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Enhanced reducing sugar production and extraction for *Chlorella vulgaris* in mixotrophic cultivation using high hydrostatic pressure processing and ultrasound

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ABSTRACT

Although extraction of polysaccharides to convert reducing sugars (RS) from microalgae by acid or alkali pretreatments and enzymatic hydrolysis has been extensively studied, few reports exploring the use of high hydrostatic pressure processing (HHP) and ultrasonication (US) as emerging technologies for the extraction of sugars from microalgae biomass exist. Thus, the present study was conducted to determine the effects of mixotrophic growth and stress conditions ($NaNO_3$ and CO_2 concentration and light intensity) on RS and protein accumulation in the unicellular green alga *Chlorella vulgaris* in addition to optimization of the effectiveness of the sequential applications of HHP and US with dilute acid as well as simultaneous enzymatic saccharification on the production of RS from microalga cells. High light intensity, high CO_2 concentration and limited nitrogen concentration promoted RS production. The maximum protein content (0.0683 mg g^{-1}) was achieved at 0.3 g l^{-1} $NaNO_3$ concentration, 7000 µmol photons m^{-2} s^{-1} and 6 l min^{-1} CO_2 concentration. The highest RS content of *C. vulgaris* after 48 h enzymatic saccharification (583.86 ± 13.23 mg g^{-1}) was obtained at 1% (w/w) acid concentration and 80% amplitude for 30 min with 79.4% RS yield. Combined US-assisted dilute acid pretreatment and enzymatic hydrolysis were also found to be more effective than HHP assisted dilute acid pretreatment and enzymatic saccharification. Therefore, microalgal biomass can be considered a suitable renewable feedstock used in fermentation.

HIGHLIGHTS

- The cultivation period of *Chlorella vulgaris* was reduced from 25 days to 14 days using mixotrophic growing conditions.
- Mixotrophic conditions enhanced reducing sugar productivity.
- Novel extraction techniques enhanced the extraction of reducing sugar from microalgae.

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KEYWORDS High hydrostatic pressure processing; light intensity; microalga; mixotrophic cultivation; nitrogen starvation; ultrasonication

Introduction

Microalgae are a good source of valuable compounds such as protein, carbohydrates, amino acids, lipids and pigments and third generation of biodiesel feedstock, and have gained much interest in the past few decades because they have starch and cellulosic content without lignin, fast-growing characteristics (Miao & Wu, 2006), and large amounts of synthesized carbohydrates (Subhadra & Edwards, 2010). There are also advantages of using microalgal biomass as a resource in comparison to plant biomass, such as the use of non-arable land, high recovery of nutrients from wastewater and high yield of biomass production (Benedetti *et al*., 2018). Moreover, microalgae are renewable, economical and sustainable biomass sources (Khan *et al*., 2018).

Some physicochemical factors such as nutrients, temperature, light source, pH and salinity have been reported to have effects on microalgal biomass cultivation. The type of cultivation conditions such as photoautotrophic, heterotrophic, mixotrophic or photoheterotrophic, affect biomass growth and cellular composition of microalgae (Brennan & Owende, 2010). Microalgae accumulate carbohydrates (55% of dry weight, DW) during the photosynthetic process (Yao *et al*., 2012). Nutrient starvation such as nitrogen and sulphur depletion (Dragone *et al*., 2011; Zhu *et al*., 2014), light intensity (Brányiková *et al*., 2011), light-dark cycle (Zhu *et al*., 2014), and CO_2 concentration (Izumo *et al*., 2007) can promote starch and carbohydrate accumulation. For instance, it has been found that increasing CO_2 concentration from 1 to 30% significantly increased carbohydrate content (Li *et al*., 2015). Moreover, carbohydrate accumulation in the haptophyte *Isochysis zhangjiangensis* increased under limited nitrogen concentration (Wang *et al*., 2014). Combining two or three stress conditions was observed to enhance carbohydrate accumulation. For example, Ho *et al*. (2012) reported that a combination of high light intensity and nitrogen starvation conditions was used to obtain carbohydrate or starch-rich

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microalgae. However, nitrogen starvation reduced the protein synthesis by increasing lipid and carbohydrate content due to a decrease in the cell growth rate of microalgae (Agirman & Cetin, 2017). Moreover, high growth ratio, high lipid, carbohydrate and protein contents have made *C. vulgaris* a renewable source for biofuel, chemicals and enzymes production (Rehman & Anal, 2018).

Utilization of effective extraction methods of polysaccharides from microalgal cells is a crucial step towards high-value-added products. Extraction of polysaccharides from microalgae cells to convert into reducing sugars (RS) was practiced by common conventional methods such as acidic or alkaline pretreatment methods (H_2SO_4, H_3PO_4 and NaOH) and enzymatic hydrolysis (Hernández *et al.*, 2015). *Chlorococcum* sp. was disrupted using different pretreatment methods such as high-pressure homogenization (73.8% disruption), sulphuric acid treatment (33.2% disruption), bead beating (33.2% disruption) and ultrasonics (4.5% disruption) (Halim *et al.*, 2012).

