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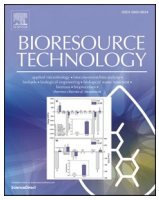

High-throughput integrated pretreatment strategies to convert high-solid loading microalgae into high-concentration biofuels

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HIGHLIGHTS

- Energy-saving microwave pretreatment required low specific energy (4.2 MJ/kg).
- Sequential fermentation completely disintegrated microalgal biomass.
- Unprecedented conversion efficiency (67%) was obtained from *C. pitschmannii*.
- Sustainable integrated pretreatment markedly enhanced high-titer biofuels production.

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ABSTRACT

The commercial feasibility of energy-efficient conversion of highly concentrated microalgal suspensions to produce high-titer biofuels is a major bottleneck due to high energy consumption. Herein, high-titer biofuels (bioethanol, higher-alcohols, and biodiesel) were generated from carbohydrate-rich *Chlamydomonas mexicana* and lipid-rich *Chlamydomonas pitschmannii* biomass through energy-saving microwave pretreatment, successive fermentation, and transesterification. Microwave pretreatment needed low specific energy (4.2 MJ/kg) for 100 g/L of microalgal suspension. Proposed sustainable integrated pretreatments method achieved unprecedented total conversion efficiency (67%) and highest biomass utilization (87%) of *C. pitschmannii* (100 g/L) with high yields of bioethanol (0.48 g-ethanol/g-carbohydrates), higher-alcohols (0.44 g-higher-alcohols/g-proteins), and biodiesel (0.90 g-biodiesel/g-lipids). Transmission electron microscopy showed the changes in the microalgal cellular integrity before and after sequential fermentations. Energy-efficient integrated pretreatments enhanced the extraction efficiency and whole utilization of high-concentration microalgae to generate high-titer biofuels with minimum waste production.

1. Introduction

Microalgal biomass is a promising renewable energy source for biofuels production [bioethanol, higher-alcohols, and biodiesel (BE, HA, and BD, respectively)] due to its rich organic contents (carbohydrates, proteins, and lipids) which are the major substrates of biofuels (Staples et al., 2017). Most microalgal biofuel research focused on the utilization of carbohydrates and lipids to produce BE and BD, respectively, with the residual protein fraction being discarded as process waste (Giwa et al., 2018). Residual proteins can be converted to high-energy-density biofuels (propanol and butanol) via the Ehrlich pathway of engineered yeast cells (Lee et al., 2019) to maximize the total microalgal biomass utilization by more than 80% (Ha et al., 2020a). Recently, an energy-saving integrated pretreatment and fermentation approach has been demonstrated to accomplish fractionation and use of highly concentrated microalgal suspensions (100 g/L) that remained a bottleneck during downstream processing and limited the commercialization of microalgal biorefinery (Ha et al., 2021). However, this approach needs a

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long fermentation period (~94 hr) to disrupt cells and utilize biochemical constituents in biomass and should improve pretreatment and fermentation efficiency to economically commercialize microalgal biofuels.

Complete utilization of microalgal biomass requires multi-layered, thick, and rigid cell walls to be disrupted using an effective pretreatment method, extracting the biomolecules into the medium for subsequent fermentation and biofuel production (Halim et al., 2019). The different physiological properties of microalgal strains, such as cell wall thickness and biocomponents, have a remarkable effect on pretreatment and fermentation efficiency, and biofuel yield (Ha et al., 2020b). Although different microalgae pretreatment methods have been developed and standardized over the last decade, there are still major drawbacks to commercializing microalgae-based biofuels on a pilot and industrial scale: 1) use of low concentration biomass suspension (~50 g/L) and high process volume, resulting in low biofuel production and high downstream processing cost 2) energy-intensive pretreatments (270–1080 MJ/kg), 3) limitation of total biomass utilization (~66%) and low conversion efficiency (~53%) (Ashokkumar et al., 2015; Marwa et al., 2019; Sivaramakrishnan & Incharoensakdi, 2018; Wang et al., 2014). The economic feasibility of existing microalgal pretreatment technologies for high-titer biofuel productions is limited as they are approximately twice as expensive as fossil fuels (Laurens et al., 2015), and accessing intracellular biocomponents in a cost-effective and environmentally friendly process remains the main challenge for economical industrial scale-up (Khoo et al., 2020). Therefore, the development of high-efficiency pretreatment technologies capable of effectively disrupting the microalgal cell walls in highly concentrated microalgal suspensions and extraction/utilization of main bioconstituents (carbohydrates, proteins, and lipids), thereby remarkably reducing the downstream process cost is necessary to overcome these challenges and improve economic viability. Microwave-based processing is an effective biomass disruption and extraction method that accelerates the deconstruct biomass structure and release of intracellular bioconstituents by utilizing the thermal/non-thermal effect (Aguilar-Reynosa et al., 2017; Hussain et al., 2021). The reported microwave processing consumes excessive specific energy (630–1680 MJ/kg) due to the high microwave power (~1400 W) and long extraction time (~60 min) to obtain only higher BD yields (80%) from a high concentration of microalgae (70 g/L) (Florentino de Souza Silva et al., 2014; Martinez-Guerra et al., 2014; Rahul et al., 2018). Hence, microwave pretreatment (MP) must be standardized to markedly decrease specific energy consumption and produce sustainable high-titer biofuels.

The present study developed an energy-saving MP to improve the bioaccessibility and bioavailability of carbohydrates, proteins, and lipids in a highly concentrated suspension of microalgae with different physiological properties. An eco-friendly, integrated sequential fermentation and transesterification approach was applied to enhance high-titer BE, HA, and BD production with minimum waste generation. The rheological determination, yeast population dynamics, microalgal cell disruption efficiency, and mass balance were examined during the biofuels production to assess the biomass utilization and conversion efficiency.

2. Materials and methods

2.1. Microalgal biomass preparation and bioconstituent characterization

Chlamydomonas mexicana GU732420 and *Chlamydomonas pitschmannii* GU732416 were grown in 4 L Bold's basal medium. The algal strains were cultivated with white fluorescent light (60 μmol/m^2/s) and an airflow rate of 0.3 vvm, at 27 °C, 200 rpm, for 14 days (Ha et al., 2020a). The biochemical compositions in microalgae were identified in a triplicate using phenol–sulfuric acid method for carbohydrates analysis, Lowry method for protein estimation, and Bligh and Dyer method for lipid analysis (Laurens et al., 2012).

