incubated at 100 ℃ for 10 min. After cooling, distilled water was added until the mixture separated into two phases, it was then mixed vigorously for 10 min and centrifuged (4000 rpm for 5 min). The chloroform layer was filtered using a 0.2 μm polyvinylidene fluoride syringe microfilter and evaluated for fatty acid methyl esters using gas chromatography (Lepage and Claude, 1986).

2.4. Microalgae cell integrity

The shape of the microalgae was evaluated using a trinocular microscope with a digital camera system (Lx400, Labomed, USA) at high magnification (×100). The microalgae cell integrity measurements were verified using Eyecam software (Labomed, USA). The microstructure and the cell wall thickness of the microalgal strains were examined using TEM (Leo 912A 8B OMEGA EF-TEM, Carl Zeiss, Germany) before and after fermentation at a 120 keV electron energy emission (El-Dalatony et al., 2019b). The microalgae cell suspension was fixed using 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), then post-fixed in 1% osmium tetroxide with 0.1 M cacodylate buffer for 1 h and rinsed with 0.1 M cacodylate buffer. The cells were pelleted, fixed in glutaraldehyde, dehydrated in a series of EtOH solutions and embedded in EPON resin. The polymerized blocks were anaerobically sectioned on a microtome and thin sections were mounted on copper grids coated with formvar and carbon for TEM analysis (Choi et al., 2011). The cell integrity and cell wall thickness in 15 cells were measured by TEM and expressed as an average value.

2.5. Analytical methods

Bioethanol was analyzed by gas chromatography on a DS 6200 (Do-Nam, Agilent, USA) equipped with a flame ionization detector (FID) and a DB-624 column (30 m, 0.53 mm, 3 μm, Agilent, USA) using N_2 as a carrier gas. The oven temperature was held at 70 °C, and the temperatures of both the injector and detector were 270 °C. The injection volume was 1 μL, and the flow rate was fixed at 5 mL/min (Eldalatony et al., 2016).

Higher alcohols were analyzed using gas chromatography GC, DS 6200 (Do-Nam, Agilent, South Korea) equipped with a FID and a DB-624 column (30 m, 0.53 mm, 3 μm, Agilent, USA), using Helium as a carrier gas. The oven temperature was initially held at 40 °C for 2 min, raised with a gradient of 5 °C min^{-1} until it reached 45 °C, and held for 4 min. Then it was raised with a gradient of 15 °C min^{-1} until it reached 230 °C and held for 4 min. The injector and detector were maintained at 225 °C. A 2 μL sample was injected using the splitless injection mode (El-Dalatony et al., 2019b).

The fatty acid methyl esters were analyzed using a gas chromatograph, GC-6890 (Agilent, USA) equipped with an FID and HP-INNO wax capillary column (30 m, 0.25 mm, 0.25 μm, Agilent, USA). Nitrogen was used as the carrier gas at 1 mL.min^{-1}. The temperature condition was set at 120 °C for 7 min and then raised to 180 °C at 4 °C per minute. Then it was raised to 250 °C at a rate of 5 °C per minute and was maintained at 250 °C for 20 min. The injection volume and split ratios were 2 μL and 45:1, respectively. The injector and detector temperatures were set at 250 and 275 °C, respectively (El-Dalatony et al., 2019b). All experiments were conducted in triplicate and the data is presented as the mean ± standard deviation (SD) of each triplicate experiment.

2.6. Calculations of parameters

The biofuels (bioethanol, higher alcohols, and biodiesel) yield, carbohydrate and protein fermentation efficiency, conversion efficiency, and total energy recovery were calculated according to Eqs. (1)–(4) (El-Dalatony et al., 2019b; Fernandes et al., 2015).