There are only a limited number of studies focusing on microalgal growth under multiple stress conditions in conjunction with novel extraction techniques of RS from microalgae, especially from the unicellular green alga *Chlorella vulgaris*. To our knowledge, novel extraction techniques of RS from microalgae followed by enzymatic saccharification which is the conversion, by enzymes, of starches, cellulose or hemicellulose into RSs and dextrins during the mashing process have not been reported so far. This study investigates the combined effect of stress conditions on *C. vulgaris* growth, and the use of two novel cell disruption techniques such as high hydrostatic pressure (HHP) and ultrasonication (US) for RS extraction. Information obtained is critical to determine the full potential of microalgal cells as a feedstock for bioprocessing applications, especially in bioethanol and enzyme production. The objectives of this study were: (1) to explore the effects of stress conditions ($NaNO_3$ concentration, light intensity and CO_2 concentration) on maximum biomass, RS and protein content, (2) to apply BBD-RSM to quantify the effect of maximum biomass, RS and protein content and (3) to analyse the sequential applications of two novel cell disruption techniques (HHP and ultrasonication) and enzymatic saccharification for RS extraction.

Materials and methods

Chlorella vulgaris *culture*

The microalga, *Chlorella vulgaris* (SAG strain Number: 211–11b) was provided by the Experimental Phycology and Culture Collection of Algae from the University of Goettingen in Germany (EPSAG).

C. vulgaris was cultivated in single beam photobioreactors (PBR) including modified BG-11 medium containing $NaNO_3$ (0.25 g l^{-1}), $MgSO_4 7H_2O$ (0.075 g l^{-1}), NaCl (0.025 g l^{-1}), K_2HPO_4 (0.075 g l^{-1}), KH_2PO_4 (0.175 g l^{-1}), $CaCl_2 2H_2O$ (0.025 g l^{-1}), H_3BO_3 (0.14 g l^{-1}), trace elements of EDTA stock (1 ml), Fe solution (1 m) and set to 30°C under continuous illumination (12:12 light:dark cycle). The cultivation time for the experiment was 25 days, with the initial biomass of microalgae of *ca*. 0.14 g l^{-1}. Light intensity was measured with a lux meter. The PBR was placed in a cabinet covered with aluminium foil to minimize the effect of light disturbance.

Determination of dry cell weight and density

C. vulgaris cells were harvested by centrifugation at 4600 × g for 20 min. After that, the cells were washed twice using 20 ml of distilled water. Cell samples were oven-dried at 80°C overnight and weighed. The initial and final weight of the filter paper was recorded, and then biomass content was calculated from dried cell weight per litre of growth medium (g l^{-1}). Cell density was measured with a spectrophotometer (PG Instruments T80, Leicestershire, UK) at 660 nm wavelength.

Determination of reducing sugar content-DNS method

RS content of the dried microalgal pellet was estimated using DNS method (Miller, 1959). A calibration curve for this method was prepared using analytical grade D-glucose (Merck Chemical companies, Deisenhofen, Germany) solutions at 0.15–1.00 g l^{-1} concentrations.

Determination of protein content

The total protein content of the algal samples was determined following Lowry *et al.* (1951) with bovine serum albumin (BSA) (Sigma-Aldrich, New York, USA) used as a protein standard. A sample of 0.5 ml was added to 0.7 ml Lowry solution, mixed thoroughly, and incubated for 20 min at room temperature in the dark before 0.1 ml Folin's reagent (Sigma-Aldrich, New York, USA) was added. After mixing, it was left for 30 min at room temperature in the dark, and then absorbance of each sample was determined at 750 nm against blank.

Determination of reducing sugar extraction of Chlorella vulgaris

Conventional pretreatment methods: acid pretreatment

0.3 g of the dried algal pellet was hydrolysed with 3 ml of 72% (w/v) H_2SO_4 at 30°C and then mixed at 150 rpm

for 60 min using a shaking water bath. Eighty-four ml of deionized water was then added to bring the concentration to 4% (w/w) before autoclaving at 121°C for 1 h. After acid hydrolysis, the solid algal residue was separated by centrifugation (10 000 rpm for 10 min) (Thermo Fisher Scientific, USA), and the pH of the supernatant was adjusted to 5.0 using 10 M NaOH.

Alternative pretreatment methods

Alternative extraction techniques such as HHP and US were carried out at optimum culture conditions of microalga to leach RS.