2.2. Rheological determination

Rheological properties of microalgal suspensions at concentrations (60, 70, 80, 90, and 100 g/L) were investigated through a rheometer (ARES-G2, TA instruments, USA). Suspensions (20 mL) were analyzed at a shear rate of 0.1–900 s^{-1} and 25 °C. Rheological parameters were determined by applying suitable rheologic equations (Herschel–Bulkley and non-Newtonian power-law) (Buchmann et al., 2018).

2.3. Microwave pretreatment of highly concentrated algal suspensions

Microalgal cell suspensions were collected by centrifugation (R515, Hanil science, South Korea) at 4000 rpm for 15 min and changed to 60, 70, 80, 90, and 100 g/L. Microalgal biomass suspensions were energy-saving microwave (EKRL202DGK; Winia Corp., South Korea) pretreated at different powers (210–700 W) to make the biomass bioavailable for the fermentation process. Microwave-pretreated biomass was centrifugated at 4000 rpm for 10 min, and bioconstituents were analyzed before and after MP.

2.4. Sequential fermentation for production of high-titer bioethanol and higher-alcohols

Sequential fermentation was carried out in batches using different yeast strains from microwave-pretreated algal biomass. Both microalgal suspensions (*C. mexicana* and *C. pitschmannii*) were treated simultaneously saccharification fermentation with Cellulase (*Trichoderma reesei*, Sigma Aldrich, USA) (1%, w/w) at pH 5, respectively. Lipase (*Rhizomucor miehei,* Sigma Aldrich, USA) was added separately to enhance the hydrolysis of lipids accumulated in the cell walls of lipid-rich *C. pitschmannii*. Precultured *Saccharomyces cerevisiae* YPH499 (ATCC 204679™, USA) was added (10%, w/w) into 530 mL serum bottles. The anaerobic condition of the bottle was made by flushing pure nitrogen gas (99.999%) for 15 min and then closed with a rubber stopper. Carbohydrate fermentation for BE was performed in a bath shaker with 200 rpm, at 37 °C for 4 days. The number of yeast cells (CFU/mL) during carbohydrate fermentation was analyzed using Petrifilm™ (3 M, USA).

Afterward, fermentation to generate HA from proteins was carried out in the same serum bottles after carbohydrate fermentation. The remaining algal suspensions were inoculated with *S. cerevisiae* S288C (ATCC 204508™, USA) previously cultured, and the anaerobic state was maintained using pure nitrogen gas. Protein fermentation was carried out at 37 °C and 200 rpm for 5 days. After the protein fermentation, the fermentation broth was distilled at 140 °C for 120 min to recover BE and HA, and evaluated by gas chromatography (GC) (El-Dalatony et al., 2019b; Ha et al., 2020b).

2.5. Transesterification for production of high-titer biodiesel

Lipids in the residual microalgal biomass (200 mg) collected after distillation of fermentation broth were transesterified in a bath shaker with 200 rpm, at 60 °C for 120 min using a mixture of methanol (3 mL), H_2SO_4 (10% of methanol layer), and tetrahydrofuran (0.1 mol/mol of methanol). Mixture of methanol (3 mL), NaCl solution (3 mL, 10% w/v), hexane (3 mL), and methyl-heptadecanoate (1 mg/mL) were used to convert to fatty acid methyl ester (FAME) after cooling. It was then mixed for 10 min and centrifugated (4000 rpm for 10 min) to split hexane containing FAME and analyzed by GC (Nguyen et al., 2020). The BD profile and properties were identified based on different FAME compositions (Yang et al., 2016).

2.6. Visualization of intercellular morphologies of microalgae

The cell morphology and cell wall thickness of microalgae were confirmed during sequential fermentation using transmission electron