$$\text{Biofuels yield (g/g)} = \frac{\text{Concentration of produced biofuel (g/L)}}{\text{Concentration of biofraction (g/L)}} \quad (1)$$

$$\text{Fermentation efficiency (\%)} = \frac{\text{Produced bioalcohol (g/L)}}{\text{Theoretical bioalcohol production (g/L)}} \times 100 \quad (2)$$

$$\text{Biomass conversion efficiency (\%)} = \frac{\text{Initial biomass} - \text{Final biomass (g/L)}}{\text{Initial biomass (g/L)}} \times 100 \quad (3)$$

$$\text{Total energy recovery (\%)} = \frac{\text{Maximum biofuels production (g/L)}}{\text{Initial biomass (g/L)}} \times 100 \quad (4)$$

3. Results and discussion

3.1. Biochemical characterization of microalgae strains

The biocomponents from three microalgae strains (*Pseudochlorella* sp., *Chlamydomonas mexicana,* and *Chlamydomonas pitschmannii*) were analyzed. The amount of total carbohydrate, protein, lipid, ash, and pigments (chlorophylls and carotenoids) for each strains were estimated to be 36, 24, 30, 10, and 0.04% for *Pseudochlorella* sp., 50, 20, 21, 9, and 0.03% for *C. mexicana*, and 23, 20, 50, 7, and 0.08% for *C. pitschmannii*, respectively (Table 1). The results of the biochemical characterization showed that *C. mexicana* has the highest carbohydrate content (50%), while *C. pitschmannii* had the highest lipid content (50%). Recent studies reported that the amount of extracted reducing sugars (14 and 32%) differs under the same pretreatment conditions (ultrasound power: 250 W, time: 60 min) depending on the microalgae (*C. mexicana, S. obliquus*) used (Choi et al., 2011; Eldalatony et al., 2016). The biocomponents of the two microalgal varieties (*C. mexicana, S. obliquus*) used in the literature were analyzed and they both have different biochemical compositions (52 and 26% carbohydrate, 36 and 54% protein, 10 and 11% lipid content). These variations in biocomposition effect the nature and efficacy of the pretreatment as well as the feasibility of using microalgae to extraction the major precursors (sugars, amino acids, and fatty acids) for biofuels production.

3.2. Carbohydrate fermentation of pretreated microalgal biomass for bioethanol production

Sonicated microalgal biomass containing 230, 360, and 500 mg-sugar/g-biomass of *C. pitschmannii*, *Pseudochlorella* sp., and *C. mexicana*, respectively, were used as feedstocks for carbohydrate fermentation by *S. cerevisiae* YPH499. The concentration of bioethanol from *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii* were 0.81, 1.06, and 0.41 g/L, respectively. This concentration increased rapidly during

Table 1
Parameters for biofuel production during the fermentation process using each of the three microalgal strains.

Parameters	*Pseudochlorella* sp.	*Chlamydomonas mexicana*	*Chlamydomonas pitschmannii*
Dry weight of carbohydrate, protein, lipid, and ash (%)	36, 24, 30, 10	50, 20, 21, 9	23, 20, 50, 7
Total carbohydrate concentration (g/L)	1.8	2.5	1.15
Total protein concentration (g/L)	1.2	1.0	1.0
Total lipid concentration (g/L)	1.5	1.05	2.5
Bioethanol production (g/L) and yield (g/g)	0.81, 0.45	1.06, 0.42	0.41, 0.36
Higher alcohols production (g/L) and yield (g/g)	0.52, 0.44	0.42, 0.42	0.39, 0.39
Biodiesel production (g/L) and yield (g/g)	0.79, 0.55	0.41, 0.48	0.39, 0.37
Carbohydrate fermentation efficiency (%)	86.26	81.8	69.94
Protein fermentation efficiency (%)	72.63	70.76	65.38
Biomass conversion efficiency (%)	84.1	85.65	63.22
Total energy recovery (%)	42.44	37.96	26.25
Utilized of total carbohydrate, protein, lipid content (%)	100, 90, 55	100, 82.89, 48	90, 74, 37.4