High hydrostatic pressure (HHP) extraction

Extraction of RS was carried out using a pilot scale HHP system with an operating pressure capacity of 690 MPa (2 l model, Avure Systems, Columbus, Ohio, USA) at optimum culture conditions for microalga. The time required to reach the maximum pressure was less than 30s. The ground microalga biomass was pretreated with 87 ml H_2SO_4 solution at the concentrations of 1, 2 and 3% (w/v), pressures of 200, 350 and 500 MPa and pretreatment times of 5, 12.5 and 20 min at biomass solid to liquid ratio of 0.3:87 (w/v, dry weight basis) according to the experimental conditions. The samples were placed in a high-density polyethylene bag (HDPE) and then vacuum packed. After vacuum packaging, each sample was placed into the HHP chamber to be processed. The initial temperature of the samples was 22°C, but the processing temperature increased to 25 or 30°C depending on the process conditions. Processed samples after HHP were hydrolysed with enzymes defined in the following enzymatic saccharification section.

Ultrasonication (US) extraction

The Optic Ivymen Systems CY-500 homogenizer (Hielscher, Germany) with the standard probe of 5.6 mm diameter was used to leach RS. It has a 500 W power and 20 kHz frequency. Extraction temperature was kept constant at 20°C using a water bath. The sample was placed into a 100 ml beaker, and an ultrasonic probe was dipped at most 1.5 cm depth into the extraction media. The ground microalgae biomass (0.3 g) was pretreated with 87 ml H_2SO_4 solution at concentrations of 1, 2 and 3% (w/v), amplitudes of 70, 80 and 90%, and pretreatment times of 5, 12.5 and 20 min according to the experimental conditions. Processed samples after US were hydrolysed with enzymes defined in the following enzymatic saccharification section. Processed samples after US and enzymatic saccharification were analysed for RS using DNS method.

Enzymatic saccharification

Recovered dried algal residues from conventional and alternative pretreatment methods were hydrolysed using cellulase and Viscozyme L enzyme cocktail (Sigma-Aldrich, Denmark) containing cellulase and xylanase enzymes. The enzyme activity of cellulase and xylanase were measured at 2250 and 1400 U ml^{-1}, respectively at 50°C, 130 rpm, and pH 5.0 for 48 h in 100 l flasks. After enzymatic saccharification, the samples were heated to 100°C for 15 min to inactive the enzymes. The samples with the equivalent enzyme loading control group were also hydrolysed. After enzymatic saccharification, the samples were centrifuged at 10 000 rpm for 10 min, and the supernatant was used to estimate RS using the DNS method.

Data analysis and experimental design

Statistical analyses were conducted to test the mean significant differences both in microalgae growth conditions in terms of biomass, RS and protein content as a function of $NaNO_3$ concentration, light intensity and CO_2 concentration, and in RS production using HHP and US conditions in terms of RS as a function of H_2SO_4 concentration, pressure, pretreatment time, H_2SO_4 concentration and amplitude, respectively. Optimization was carried out using the Box-Behnken Design (BBD) as the response surface methodology with a quadratic model. $NaNO_3$ concentration (χ_1; 0.1, 0.2 and 0.3 g l^{-1}, w/v), light intensity (χ_2; 6500, 7000 and 7500 μmol photons m^{-2} s^{-1}), and CO_2 concentration (χ_3; 6, 12 and 18 l min^{-1}) were chosen to be optimized for biomass, RS and protein content. The chosen ranges of factors for RS production using HHP were H_2SO_4 concentrations of 1, 2 and 3% (w/v), pressures of 200, 350 and 500 MPa, and pretreatment times of 5, 12.5 and 20 min. The factors and levels for RD production using US were selected as amplitudes of 70, 80 and 90%, H_2SO_4 concentrations of 1, 2 and 3%, and pretreatment times of 15, 22.5 and 30 min.

The levels of these variables were determined using preliminary experiments. The uncoded predictors and the overall BBD are given in Tables 1 and 2. Biomass, protein content, and RS using HHP and US were reported by averaging three replicates of each run (total of 15 runs). All the statistical analyses were performed using JMP PRO software package.

Equation (1) was used to fit the experimentally collected data:

Table 1. (Un)coded variables of Box-Behnken design of culture conditions for biomass, reducing sugars (RS) and protein content (n = 3).