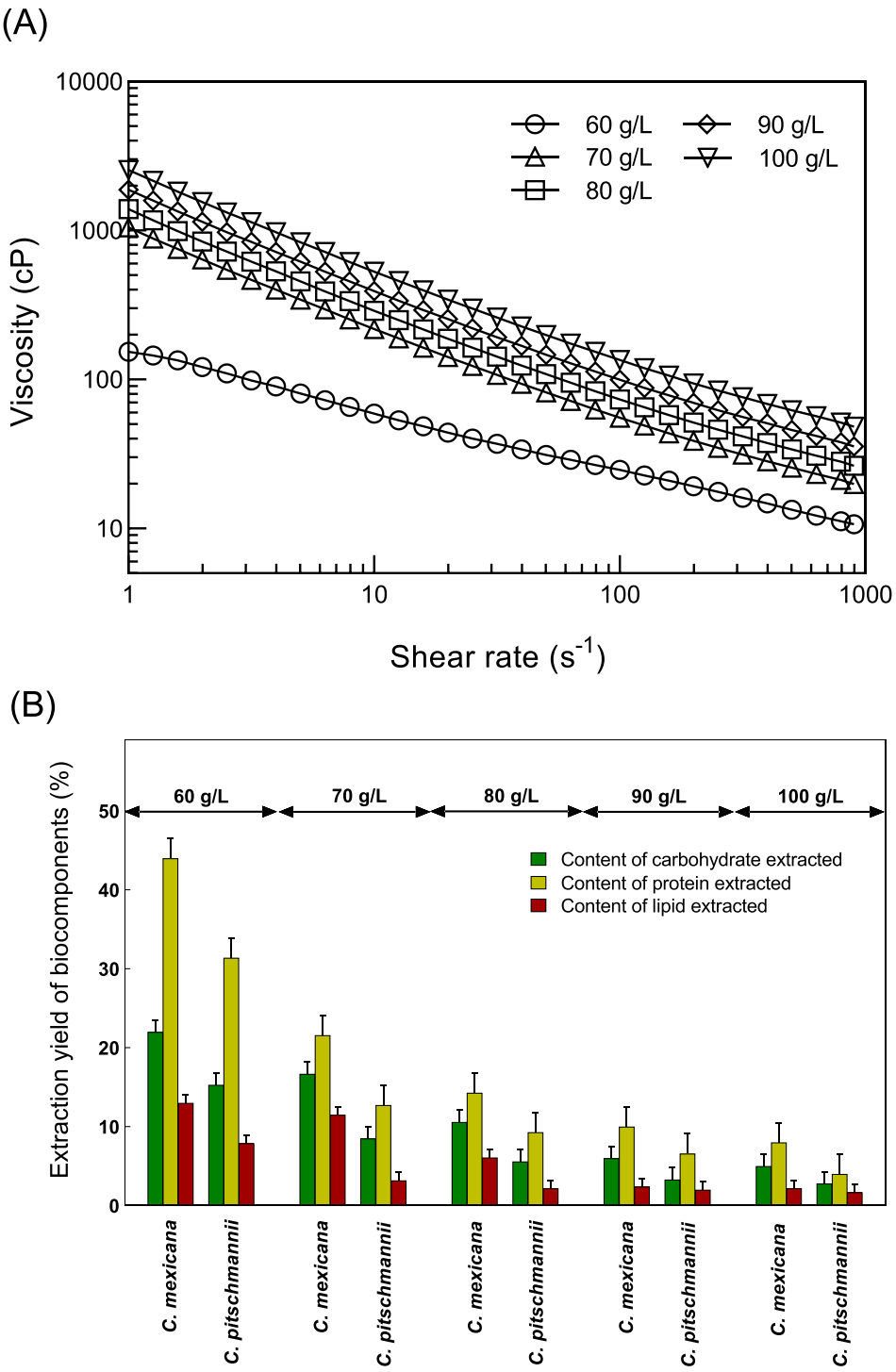

Fig. 1. Viscosities and shear rate (A), and extraction yield of bioconstituents under microwave pretreatment at 210 W for 2 min (B) from different initial microalgal biomass concentrations.

microscopy (Tecnai F20 G2; USA). The sampling procedure was carried out through biological specimens (fixation using glutaraldehyde (4%) and osmium tetroxide (1%), dehydration using ethanol solutions, embedding in EPON resin, microtomy, staining) (Choi et al., 2011; Ha et al., 2020b).

2.7. Calculation of parameters

Specific energy consumption, each biofuel (BE, HA, and BD) yield, fermentation efficiencies, biomass utilization, conversion efficiency, and power density were calculated by formulas (1) to (7) (Ha et al., 2020a). The kinetic parameters of substrate consumption and yeast population dynamics during carbohydrate fermentation were analyzed using the reported Luedeking-Piret model (Tan et al., 2019).

$$\text{Specific energy consumption (MJ/kg·TS)} = \frac{\text{Power (W)} \times \text{time (s)}}{\text{Biomass weight (kg·TS)}} \quad (1)$$

$$\text{Biofuel yield (g/g)} = \frac{\text{Concentration of produced biofuel (g/L)}}{\text{Concentration of biofraction (g/L)}} \quad (2)$$

$$\text{Fermentation efficiency}\ (\%) = \frac{\text{Produced bioalcohol}\ (g/L)}{\text{Theoretical bioalcohol production}\ (g/L)} \times 100 \quad (3)$$

$$\text{Total biomass utilization}\ (\%) = \frac{\text{Initial biomass}\ (g/L) - \text{Final biomass}\ (g/L)}{\text{Initial biomass}\ (g/L)} \times 100 \quad (4)$$

$$\text{Total conversion efficiency}\ (\%) = \frac{\text{Maximum biofuels production}\ (g/L)}{\text{Initial biomass}\ (g/L)} \times 100 \quad (5)$$

$$\text{Cell disruption efficiency}\ (\%) = (1 - \frac{\text{Intact cells counted after treatment}}{\text{Initial count of the intact cells}}) \times 100 \quad (6)$$

$$\text{Power density}\ (kW/m^3) = \frac{\text{Microwave power}}{\text{Volume of liquid}} \quad (7)$$

3. Results and discussion

3.1. Rheological measurement of highly concentrated biomass

Rheological equations were used to estimate the viscosity, mass flow rate, yield stress and shear rate, flow consistency, and flow behavior indices of untreated *C. mexicana* and *C. pitschmannii* biomass with various initial concentrations (60, 70, 80, 90, and 100 g/L) (Buchmann et al., 2018). The shear-dependence of the viscosity and the rheological parameters of the biomass suspension are essential for understanding the effects of changes in shear and viscosity in cell disruption efficiency during the pretreatment and fermentation process for biofuel production (Law et al., 2017; Mettu et al., 2019). Viscosities decreased as the shear rate increased, implying shear-thinning and non-Newtonian fluid behavior in biomass suspensions of different concentrations (Fig. 1A). The highly concentrated microalgal suspension (100 g/L) showed the highest viscosity, yield stress, and shear rate, and flow consistency index of 135.6 cP, 0.1710, and 0.1204, respectively, with the low mass flow rate (1.63×10^{-6} kg/s) and flow behavior index (0.0010) (Table 1). Viscosity, yield stress and shear rate, and flow consistency index increased exponentially at 60 g/L, while mass flow and flow behavior index decreased (Fig. 1A and Table 1). These findings suggest that as biomass concentration increased, the shear-thinning of the suspensions increased, and mass transfer decreased due to aggregate structure formation. These trends are consistent with results on the rheological properties of high concentration suspensions reported in previous studies (Buchmann et al., 2018; Mettu et al., 2019). Hence, the limitations of utilizing highly concentrated microalgal suspensions with high viscosity and low mass transfer that affect pretreatment and fermentation efficiency, and effectively converting them to high-titer biofuels can be overcome through energy-efficient microwave pretreatment.

3.2. Effect of initial concentrations and bioconstituents on extraction efficiency

The cell bioconstituents and cell wall thickness of employed microalgae species were measured. Total carbohydrates, proteins, lipids, and

Table 1
Rheological factors of different microalgal biomass concentrations.

Initial biomass concentration (g/L)	Viscosity (cP)		Mass flow rate (kg/s)		Yield stress × shear rate ($\tau_0 \gamma^{-1}$)		Flow consistency index (K)		Flow behavior index (n)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
60	23.2 ± 1.1	3.1 ± 0.4	$2.10 \cdot 10^{-6}$	$2.38 \cdot 10^{-6}$	0.0193	0.0010	0.0233	0.0009	0.0331	0.1310
70	45.4 ± 1.4	4.5 ± 0.2	$1.97 \cdot 10^{-6}$	$2.28 \cdot 10^{-6}$	0.0441	0.0034	0.0384	0.0025	0.0231	0.1252
80	70.5 ± 2.1	4.8 ± 0.6	$1.87 \cdot 10^{-6}$	$2.21 \cdot 10^{-6}$	0.0720	0.0048	0.0545	0.0036	0.0132	0.1227
90	101.5 ± 2.4	5.0 ± 0.5	$1.78 \cdot 10^{-6}$	$2.15 \cdot 10^{-6}$	0.1220	0.0061	0.0871	0.0051	0.0072	0.1198
100	135.6 ± 2.3	5.3 ± 0.4	$1.63 \cdot 10^{-6}$	$2.08 \cdot 10^{-6}$	0.1710	0.0083	0.1204	0.0068	0.0010	0.1165

Table 2
Parameters of high titer multiple biofuels generation from microalgal strains.