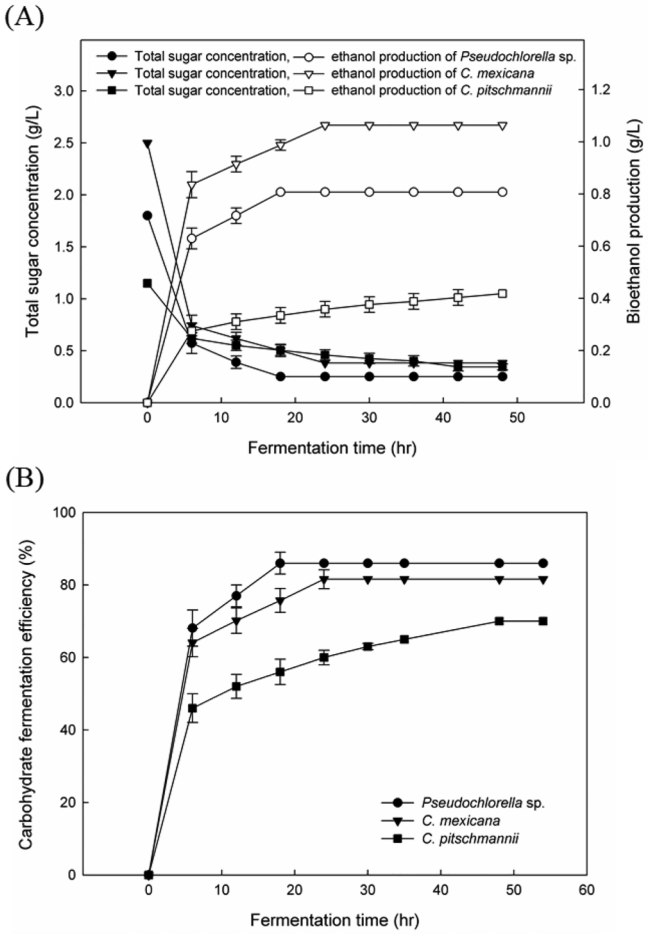

Fig. 1. Residual sugar concentration and bioethanol production during fermentation of the carbohydrate fraction (A), and the efficiency of the carbohydrate fermentation process (B) using each of the three microalgal strains as biomass.

the first 6 h and reached a constant equilibrium at 18, 24, and 48 h (Fig. 1A), with an ethanol yield of 0.45, 0.42, and 0.36 g-ethanol/g-carbohydrates, respectively (Table 1). The total reducing sugar concentration was 1.80, 2.50, and 1.15 g/L of *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii*, which decreased significantly during the first 6 h of carbohydrate fermentation, and reached equilibrium at 18, 24, and 48 h, respectively. The carbohydrate fermentation efficiency of *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii* during the bioethanol production was 86.26, 81.80, and 69.94% respectively (Fig. 1B). These results indicate that these microalgae contained higher amounts of carbohydrates and lipids. The higher density of the cell wall structure reduced the availability of substrate, consequently lowering the yield of bioethanol (Aligata et al., 2018). The microalgal cell walls are difficult to be disrupted efficiently by simple sonication process in order to release all biocomponents due to the tough interlinking biopolymers structure of microalgae cell walls (Gunerken et al., 2015; Velazquez-Lucio et al., 2018). Most of the biocomponents can be utilized through the biological pretreatment of microorganisms during the fermentation process after ultrasound pretreatment. Lipid-rich *C. pitschmannii* is composed of neutral lipids (triacylglycerides, TAGs) and polar lipids (phospholipids and glycolipids), which have multiple double bonds and higher carbon chain lengths that reduced the extraction efficiency of these biofractions (Ma et al., 2014; Olmstead et al., 2013). These results suggest that the variation in the biochemical compositions of the microalgae is closely correlated with biofuel production (Breuer et al., 2012; Michalak andand Chojnacka, 2014).