	Variables			Results**		
Levels/ run order	A ($g\ l^{-1}$)	B (μmol photons $m^{-2}\ s^{-1}$)	C ($l\ min^{-1}$)	Biomass ($g\ l^{-1}$)	RS ($mg\ g^{-1}$)	Protein content ($mg\ g^{-1}$)
M1*	0.2 **(0)**	7000 **(0)**	12 **(0)**	0.566 ± 0.000^{c}	587.34 ± 4.05^{a}	0.040 ± 0.001^{cd}
M2	0.1 **(−1)**	7000 **(0)**	18 **(+1)**	0.351 ± 0.001^{f}	388.12 ± 6.95^{ef}	0.016 ± 0.001^{i}
M3	0.1 **(−1)**	7000 **(0)**	6 **(−1)**	0.819 ± 0.007^{b}	331.52 ± 15.86^{f}	0.020 ± 0.005^{hi}
M4	0.1 **(−1)**	6500 **(−1)**	12 **(0)**	0.312 ± 0.002^{g}	385.49 ± 7.18^{ef}	0.025 ± 0.002^{fgh}
M5	0.2 **(0)**	6500 **(−1)**	18 **(+1)**	0.302 ± 0.002^{g}	398.83 ± 0.00^{def}	0.029 ± 0.001^{fg}
M6	0.2 **(0)**	6500 **(−1)**	6 **(−1)**	0.433 ± 0.001^{e}	465.13 ± 13.29^{cd}	0.030 ± 0.000^{ef}
M7*	0.2 **(0)**	7000 **(0)**	12 **(0)**	0.558 ± 0.005^{c}	598.23 ± 30.55^{a}	0.043 ± 0.000^{c}
M8	0.3 **(+1)**	7500 **(+1)**	12 **(0)**	0.865 ± 0.003^{a}	436.62 ± 20.86^{cde}	0.033 ± 0.001^{def}
M9	0.2 **(0)**	7500 **(+1)**	18 **(+1)**	0.489 ± 0.001^{d}	466.86 ± 12.14^{cd}	0.018 ± 0.003^{hi}
M10	0.3 **(+1)**	7000 **(0)**	18 **(+1)**	0.545 ± 0.004^{c}	454.32 ± 40.20^{cde}	0.054 ± 0.001^{b}
M11	0.1 **(−1)**	7500 **(+1)**	12 **(0)**	0.528 ± 0.023^{c}	417.41 ± 17.44^{cde}	0.021 ± 0.001^{hi}
M12	0.3 **(+1)**	7000 **(0)**	6 **(−1)**	0.876 ± 0.009^{a}	482.74 ± 23.00^{bc}	0.068 ± 0.003^{a}
M13	0.3 **(+1)**	6500 **(−1)**	12 **(0)**	0.867 ± 0.002^{a}	459.73 ± 11.50^{cd}	0.037 ± 0.000^{cde}
M14	0.2 **(0)**	7500 **(+1)**	6 **(−1)**	0.863 ± 0.003^{a}	546.62 ± 1.34^{ab}	0.022 ± 0.001^{ghi}
M15*	0.2 **(0)**	7000 **(0)**	12 **(0)**	0.536 ± 0.021^{c}	555.25 ± 10.39^{a}	0.031 ± 0.009^{ef}
	A = $NaNO_3$ concentration		**B** = Light intensity		**C** = CO_2 concentration	

*Centre-points.
**Different letters in the same column show statistically significance between the mean values ($p < 0.05$).

Table 2. Revised-analysis of variance (ANOVA) results and estimated regression coefficients for coded biomass ($g\ l^{-1}$), RS ($mg\ g^{-1}$), and protein content ($mg\ g^{-1}$) based on RSM-BBD conditions.

	Biomass ($g\ l^{-1}$)		RS ($mg\ g^{-1}$)		Protein content ($mg\ g^{-1}$)	
Terms	Coeff	p value	Coeff	p value	Coeff	p value
Linear						
A	0.1428	0.000	38.86	0.000	0.0138	0.000
B	0.1040	0.000	19.79	0.036	−0.0035	0.087*
C	−0.1631	0.000	−14.74	0.110*	-	-
Square						
A^2	0.1108	0.005	−105.30	0.000	0.0031	0.298*
B^2	-	-	−50.10	0.001	−0.0119	0.000
C^2	-	-	−60.80	0.000	-	-
Interaction						
A*B	-	-	-	-	-	-
A*C	-	-	-	-	-	-
B*C	-	-	-	-	-	-
Lack-of-fit	-	**0.001**	-	**0.243**	-	**0.327**
Constant	0.5345	0.000	580.30	0.000	0.0373	0.000
R^2	0.81		0.84		0.75	
R^2 ***(adj)***	0.78		0.79		0.70	
R^2 ***(pred)***	0.69		0.70		0.60	

A = $NaNO_3$ concentration, B = Light intensity, C = CO_2 concentration.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}{X_1}^2 + b_{22}{X_2}^2 + b_{33}{X_3}^2 \quad (1)$$

where Y_1, Y_2 and Y_3 are the response variables of biomass, protein content and extraction of RS using HHP and US; *b*s are regression slope coefficients; and *A*, *B*, *C*, *D*, *E*, *F*, *G*, *H* and *J* are the $NaNO_3$ concentration, light intensity and CO_2 concentration for growth conditions; H_2SO_4 concentration, pressure and pretreatment time for HHP; amplitude, H_2SO_4 concentration and pretreatment time for US, respectively. The culture conditions of microalga, biomass and extraction of RS using HHP and US were optimized using the response optimizer function under DOE-RSM. To validate the models, additional experiments were conducted in triplicate under the optimal conditions of biomass and RS using HHP and US. Analysis of variance (ANOVA) and regression models were performed at 95% confidence interval ($p < 0.05$) to define the significant terms of the predictive model. ANOVA was performed to determine the statistically significant effects of the three predictors ($p < 0.05$). Multiple comparisons were made using Tukey's test. The coefficient of variation (CV) value was computed to verify the predicted model using Equation (2):

$$CV = \frac{\sigma}{\bar{X}} 100 \quad (2)$$

where σ is sample standard deviation, and X is sample mean.