Parameters	*Chlamydomonas mexicana*					*Chlamydomonas pitschmannii*				
Initial biomass concentration (g/L)	60	70	80	90	100	60	70	80	90	100
Dry weight of carbohydrates (%)	53.3					21.4				
Dry weight of proteins (%)	18.2					18.3				
Dry weight of lipids (%)	21.1					53.8				
Dry weight of ash (%)	7.4					6.5				
Yield of bioethanol (g/g)	0.48	0.47	0.48	0.47	0.48	0.48	0.48	0.47	0.48	0.48
Yield of higher alcohols (g/g)	0.43	0.43	0.44	0.44	0.44	0.44	0.43	0.44	0.44	0.44
Yield of biodiesel (g/g)	0.83	0.82	0.82	0.83	0.83	0.89	0.90	0.89	0.90	0.90
Fermentation efficiency of carbohydrates (%)	96.9	94.8	96.6	94.6	96.8	96.5	96.1	94.5	96.5	96.2
Fermentation efficiency of proteins (%)	71.7	71.9	73.2	73.2	73.2	72.8	71.5	73.9	73.8	73.7
Utilized total carbohydrate contents (%)	99.3	99.1	99.1	99.3	99.2	99.3	99.2	99.1	99.3	99.2
Utilized total protein contents (%)	91.1	90.7	90.8	91.0	91.2	90.5	90.7	90.5	91.2	91.5
Utilized total lipid contents (%)	83.2	82.1	82.2	83.3	83.7	89.4	90.0	89.2	90.4	90.5
Utilized total organic matters (%)	94.4	94.1	94.2	94.8	94.9	92.5	93.1	92.6	93.3	93.2
Total biomass utilization (%)	87.4	87.1	87.2	87.4	87.5	86.0	86.6	86.1	86.8	86.7
Total conversion efficiency (%)	51.1	50.4	51.1	50.7	51.4	66.3	66.7	66.4	67.1	67.0

ashes were 53.3, 18.2, 21.1, and 7.4% for *C. mexicana* and 21.4, 18.3, 53.8, and 6.5% for *C. pitschmannii*, respectively (Table 2). The biochemical characterization confirmed the maximum carbohydrates (53.3%) in *C. mexicana* as compared to *C. pitschmannii* which had the maximum lipid (53.8%) content. Because of lipidic accumulation in large amounts, *C. pitschmannii* cell walls showed much thickness (364 nm) than that of *C. mexicana* (261 nm) (Table 2) (Breuer et al., 2012).

The extraction yields of bioconstituents for the various initial concentrations (60, 70, 80, 90, and 100 g/L) of *C. mexicana* and *C. pitschmannii* biomass were assessed under established optimal MP conditions (210 W and 2 min) (Ha et al., 2020a). The extraction yield of three bioconstituents (carbohydrates, proteins, and lipids) decreased remarkably to 22%−5% and 15%−3%; 44%−8% and 31%−4%; 13%−2% and 8%−1.7% at 60–100 g/L for *C. mexicana* and *C. pitschmannii*, respectively (Fig. 1B), possibly because of the high viscosity (23–135 cP) and low mass flow rate (2.1×10^{-6}–1.63×10^{-6} kg/s) of the highly concentrated suspensions (Table 1). The cell wall of *C. pitschmannii* accumulates neutral and polar lipids, reducing the extraction efficiency (Olmstead et al., 2013). The highly concentrated and viscous suspension of microalgal strains with different physiological properties could significantly influence the extraction efficacy of bioconstituents, its subsequent transformation to high-titer biofuels, and ultimately economic feasibility of the process.

3.3. Standardization of microwave pretreatment for extraction of biocomponents from high concentration suspensions

The extraction efficiency of bioconstituents from *C. mexicana*, and *C. pitschmannii* was investigated under various microwave powers (210, 280, 350, 420, 490, 560, 630, and 700 W) and pretreatment times (1–5 min) for improving the utilization of microalgal bioconstitutents. The maximum extraction yields of carbohydrates, proteins, and lipids from *C. mexicana* and *C. pitschmannii* (100 g/L) were 32.8%, 57.4%, and 22.6%; 26.6%, 47.5%, and 17.4%, respectively, at 700 W and 1 min (Table 3). The extraction yields gradually increased with microwave power and time and attained the equilibrium at 560 W and 5 min; 630 W and 2 min; and 700 W and 1 min, respectively, from 100 g/L of microalgal suspensions. After MP, the temperature of the microalgal suspensions (60–100 g/L) reached 63–70 °C under optimal microwave conditions. These observations suggest that microwave irradiation/heating accelerates the disruption of the cell walls, resulting in the improved release of biocomponents (Chew et al., 2019). The extraction yield of lipid-rich *C. pitschmannii* was lower than that of carbohydrate-rich *C. mexicana*, which is caused by the high density, thicker cell wall structure of *C. pitschmannii*, reducing the bioaccessibility of a substrate and pretreatment efficiency (Aligata et al., 2019). The extraction yield of protein in both microalgal strains of 100 g/L decreased at 560 W for 6 min, 630 W for 3 min, and 700 W for 2 min, respectively. This may be due to the high sensitivity of proteins that may denature when exposed to microwave radiation and heat at higher power and longer durations (Fu et al., 2018). The power density (567–1891 kW/m^3), a scalable microwave process parameter, increased with the extraction efficiency of biocomponents. Thus, the optimal MP conditions for obtaining the highest extraction yields without protein degradation from both microalgal strains were 350 W and 1 min for 60 g/L; 490 W and 1 min for 70 g/L; 560 W and 1 min for 80 g/L; 630 W and 1 min for 90 g/L; 700 W and 1 min for 100 g/L, respectively (Table 3).

Specific energy consumptions of the MP with optimal conditions on biomass concentration of 60, 70, 80, 90, and 100 g/L were 3.50, 4.20, 4.20, 4.20, and 4.20 MJ/kg by Eq. (1), respectively (Table 3). The energy consumption (4.2 MJ/kg) of the developed MP was 150–400 times lower than the higher energy (630 ~ 1680 MJ/kg) of the previous studies (Kapoore et al., 2018), which required higher power (1000 W) and longer time (~60 min) and caused degradation of the protein. Energy-

Table 3
Extraction yields of biocomponents and specific energy consumption for different microalgal biomass concentrations under corresponding optimum microwave pretreatment conditions.