3.3. Transmission electron microscopic examinations

TEM images of the internal cell structure and cell walls of the untreated microalgal strains before and after carbohydrate fermentation reveal that they all have a round shape. *C. mexicana* with the highest carbohydrate content (50%), had stronger and thicker cell walls (250 nm) than *Pseudochlorella* sp. (150 nm). However, *C. pitschmannii,* which has lower carbohydrate content and higher lipid content than *C. mexicana*, had much thicker cell walls (351 nm), as a result of the accumulation of large amounts of lipids in the cell wall (Breuer et al., 2012). Similar results have been reported when observing thick cell walls in sectioned microalgal cells with high lipid content (Simionato et al., 2013; Yap et al., 2016). Fermentation altered the cell morphology of all microalgal strains the extent of these changes reflects the substrate extraction and utilization efficiency for each species. The cell walls were lysed after carbohydrate fermentation resulting in the release of cell wall-associated carbohydrates and proteins into the extracellular medium. The thickness of the cell walls for each of the microalgae (*Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii*) was measured before and after carbohydrate fermentation to confirm its effect on fermentation efficiency (Fig. 2). *Pseudochlorella* sp. showed a collapsed shape with a 90% decrease in cell wall thickness after carbohydrate fermentation, and most of the total sugars (86%) were utilized. While, *C. pitschmannii* showed a softened shape, with a 23% decrease in cell wall thickness after carbohydrate fermentation (Fig. 2). These results suggest that microalgae have different physiological properties including cell wall thickness and composition and that these differences have a significant effect on pretreatment and fermentation (Yap et al., 2016).

3.4. Monitoring of protein content during carbohydrate fermentation

The protein content for each microalgal strain was evaluated in the

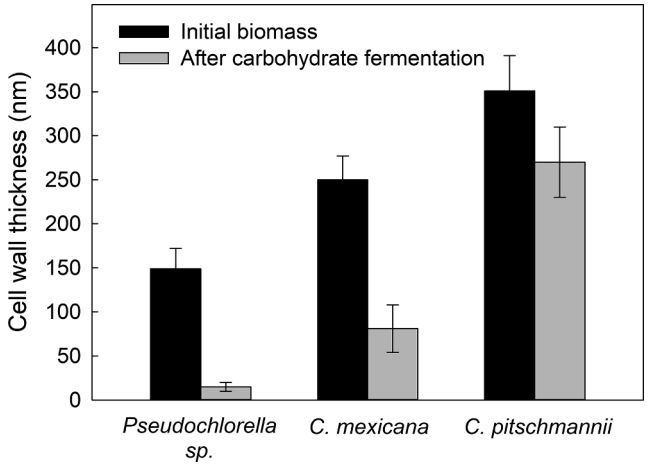

Fig. 2. Cell wall thickness of initial biomass (untreated) before and after carbohydrate fermentation of the three microalgal strains.