The absolute error was calculated based on Equation (3) as shown below:

$$Absolute\ error(\%) = \left| \frac{Experimental\ value - Predicted\ value}{Predicted\ value} \right| \times 100 \quad (3)$$

Results

Effect of the relationship between Chlorella vulgaris *culture conditions and biomass*

The growth profile of *C. vulgaris* in terms of optical density (OD at 600 nm), which is a good indicator of how algal biomass is cultivated, is shown in Fig. 1. The cultivation period was approximately 25 days under the different growth conditions. *C. vulgaris* biomass was measured and used to evaluate the overall culture conditions efficacy.

Various culture conditions of *C. vulgaris* were performed to generate biomass, protein content and RS (Table 1). The microalgal biomass ranged from 0.302 ± 0.002 to 0.876 ± 0.009 g l^{-1} under different culture conditions. The highest biomass was found as 0.876 ± 0.009 g l^{-1} under 0.3 g l^{-1} $NaNO_3$ concentration, 7000 μmol photons m^{-2} s^{-1} and 6 l min^{-1} CO_2 concentration (M12), whereas the lowest biomass was found as 0.302 ± 0.002 g l^{-1} under 0.2 g l^{-1} $NaNO_3$ concentration, 6500 μmol photons m^{-2} s^{-1} and 18 l min^{-1} CO_2 concentration (Table 1). With the increased $NaNO_3$ concentration during culture growth, the biomass increased by 2.8-fold (Table 1, runs M4 & M13). When $NaNO_3$ concentration decreased from 0.3 to 0.1 g l^{-1}, biomass decreased from 0.865 ± 0.003 to 0.528 ± 0.023 g l^{-1} under 7500 μmol photons m^{-2} s^{-1} and 12 l min^{-1} CO_2 concentration (Table 1, runs M8 & M11). When the light intensity was increased from 6500 to 7500 μmol photons m^{-2} s^{-1}, biomass increased from 0.302 ± 0.002 to 0.489 ± 0.001 g l^{-1} (Table 1, runs M5 & M9). Also, when CO_2 concentration was decreased from 18 to 6 l min^{-1}, biomass increased from 0.351 ± 0.001 to 0.819 ± 0.007 g l^{-1} (Table 1, runs M2 & M3). Therefore, CO_2 concentration played the most crucial role in enhancing biomass, given the high F (78.18) and very low p values (< 0.000) (data not shown). Results from ANOVA also supported these results in that the significant linear terms were found for $NaNO_3$ concentration ($p < 0.000$) and light intensity ($p < 0.000$) with a positive effect on biomass but the CO_2 concentration ($p < 0.000$) with a negative effect (Table 2).

The relationship between Chlorella vulgaris *culture conditions and protein content*

Protein content of microalgae ranged from 0.016 ± 0.001 to 0.068 ± 0.003 mg g^{-1} under the different culture conditions. The highest protein content of *C. vulgaris* was found as 0.068 ± 0.003 mg g^{-1} under 0.3 g l^{-1} $NaNO_3$ concentration, 7000 μmol photons m^{-2} s^{-1}, and 6 l min^{-1} CO_2 concentration (run M12), whereas the lowest protein content was found as 0.016 ± 0.001 mg g^{-1} under 0.1 g l^{-1} $NaNO_3$ concentration, 7000 μmol photons m^{-2} s^{-1}, and 18 l min^{-1} CO_2 concentration (run M2) (Table 1). With increased $NaNO_3$ concentration during culture growth, the protein content increased from 0.016 ± 0.001 to 0.054 ± 0.001 mg g^{-1} (*ca.* 3.5-fold) under 7000 μmol photons m^{-2} s^{-1} and 18 l min^{-1} CO_2 concentration (Table 1, runs M2 & M10). Moreover, during the nitrogen starvation, the protein content decreased from 0.033 ± 0.001 to 0.021 ± 0.001 mg g^{-1} under 7500 μmol photons m^{-2} s^{-1} and 12 l min^{-1} CO_2 concentration (Table 1, runs M8 & M11).