Initial microalgal biomass concentration (g/L)	Optimum conditions (power and extraction time)	Parameters	*Chlamydomonas mexicana*	*Chlamydomonas pitschmannii*
60	350 W and 1 min	Extraction yield of carbohydrates (%)	28.6 ± 1.2	24.1 ± 1.7
		Extraction yield of proteins (%)	47.2 ± 1.0	43.5 ± 1.3
		Extraction yield of lipids (%)	21.6 ± 1.7	13.0 ± 1.8
		Specific energy consumption (MJ/kg)	3.50	
70	490 W and 1 min	Extraction yield of carbohydrates (%)	29.6 ± 1.8	25.0 ± 1.1
		Extraction yield of proteins (%)	49.5 ± 1.0	43.5 ± 1.8
		Extraction yield of lipids (%)	20.5 ± 1.4	15.3 ± 1.9
		Specific energy consumption (MJ/kg)	4.20	
80	560 W and 1 min	Extraction yield of carbohydrates (%)	31.7 ± 1.0	27.2 ± 1.9
		Extraction yield of proteins (%)	46.9 ± 1.5	47.1 ± 1.3
		Extraction yield of lipids (%)	22.0 ± 1.2	16.0 ± 1.1
		Specific energy consumption (MJ/kg)	4.20	
90	630 W and 1 min	Extraction yield of carbohydrates (%)	33.4 ± 1.8	27.7 ± 1.0
		Extraction yield of proteins (%)	56.2 ± 1.9	48.5 ± 1.4
		Extraction yield of lipids (%)	23.2 ± 1.3	17.6 ± 1.3
		Specific energy consumption (MJ/kg)	4.20	
100	700 W and 1 min	Extraction yield of carbohydrates (%)	32.8 ± 1.2	26.6 ± 1.7
		Extraction yield of proteins (%)	57.4 ± 1.4	47.5 ± 1.2
		Extraction yield of lipids (%)	22.6 ± 1.1	17.4 ± 1.0
		Specific energy consumption (MJ/kg)	4.20	

efficient MP facilitated higher extraction yields of biocomponents from highly concentrated microalgal suspensions without significant protein degradation.

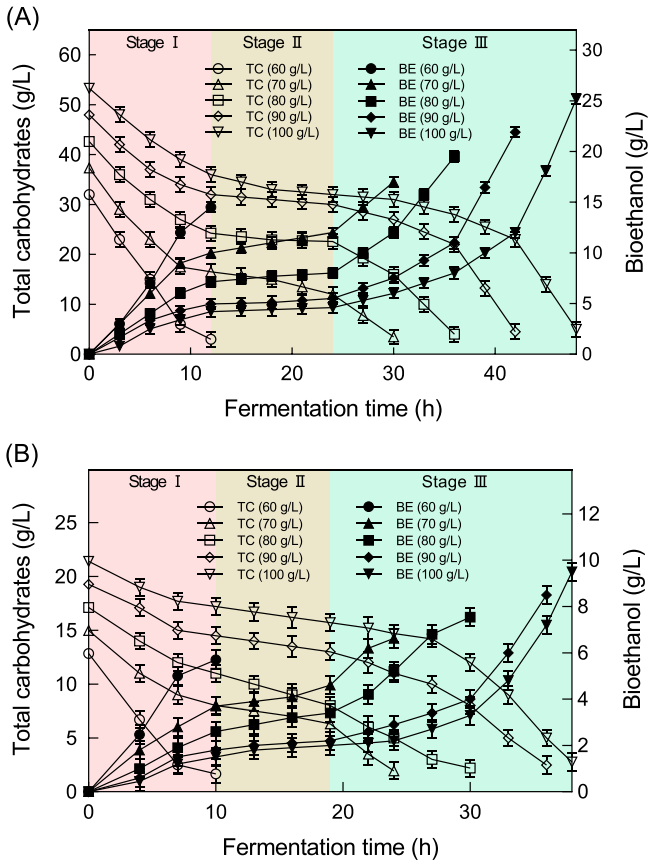

Fig. 2. Total carbohydrate (TC) concentration and bioethanol (BE) production at different concentrations of microwave pretreated *C. mexicana* (A) and *C. pitschmannii* (B) during carbohydrate fermentation.

3.4. Sequential fermentation for high-concentration bioethanol and higher alcohol production

Different concentrations (60, 70, 80, 90, and 100 g/L) of microwave pretreated suspensions were utilized in sequential carbohydrate and protein fermentation for the production of BE and HA. The highest BE concentrations and yields were attained in 100 g/L of *C. mexicana* (25.2 g/L and 0.48 g-ethanol/g-carbohydrates) and *C. pitschmannii* (9.5 g/L and 0.48 g-ethanol/g-carbohydrates) after 48 and 38 hr fermentation, respectively (Fig. 2 and Table 2). The number of *S. cerevisiae* for 100 g/L of biomass were 4.6×10^6 and 4.6×10^6; 3.0×10^7 and 1.2×10^7; 3.1×10^7 and 1.3×10^7; 2.9×10^8 and 1.7×10^8 CFU/mL at initial, stage I, stage II, and stage III, with growth associated constant of 4.296 and 2.85; 0.455 and 0.252; and 8.535 and 5.683 ($R^2 = 0.9417–0.9994$), respectively (Table 4). These results suggest that the biopretreatment of *S. cerevisiae* cells that allow co-extraction of biomolecules, and hydrolytic actions of cellulase and lipase further enhanced bioaccessibility [degrees of cell disruption (stage II)] and bioavailability [availability of dissolved nutrients (stage III)] of highly concentrated and viscous biomass with low mass transfer (at 70–100 g/L) throughout reasonable fermentation period (Fig. 2 and Table 1) (de Carvalho et al., 2020). Total carbohydrate utilization, from an initial concentration of 32–53 and 13–21 g/L for *C. mexicana* and *C. pitschmannii*, respectively, corresponded to carbohydrate fermentation efficiency of 96.8% and 96.2% during BE production (Fig. 2 and Table 2). The thick cell wall of the highly concentrated *C. mexicana* and *C. pitschmannii* was effectively disrupted due to the temperature and pressure of the standardized energy-efficient microwave pretreatment, improving the bioaccessibility and bioavailability of biomass for *S. cerevisiae* (Li et al., 2021), and significantly reducing the fermentation time by 51–62% (Fig. 2). Furthermore, the hydrolytic activity of enzymes facilitated the disruption of the high-density cell wall, resulting in higher biofuel yields (He et al., 2018).

The remaining biomass after carbohydrate fermentation was utilized in subsequent protein fermentation to produce two different HAs (propanol and butanol). Residual protein, regarded as a process waste in the earlier microalgae biorefinery (Giwa et al., 2018), was utilized (91%) and converted into these alternative fuels through protein fermentation by a metabolically engineered *S. cerevisiae* S288C, which improves protein waste management. The highest concentrations of HAs

Table 4
The number of *Saccharomyces cerevisiae* YPH499 and kinetic parameters of Luedeking-Piret model during carbohydrate fermentation at various initial biomass concentrations.