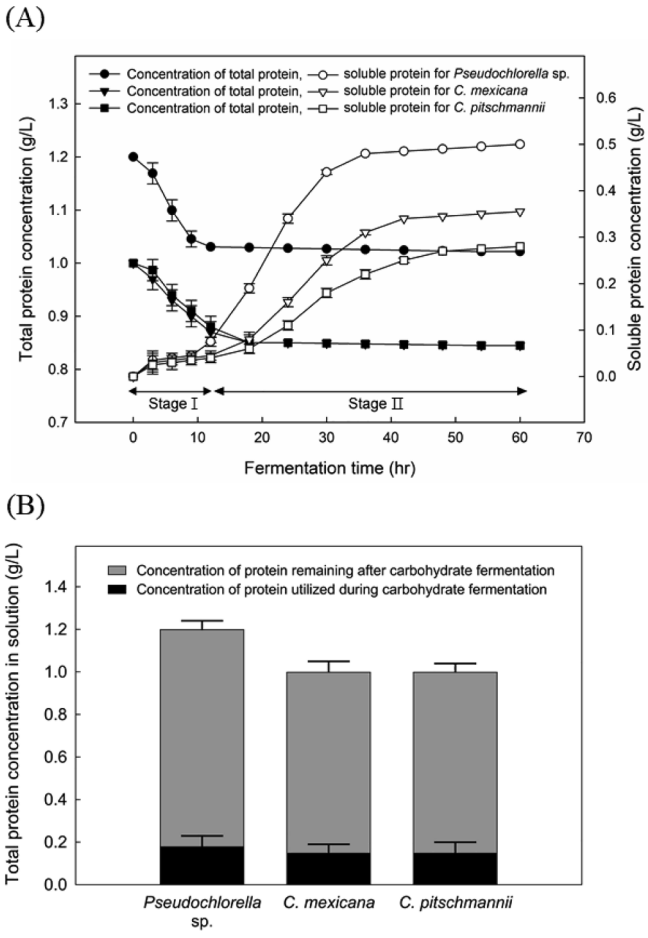

Fig. 3. Effect of carbohydrate fermentation on protein release from *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii* (A). Amount of protein utilized during carbohydrate fermentation and the amount remaining for higher alcohols production (B). Stage I and II protein utilization to enhance growth and sugar use in *S. cerevisiae*, and the proteins released from the microalgae, respectively.

initial biomass and in the leftover biomass following carbohydrate fermentation. The soluble protein concentration increased very slowly at stage I (at a high sugar utilization rate) and then rapidly increased from 9 and 12 h at stage II (proteins released into the media) (Fig. 3A). These results indicate that the cell wall was highly disrupted by bio-pretreatment with yeast cells that reflects the co-extraction of proteins during carbohydrate fermentation enhancing extraction efficiency and bioavailability (El-Dalatony et al., 2019b; Eldalatony et al., 2016). Carbohydrate fermentation can be used as bio-pretreatment for economically feasible extraction of proteins and is associated with improved hydrolysis of proteins into amino acids, which can be used in downstream processes (El-Dalatony et al., 2019a; Lai et al., 2016a). The proportion of proteins used from *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii* were all 15% at stage I, regardless of their starting proportion (Fig. 3B). When reducing sugars are used for bioethanol production, *S. cerevisiae* consumes some of the amino acids as a nitrogen source to enhance growth and sugar metabolism (Wernick and Liao, 2013; Yin et al., 2015). The total remaining protein content from *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii* after carbohydrate fermentation was 85% (Fig. 3B), which would normally be considered waste. However, in this study, the leftover biomass, containing these proteins was used for higher alcohols production (C3-C5) by a protein fermenting microorganism (*S. cerevisiae* S288C). In this microorganism the amino acids were converted into higher alcohols through Ehrlich pathway where the amino acids have gone through deamination/transamination, decarboxylation, and reduction steps (Eldalatony et al., 2016; Huo et al., 2011). The biological pretreatment has a potential for co-extracting protein contents which can be further utilized in a downstream process toward the production of higher alcohols.

3.5. Protein fermentation of leftover biomass after carbohydrate fermentation for higher alcohols production

Bioethanol production of *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii* during the carbohydrate fermentation was 0.81, 1.06, and 0.41 g/L, respectively. The total carbohydrate concentration of microalgal strains decreased slowly and reached equilibrium, respectively (Fig. 4A). The leftover biomass recovered after carbohydrate fermentation was used to produce higher alcohols by *S. cerevisiae* S288C. Four different higher alcohols (propanol, *iso*-butanol, 3-methyl-1-butanol, and butanol) were generated from these microalgal strains. *Pseudochlorella* sp. showed the highest overall higher alcohols production (0.52 g/L) when compared to *C. mexicana* (0.42 g/L) and *C. pitschmannii* (0.39 g/L). These products increased slowly and reached a constant equilibrium at 132, 144, and 168 h (Fig. 4B) with a higher alcohols yield of 0.44, 0.42, and 0.39 g-higher alcohols/g-proteins, respectively (Table 1). These results indicate that the thick cell walls of microalgae resist biological pretreatment, and reduce the bioaccessibility of various substrates (Lee et al., 2017). The total protein concentration was 1.02, 0.90, and 0.95 g/L from *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii*, which decreased slowly during the protein fermentation (Fig. 4B). The protein fermentation efficiency of *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii* during higher alcohols production was 72.63, 70.76, and 65.38%, respectively (Table 1). Using amino acids from the remaining proteins to produce energy-dense biofuels which improves overall yield, reduces process costs, increases the longevity of transportation fuels, and advances protein waste management. Protein portion was usually utilized as fertilizer and was left as waste from biorefinery processing. However, with the current approach of this study, the protein leftover after bioethanol or biodiesel production can be the used for the production of not only higher alcohol, but also various chemicals and pharmaceutical intermediates (succinate, itaconate, etc.) through metabolically engineered microorganisms. This approach, resulted in reduced protein waste from the fermentation industry (El-Dalatony et al., 2019a; Huo et al., 2012).