When CO_2 concentration decreased from 18 to 6 l min^{-1}, protein content increased from 0.054 ± 0.001 to 0.068 ± 0.003 mg g^{-1} under 0.3 g l^{-1} $NaNO_3$ concentration and 7000 μmol photons m^{-2} s^{-1} (Table 1, runs M10 & M12). Light intensity did not influence the protein content significantly ($p > 0.05$). Also, $NaNO_3$ concentration affected protein content positively, but the quadratic effect of light intensity ($p < 0.05$) affected protein content negatively (Table 2).

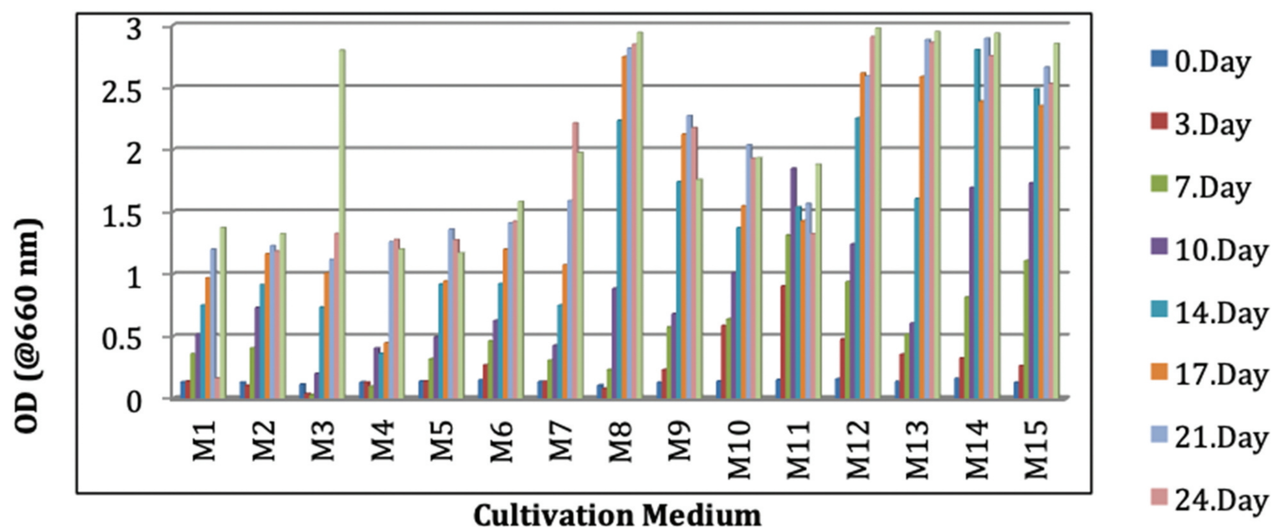

Fig. 1. Growth profile of *Chlorella vulgaris* in terms of optical density (OD at 660 nm).

Effect of the relationship between Chlorella vulgaris *culture conditions and reducing sugars during dilute acid conventional extraction and enzymatic saccharification*

With increased $NaNO_3$ concentration during growth culture, the RS content was increased from 388.12 ± 6.95 to 454.32 ± 40.20 mg g^{-1} under 7000 μmol photons m^{-2} s^{-1} and 18 l min^{-1} CO_2 concentration (Table 1, Runs M2 & M10). When light intensity increased from 6500 to 7500 μmol photons m^{-2} s^{-1}, RS content increased from 465.13 ± 13.29 to 546.62 ± 1.34 mg g^{-1} under 0.2 g l^{-1} $NaNO_3$ and 6 l min^{-1} CO_2 concentrations (Table 1, Runs M6 & M14). According to ANOVA results, $NaNO_3$ concentration ($p < 0.000$) and light intensity ($p < 0.042$) affected RS content after enzymatic saccharification positively, but the quadratic effect of $NaNO_3$ concentration, light intensity and CO_2 concentration ($p < 0.05$) affected RS content after enzymatic saccharification negatively (Table 2).

Before enzymatic saccharification, dried algal biomass was pretreated with dilute acid using conventional or alternative methods. For enzymatic saccharification, recovered dried algal residues after conventional or alternative pretreatment methods were hydrolysed using the enzyme. The RS content of *C. vulgaris* under different culture conditions during dilute acid (conventional) pretreatment ranged from 165.74 ± 10.55 to 284.14 ± 1.23 mg g^{-1}, which varied with $NaNO_3$ concentration, light intensity and CO_2 concentration (Table 3). The highest amount of RS before enzymatic saccharification (284.14 ± 1.23 mg g^{-1}) was obtained at $NaNO_3$ concentration of 0.2 g l^{-1}, 7000 μmol photons m^{-2} s^{-1} and CO_2 concentration of 12 l min^{-1} (Table 3, Run M15). However, the lowest amount of RS before enzymatic saccharification (165.74 ± 10.55 mg g^{-1}) was obtained at $NaNO_3$ concentration of 0.1 g l^{-1}, 7000 μmol photons m^{-2} s^{-1} and CO_2 concentration of 6 l min^{-1} (Table 3, Run M3). The RS content significantly ($p < 0.05$) increased with an increase in light intensity during conventional pretreatment (Table 3, Run M5 and M9), whereas the RS content significantly ($p < 0.05$) increased with a decrease in CO_2 concentration (Table 3, Run M10 and M12).