Microalgal strains	Initial biomass concentration (g/L)	Number of yeast cells (CFU/mL)				Growth associated constant			Non-growth associated constant			Coefficient of determination (R^2)
		Initial	Stage I	Stage II	Stage III	Stage I	Stage II	Stage III	Stage I	Stage II	Stage III	
C. mexicana	60	4.6×10^6	7.2×10^7	–	–	7.743	–	–	0.0204	–	–	0.9986
	70	4.5×10^6	5.9×10^7	6.3×10^7	1.1×10^8	6.452	2.033	5.582	0.0133	0.0091	0.0056	0.9742–0.9999
	80	4.6×10^6	5.1×10^7	5.4×10^7	1.8×10^8	5.016	1.138	6.372	0.0125	0.0018	0.0012	0.9685–0.9975
	90	4.5×10^6	4.2×10^7	4.4×10^7	2.5×10^8	4.777	0.849	8.127	0.0013	0.0353	0.0010	0.9721–0.9930
	100	4.6×10^6	3.0×10^7	3.1×10^7	2.9×10^8	4.296	0.455	8.535	0.0019	0.0011	0.0001	0.9417–0.9847
C. pitschmannii	60	4.5×10^6	3.7×10^7	–	–	5.924	–	–	0.0345	–	–	0.9630
	70	4.6×10^6	3.2×10^7	4.0×10^7	8.2×10^7	4.197	1.594	3.772	0.0210	0.0925	0.2361	0.9649–0.9842
	80	4.6×10^6	2.1×10^7	2.8×10^7	9.8×10^7	3.258	0.988	4.882	0.0067	0.0189	0.0061	0.9783–0.9884
	90	4.5×10^6	1.7×10^7	2.0×10^7	1.2×10^8	2.899	0.618	5.598	0.0034	0.0179	0.0041	0.9669–0.9943
	100	4.6×10^6	1.2×10^7	1.3×10^7	1.7×10^8	2.485	0.252	5.683	0.0012	0.0653	0.0025	0.9785–0.9994

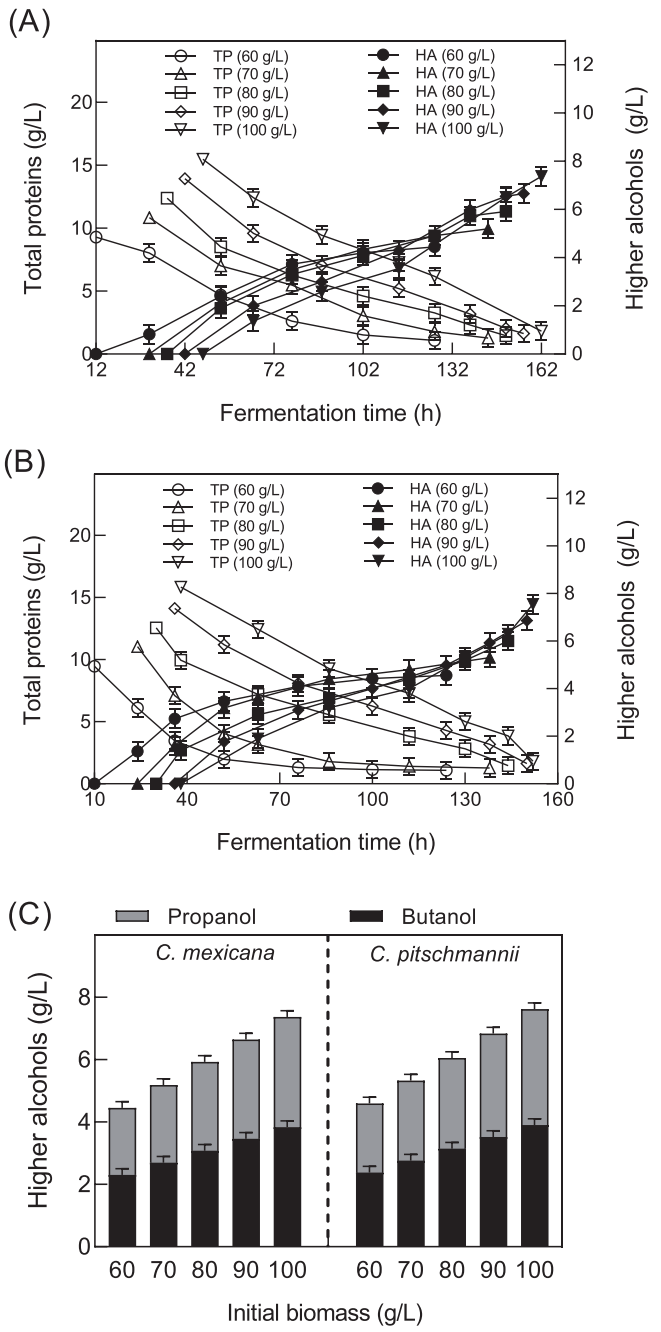

Fig. 3. Total protein (TP) concentration and higher-alcohols (HA) production from *C. mexicana* (A) and *C. pitschmannii* (B) after carbohydrate fermentation at various initial concentrations. Production of HA (propanol and butanol) during protein fermentation (C).

attained in 100 g/L of *C. mexicana* and *C. pitschmannii* were 7.4 and 7.6 g/L with yields of 0.44 and 0.44 g-higher-alcohols/g-proteins in 48–162 and 38–152 h, respectively (Fig. 3 and Table 2). The propanol and butanol generated from *C. mexicana* and *C. pitschmannii* (100 g/L) were 3.5 and 3.8 g/L, and 3.7 and 3.9 g/L, respectively, corresponding to 73–74% of protein fermentation efficiencies (Fig. 3C and Table 2). These HAs are useful in blending with conventional fossil fuels without engine retrofit to provide higher octane numbers, improve energy use efficiency, and reduce pollutant emissions (El-Dalatony et al., 2019a). The rigid cell walls with a high tensile strength of carbohydrate-rich *C. mexicana* and lipid-rich *C. pitschmannii* with high viscosities were completely ruptured by cost-effective biological pretreatment of microorganisms, improved extraction efficiency, and bioavailability. These sequential fermentations provide an economically viable option for high-titer biofuel production by minimizing downstream process costs and maximizing utilization of biocomponents in high concentrations of microalgal biomass (Li et al., 2018).