3.6. Transesterification from unfermented leftover biomass for biodiesel production

The remaining biomass was made up predominantly by lipids 1.5,

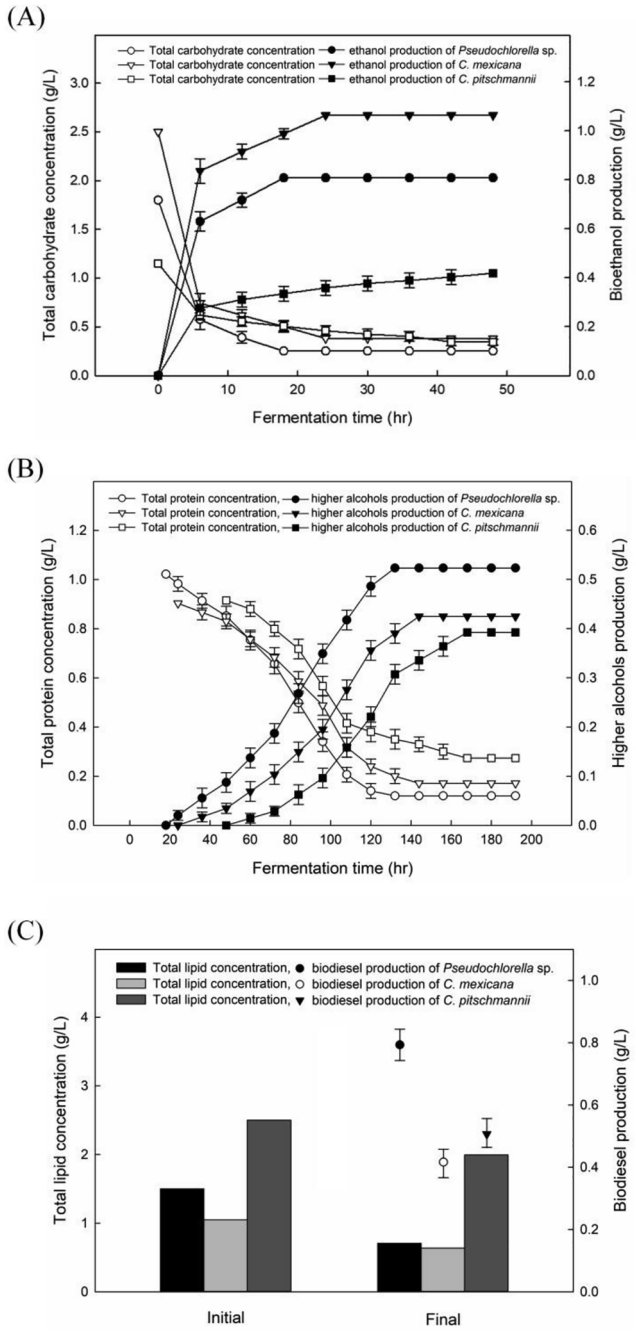

Fig. 4. Total carbohydrate concentration consumption and bioethanol production (A), total protein concentration consumption and higher alcohols production (B), total lipid consumption and biodiesel production (C) using each of the three microalgal strains subjected to carbohydrate-, protein fermentation, and transesterification, sequentially.