Enzymatic saccharification of the pretreated *C. vulgaris* was performed to generate RS (Table 3). The RS of microalga ranged from 331.52 ± 15.86 to 598.23 ± 30.55 mg g^{-1} under the different culture conditions of *C. vulgaris*. The highest RS content of *C. vulgaris* after enzymatic saccharification was found as 598.23 ± 30.55 mg g^{-1} under 0.2 g l^{-1} $NaNO_3$ concentration, 7000 μmol photons m^{-2} s^{-1} and 12 l min^{-1} CO_2 concentration (Run M7), whereas the lowest RS content after enzymatic saccharification was found as 331.52 ± 15.86 mg g^{-1} under 0.1 g l^{-1} $NaNO_3$ concentration, 7000 μmol photons m^{-2} s^{-1} and 6 l min^{-1} CO_2 concentration (Run M3) (Table 3).

The highest enzymatic conversion efficiency was obtained as 61.6% at $NaNO_3$ concentration of 0.3 g l^{-1}, 6500 μmol photons m^{-2} s^{-1} and CO_2 concentration of 12 l min^{-1} (Table 3, Run 13). However, this enzymatic conversion efficiency was not significantly different ($p > 0.05$) from run M10, which resulted in 60.8% enzymatic conversion efficiency with higher light intensity and CO_2 concentration. Pretreatments with 0.3 g l^{-1} $NaNO_3$ concentration provided significantly ($p < 0.05$) higher RS conversion efficiency than those with 0.1 and 0.2 g l^{-1} $NaNO_3$ concentrations (Table 3).

Selection of suitable models for biomass, reducing sugar and protein content of microalgae

First (linear), second (quadratic) and third (cubic) order models with interaction terms revealed that the suitability of the model, R^2 must be closer to 1, VIF must be 1, lack of fit value must be $p > 0.05$ and RMSE must be low. Based on R^2, VIF, lack of fit values, RMSE, and AICc for RS and protein content responses, a quadratic model was chosen (Table 4).

Table 3. Comparison of reducing sugars of *Chlorella vulgaris* under different culture conditions during conventional (dilute acid) pretreatment and enzymatic saccharification (ES).

Run number	RS before ES (mg g^{-1})**	RS after ES (mg g^{-1})**	Enzymatic conversion efficiency (%)
M1*	282.60 ± 3.05^{a}	587.34 ± 4.05^{a}	51.9
M2	172.57 ± 11.34^{fg}	388.12 ± 6.95^{ef}	55.6
M3	165.74 ± 10.55^{g}	331.52 ± 15.86^{f}	50.0
M4	188.39 ± 5.08^{efg}	385.49 ± 7.18^{ef}	51.1
M5	215.52 ± 0.00^{de}	398.83 ± 0.00^{def}	45.9
M6	247.78 ± 12.23^{bc}	465.13 ± 13.29^{cd}	46.7
M7*	282.84 ± 6.25^{a}	598.23 ± 30.55^{a}	52.7
M8	215.47 ± 4.44^{de}	436.62 ± 20.86^{cde}	50.7
M9	239.30 ± 6.88^{cd}	466.86 ± 12.14^{cd}	48.7
M10	178.14 ± 10.23^{fg}	454.32 ± 40.20^{cde}	60.8
M11	214.17 ± 14.93^{de}	417.41 ± 17.44^{cde}	48.7
M12	198.53 ± 6.53^{ef}	482.74 ± 23.00^{bc}	58.9
M13	176.62 ± 3.33^{fg}	459.73 ± 11.50^{cd}	61.6
M14	274.19 ± 7.79^{ab}	546.62 ± 1.34^{ab}	49.8
M15*	284.14 ± 1.23^{a}	555.25 ± 10.39^{a}	48.8

*Center-points.
**Different letters in the same column show statistically significance between mean values ($p < 0.05$).

Table 4. Selection of best model for biomass, RS and protein content of *Chlorella vulgaris* after conventional pretreatment methods and enzymatic saccharification.

Response	Models	R^2	VIF	Lack of fit (LOF)	RMSE	AICc
Biomass (g l^{-1})	Linear	0.79	> 1.0	0.000	0.108	−34.26
	Quadratic (BBD based)	0.87	= 1.0	0.000	0.091	−33.94
	Cubic	0.87	> 1.0	0.000	0.713	-85.69
RS (mg g^{-1})	Linear	0.22	> 1.0	0.000	76.72	360.43
	Quadratic (BBD based)	**082**	**= 1.0**	**0.219**	**31.61**	**316.85**
	Cubic	0.96	> 1.0	0.006	34.27	312.08
Protein content (mg g^{-1})	Linear	0.58	> 1.0	0.000	0.010	−174.47
	Quadratic (BBD based)	**0.75**	**= 1.0**	**0.327**	**0.008**	**−181.51**
	Cubic	0.78	> 1.0	0.000	0.007	−194.65

However, linear, quadratic and cubic models for biomass were not suitable according to R^2, VIF, lack of fit values, RMSE and AICc (Table 4).