3.5. *Lipid transesterification for high-concentration biodiesel production, and its profiling and properties*

Residual biomass containing lipids after sequential fermentation was carried out to transesterification for BD production. The remaining lipids from *C. mexicana* and *C. pitschmannii* biomass (100 g/L suspensions) were converted into 18 and 49 g/L of BD with yields of 0.83 and 0.90 g-biodiesel/g-lipids, respectively (Fig. 4 and Table 2). The complete disintegration of the protective layer of algae during sequential fermentations improved the solvent penetration, increasing the lipid extraction efficiency (Taqi et al., 2020). Unconverted residual lipids and glycerol, a by-product after transesterification, can be reused as a low-cost alternative carbon source for the microalgal cultivation process (Chen et al., 2020). The final viscosities and mass flow rate of the different microalgal suspensions (60–100 g/L) decreased to 3.1–5.3 cP and 2.08–2.38·10^{-6} kg/s, respectively after the sequential fermentation and transesterification (Table 1). Microalgal cell walls with stiff microfibrils can be completely disintegrated/transformed into various biofuels via a sustainable integrated approach.

The fatty acid profile and properties of algal BD were analyzed to define its quality. High-quality BDs were obtained from residual *C. mexicana* and *C. pitschmannii* biomass as highest saturated (61 and 63%, respectively) and monounsaturated (37 and 36%, respectively) fatty acids, including predominant palmitic (43% and 40%, respectively), and oleic acid (31 and 30%, respectively) were identified (Table 5). This composition of the BD could be linked to the microwave

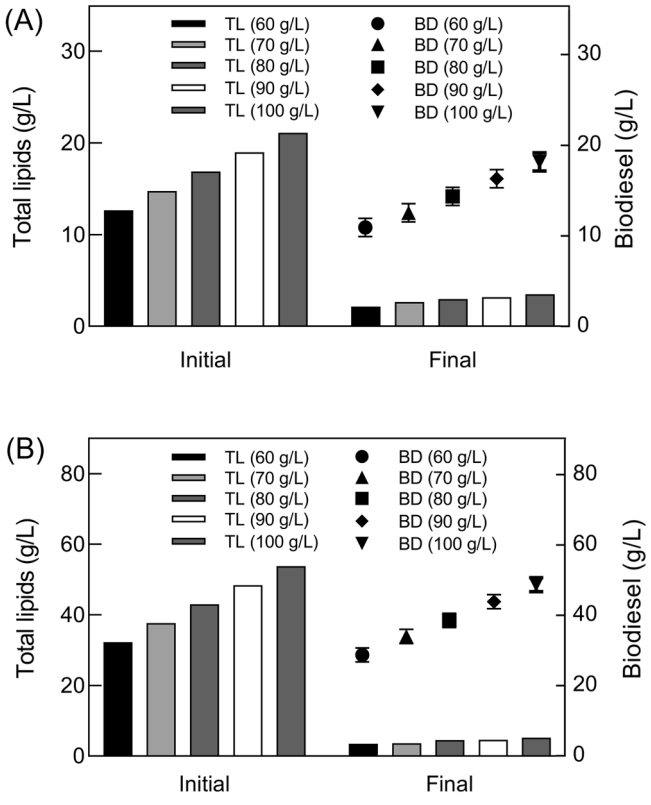

Fig. 4. Total lipid (TL) concentration and biodiesel (BD) production from residual biomass of *C. mexicana* (A), and *C. pitschmannii* (B) after protein fermentation.

Table 5
Fatty acid profiling of biodiesel after transesterification microalgal biomass (at 100 g/L).

Fatty acid	Fatty acid composition (%, w/w)	
	C. mexicana	*C. pitschmannii*
Lauric acid (C12:0)	0.51 ± 0.5	0.46 ± 0.3
Tridecylic acid (C13:0)	1.49 ± 0.6	1.66 ± 0.4
Myristic acid (C14:0)	2.51 ± 0.4	5.40 ± 0.5
Myristoleic acid (C14:1)	4.24 ± 0.8	3.0 ± 0.3
Pentadecanoic acid (C15:0)	0.07 ± 0.6	0.04 ± 0.8
cis-10-Pentadecenoic acid (C15:1n5)	0.19 ± 0.5	0.20 ± 0.6
Palmitic acid (C16:0)	42.6 ± 0.3	40.2 ± 1.3
Palmitoleic acid (C16:1)	1.30 ± 0.3	1.24 ± 0.6
Heptadecanoic (C17:0)	1.40 ± 0.5	1.77 ± 0.7
cis-10-Heptadecenoic (C17:1n7)	0.44 ± 0.4	1.50 ± 0.9
Stearic acid (C18:0)	6.12 ± 0.8	7.65 ± 0.3
Oleic acid (C18:1n9)	31.3 ± 0.7	30.4 ± 1.1
Linoleic acid (C18:2n6)	1.05 ± 0.6	0.98 ± 0.2
γ-Linolenic acid (C18:3n6)	ND	ND
α-Linolenic acid (C18:3n3)	ND	ND
Arachidic acid (C20:0)	ND	ND
Others	6.78 ± 1.4	5.50 ± 1.3
Saturated fatty acid	61.48	62.68
Monounsaturated fatty acid	37.47	36.34
Polyunsaturated fatty acid	1.05	0.98
Total (%)	100	100

ND: not detected.

and biological pretreatment during sequential fermentation which boosted chain scission, decreasing the content of polyunsaturated fatty acids (1%) (Table 5) (El-Dalatony et al., 2019b). Iodine value and cetane number of BD were lower (⟨120) and higher (>47), respectively than the reported EN-14214 standard, indicating high lubrication properties and ignition quality (Yang et al., 2016). Higher long-chain saturation factor, cold filter plugging point, and cloud point indicate better oxidation stability (Chee Loong & Idris, 2017). The higher contents of saturated and monounsaturated fatty acid in BD derived from the microwave and biologically pretreated microalgal biomass will offer high gel points that are appropriate for the utilization in meso/mega-thermal climatical areas (Salama et al., 2017).

3.6. Cell disruption examinations and quantification

Untreated biomass, before and after MP, and carbohydrate and protein fermentation were analyzed through TEM and cell counter to measure the cell wall thicknesses, verify the cellular integrity of the microalgal cells, and the degree of the cell rupture (Figs. 5 and 6). Untreated biomass appeared as intact and spherical microalgal cells. The thicker and stronger cell walls of *C. mexicana* (261 nm) and *C. pitschmannii* (364 nm) showed lysed cells with significantly decreased cell wall thickness (95 and 93 %) due to utilization of carbohydrates (96–97%) during carbohydrate fermentation. Microalgal cells were invisible after protein fermentation, indicating that the algal cell walls were completely disintegrated (Table 2). These indicate that the microwave and biopretreatment of yeast cells effectively lysed rigid cell walls, which improved the bioaccessibility and bioavailability, allowing co-extraction of microalgal biocomponent (de Carvalho et al., 2020).