1.05, and 2.5 g/L for *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii*, respectively. The transesterification process was used to convert these lipids to biodiesel, 0.79, 0.41, and 0.5 g/L (Fig. 4C), with a biodiesel yield of 0.55, 0.48, and 0.37 g-biodiesel/g-lipids, respectively (Table 1). These results indicate that the presence of larger amounts of carbohydrates and lipids in the cell wall reduced solvent penetration resulting in reduced biofuel yields (Nagappan et al., 2019; Pasaribu et al., 2016). Analysis of the fatty acid methyl esters (FAME) composition for each of the microalgal strains showed that palmitic acid (C16:0), oleic acid (C18:1n9c), and γ-Linolenic acid (C18:3n6) were the predominant lipids, estimated to be 21.18, 19.32, and 13.15% for *Pseudochlorella* sp., 25.62, 19.68, and 8.13% for *C. mexicana*, 22.94,

Table 2
Fatty acid profile of the three microalgal strains.

Fatty acids	Fatty acids composition (%, w/w)		
	Pseudochlorella sp.	*C. mexicana*	*C. pitschmannii*
Lauric acid (C12:0)	1.57	1.62	1.97
Tridecylic acid (C13:0)	5.73	5.53	6.47
Myristic acid (C14:0)	4.19	1.96	3.02
Myristoleic acid (C14:1)	1.5	3.32	1.11
Pentadecanoic acid (C15:0)	0.55	0.69	0.13
cis-10-Pentadecenoic acid (C15:1)	0.01	0.09	0.02
Palmitic acid (C16:0)	21.18	25.62	22.94
Palmitoleic acid (C16:1)	1.60	1.3	2.66
Heptadecanoic (C17:0)	6.52	5.19	1.51
Cis-10-Heptadecenoic (C17:1)	2.86	2.55	4.26
Stearic acid (C18:0)	2.98	4.93	6.13
Oleic acid (C18:1n9c)	19.32	19.68	28.72
Linoleic acid (C18:2n6c)	6.85	5.36	3.38
γ-Linolenic acid (C18:3n6)	13.15	8.13	4.88
α-Linolenic acid (C18:3n3)	0.42	0.57	3.44
Arachidic acid (C20:0)	0.2	0.05	0.06
Others	11.37	13.41	9.3
Saturated fatty acids	54.29	60.0	51.53
Monounsaturated fatty acids	25.29	25.94	36.77
Polyunsaturated fatty acids	20.42	14.06	11.7
Totals (%)	100	100	100

28.72, and 4.88% for *C. pitschmannii*, respectively (Table 2). Since the quality of biodiesel depends on the oleic and palmitic acid content the high proportion of these two fatty acids in microalgae indicates that it is a high-quality feedstock for biodiesel production (Lai et al., 2016b). The amounts of saturated (54, 60, and 51%), monounsaturated (25, 25, and 36%), and polyunsaturated (20, 14, and 11%) fatty acids were determined in *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii,* respectively. The highest concentration of saturated fatty acids (60%), including palmitic acid (25.62%), was observed in *C. mexicana*, while the highest concentration of monounsaturated fatty acids (36.77%), including oleic acid (28.72%), was observed in *C. pitschmannii* (Table 2). It has been reported that high saturated fatty acid (such as palmitic acid) content can provide biodiesel with a higher cetane number and oxidative stability, and lower NO_x emissions (Cheirsilp and Torpee, 2012). While, high monounsaturated fatty acids (including oleic acid) can provide biodiesel with a reasonable balance of fuel properties, including ignition quality, combustion heat, cold filter plugging point, oxidative stability, viscosity, and lubricity (Liu et al., 2011).