The MP, sequential fermentation, and transesterification enhanced efficiency of cell disruption for *C. mexicana* and *C. pitschmannii* (at biomass concentration of 100 g/L) in the ranges of 29–40%, 86–88%, 94–95%, and 99%, respectively (Fig. 5). The microalgal cell walls, which are complex biomaterials with biomechanical properties, are effectively disrupted in pectin and cellulose structures in the cell walls by MP. Furthermore, disrupted cell walls accelerated hydrolysis of biocomponents and nutrient bioavailability during fermentation (Zabed et al., 2019).

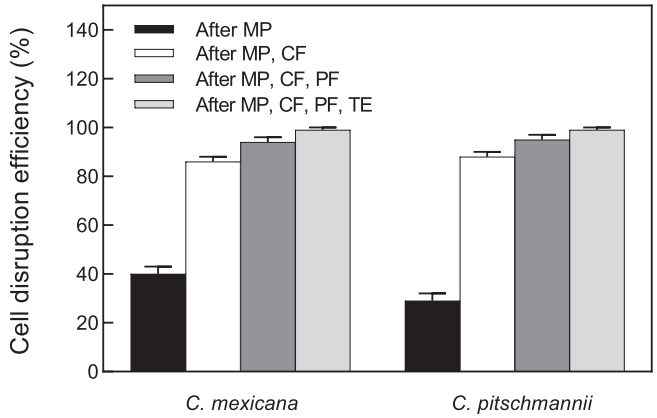

Fig. 5. Microalgal cell disruption efficiency after microwave pretreatment (MP), carbohydrate fermentation (CF), protein fermentation (PF), and transesterification (TE) at 100 g/L.

3.7. Mass balance for biochemical utilization and conversion of microalgal biomass

The compositional analysis for residual biocomponents in microalgal suspension was done for the mass balance of sequential fermentations and transesterification processes. The mass balance indicated the potential of microwave pretreated biomass as a promising feedstock for multiple high-titer biofuel generation. A gradual reduction of biomass in dry weight occurred after each process, and utilization of biochemical components succeeded in the order of carbohydrates > proteins > lipids for both microalgal strains. Total biomass components (carbohydrate, protein, lipid, and ash) at 100 g/L were decreased to 47, 30, and 13 g/L for *C. mexicana*, and 78, 62, and 12 g/L for *C. pitschmannii* after carbohydrate and protein fermentation, and transesterification, respectively (Fig. 6). Total organic matter (93–94%), including carbohydrates (99%), proteins (91–92%), and lipids (84–91%) in both microalgal strains were utilized during all biofuels production (Fig. 6 and Table 2). Unused ash ingredients can be used for soil improvement, pollutant remediation, and biocomposite production to enhance biomass conversion to realize zero-waste algal biorefinery (De Bhowmick et al., 2019). The highest total biomass utilization of 87–88% and conversion efficiencies of 51–67% with *C. mexicana* and *C. pitschmannii* biomass at 100 g/L were obtained for high-titer biofuels, respectively (Table 2). Therefore, regardless of the

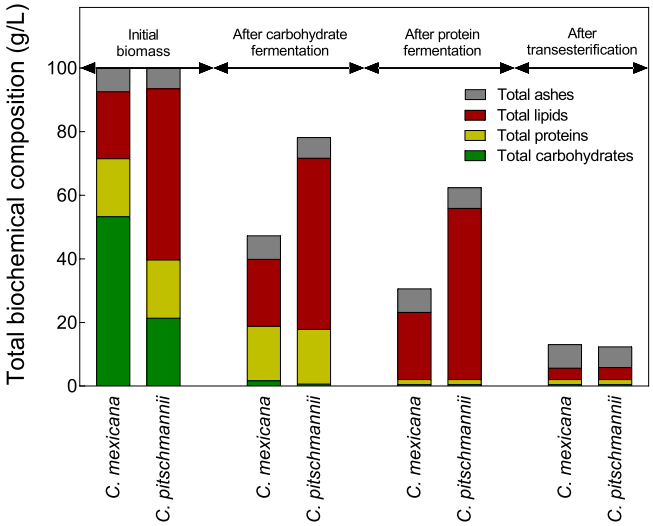

Fig. 6. Mass balances of microalgal biomass during sequential fermentation, and transesterification at 100 g/L.

physiological properties of highly concentrated microalgal suspensions (100 g/L), the sustainable high-throughput integrated pretreatment strategies greatly increase overall biomass utilization and conversion efficiency with the lowest specific energy consumption (4.2 MJ/kg). This strategy could be regarded as an eco-friendly, and cost-effective method for commercialization of high-concentration multiple biofuels generation from microalgal biomass when compared with reported conventional pretreatment methods that are associated with by-products formation and higher energy requirement (Florentino de Souza Silva et al., 2014; Martinez-Guerra et al., 2014).

4. Conclusions

Sustainable high-throughput integrated pretreatments involving energy-efficient MP (4.2 MJ/kg) and sequential fermentation completely disintegrated thick cell walls in highly concentrated (100 g/L) suspension of microalgae with different physiological properties and high viscosities. This approach improved the transformation of bioconstituents to high-titer multiple biofuels with minimum waste production. Overall biomass utilization and conversion efficiency for *Chlamydomonas pitschmannii* were 87 and 67%, respectively, corresponding to the highest BE, HA, and BD yields of 0.48, 0.44; and 0.90 g/g, respectively. The proposed integrated strategies can be useful in overcoming bottleneck of converting highly concentrated microalgal suspension into high-titer biofuels toward biofuel commercialization.

CRediT authorship contribution statement

Geon-Soo Ha: Conceptualization, Funding acquisition, Resources, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Shouvik Saha:** Validation, Investigation, Writing - review & editing. **Bikram Basak:** Validation, Investigation, Writing - review & editing. **Mayur B. Kurade:** Resources, Supervision, Writing - review & editing. **Gyeong-Uk Kim:** Methodology, Validation, Formal analysis, Investigation. **Min-Kyu Ji:** Resources, Investigation, Writing - review & editing. **Yongtae Ahn:** Resources, Writing - review & editing. **El-Sayed Salama:** Resources, Writing - review & editing. **Soon Woong Chang:** 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.

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Appendix A. Supplementary data

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