3.7. Mass balance of the biochemical composition of microalgae strains

The mass balance examination of the biochemical composition of microalgal strains during sequential fermentation and transesterification processes indicates that the biomass of selected microalgal species can serve as potential feedstock in the production of multiple biofuels. The initial carbohydrate, protein, and lipid contents were estimated to be 1.8, 1.2, and 1.5 g/L for *Pseudochlorella* sp.; 2.5, 1.0, and 1.05 g/L for *C. mexicana*; and 1.15, 1.0, and 2.5 g/L for *C. pitschmannii*, respectively. The total biomass (4.5, 4.55, and 4.65 g/L) decreased gradually during each process as a result of the sequential utilization of each family of compounds. The biomass was reduced to 2.8, 1.6, and 0.79 g/L for *Pseudochlorella* sp., 2.4, 1.2, and 0.64 g/L for *C. mexicana*, 3.8, 2.8, and 1.6 g/L for *C. pitschmannii* after carbohydrate and protein fermentation, and transesterification, respectively (Fig. 5). The amount of total carbohydrate, protein, and lipid used was determined to be 100/90/55%, 100/82.89/48%, and 90/74/37.4% in *Pseudochlorella* sp., *C. mexicana*, and *C. pitschmannii*, respectively. The total energy recovery and

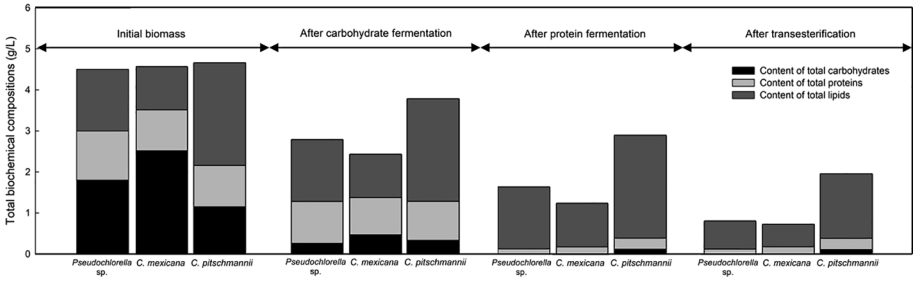

Fig. 5. Mass balance of each of the three microalgal strains during carbohydrate-, protein fermentation, and transesterification.

conversion efficiency was 42.44 and 84.1% for *Pseudochlorella* sp., 37.96 and 85.65% for *C. mexicana*, 26.25 and 63.22% for *C. pitschmannii*, respectively (Table 1). The limitations in biomass utilization resulting from various physiological properties including cell wall thickness and biocomponent content can be overcome through the use of an energy-efficient pretreatment. Proper pretreatment means that it is possible to get good energy conversion and biofuels production using these microalgae as biomass. Finally, the highest bioethanol, higher alcohols, and biodiesel yield (0.45, 0.44, and 0.55 g/g), carbohydrate and protein fermentation efficiency (86 and 72%), and total energy recovery (42.44%) were all obtained using *Pseudochlorella* sp. (Table 1). Therefore, *Pseudochlorella* sp. has been identified as a suitable candidate for future evaluation for future industrial applications, including commercial production of various biofuels.

4. Conclusions

The influence of the different bioconstituents on biofuels production from biomass of three microalgal strains was evaluated. The highest biofuel yields were obtained from *Pseudochlorella* sp. with effective utilization of carbohydrate and protein. Changes in cell wall structure and thickness were observed before and after fermentation using transmission electron microscopy. The biocomponent content of microalgae has a significant influence on both pretreatment and fermentation efficiency and is closely correlated with various parameters for biofuel production. This study provides additional insight into the pretreatment and fermentation and could be useful in scaling-up of fermentation to improve commercial biofuel production.

CRediT authorship contribution statement

Geon-Soo Ha: Conceptualization, Funding acquisition, Resources, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Marwa M. El-Dalatony:** Validation, Visualization, Writing - review & editing. **Do-Hyeon Kim:** Resources, Investigation, Writing - review & editing. **El-Sayed Salama:** Resources, Writing - review & editing. **Mayur B. Kurade:** Resources, Supervision, Writing - review & editing. **Hyun-Seog Roh:** Resources, Writing - review & editing. **Abd El-Fatah Abomohra:** Resources, Writing - review & editing. **Byong-Hun Jeon:** Funding acquisition, Resources, Validation, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Mid-Career Researcher Program No. (2017R1A2B2004143) through the National Research Foundation (NRF) of Republic of Korea.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.122809.

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