BBD-based quadratic modelling of culture conditions, reducing sugar production and protein content

The selection of the most accurate model involved several criteria such as R^2, lack-of-fit value and p-value. The goodness-of-fit (R^2_{adj}) of the models showed 78, 79 and 70% variation in biomass, RS, and protein content, respectively. The non-significant lack-of-fit values for RS (p = 0.243), protein content (p = 3.27) and high values of R^2 proved that the model fitted to the experimental data well (Table 2). It was also observed that data analysis by BBD-RSM did not provide good correlations between the process variables ($NaNO_3$ concentration, light intensity and CO_2 concentration) and the explanatory variable (biomass) (Table 2). Therefore, the best fit quadratic models fitted for both RS and protein content.

Quadratic models were the best model for regression of the experimental data for both RS and protein content optimization (Table 4). The degree of influence of the operational conditions on RS and protein content can be inferred from comparing the magnitudes of the coefficients of the quadratic regression models. $NaNO_3$ concentration showed maximum influence in RS followed by light intensity and CO_2 concentration with a relative impact of 38.86, 19.79 and 14.74, respectively. $NaNO_3$ concentration was the most important factor for protein content (0.0138) (Tables 2 and 5).

The surface plots were used to visualize how the operational settings simultaneously influenced the multiple responses of the cultural conditions (Fig. 2). The three-dimensional (3D) response surface was plotted to study the interaction of the three factors on the RS by *C. vulgaris* (Fig. 2a–c). It was observed from Fig. 2a that the lowest light intensity (6500 µmol photons m^{-2} s^{-1}) minimized the RS at the lowest $NaNO_3$ concentration (0.1 g l^{-1}). The centre point of $NaNO_3$ concentration (0.2 g l^{-1}) also maximized RD at 7000 µmol photons m^{-2} s^{-1} (Fig. 2a). Maximum RS was obtained when both factors (light intensity and CO_2 concentration) were at 7000 µmol photons m^{-2} s^{-1} and 12 l min^{-1} CO_2 concentration, respectively (Fig. 2b). RS increased with increased light intensity under the centre point of CO_2 concentration at an increasing rate (Fig. 2b). Figure 2c showed the 3D plot of the interaction between $NaNO_3$ concentration (A) and CO_2 concentration (C) on RS of *C. vulgaris*. The $NaNO_3$ concentration of 0.2 g l^{-1} maximized the RS at the CO_2 concentration of 12 l min^{-1} (Fig. 2c). RS increased with increasing concentrations of CO_2 and $NaNO_3$ at the light intensity of 7000 µmol photons m^{-2} s^{-1} (Fig. 2c). An increase in $NaNO_3$ concentration from 0.1 to 0.2 g l^{-1} gave the highest RS of 598.23 ± 30.55 mg g^{-1} at about 12 l min^{-1}, but a decrease was observed above 0.2 g l^{-1}.

The operational settings were optimized to maximize RS content of *C. vulgaris* under the different culture conditions. Due to their beneficial biotechnological effects, high RS and protein content are desired. The optimum operational conditions were achieved with 0.22 g l^{-1} $NaNO_3$ concentration, 7100 µmol photons m^{-2} s^{-1} and 11 l min^{-1} CO_2 concentration (Table 5). The maximum RS (542.63 mg g^{-1}) was obtained with the optimum operational conditions (d = 1.000) (Table 5). These conditions were experimentally tested to validate the predictive power of the model. The resultant RS content value of 537.34 ± 19.41 mg g^{-1} indicated no significant difference between the measured and predicted values (Table 5). The CV value for RS model was found as 3.61%; smaller CV values reveal the better reproducibility of the model, which is true for this model. The absolute error was also calculated as 0.97 and 16% for RS (Table 5).

Table 5. Best operating Box-Behnken design conditions with respect coded RS and protein content models.

Response	Equation	Experimental verified value at best condition	Predicted value at best condition	Absolute error (%)	CV (%)
RS (mg g^{-1})	$Y_1 = 580.3 + 38.86{*}A + 19.79{*}B - 105.3{*}A^2 - 50.1{*}B^2 - 60.8{*}C^2$	537.34 ± 19.41	542.63	0.97	3.61
Protein content (mg g^{-1})	$Y_2 = 0.037 + 0.014{*}A - 0.012{*}B^2$	0.042 ± 0.001	0.050	16	12.29

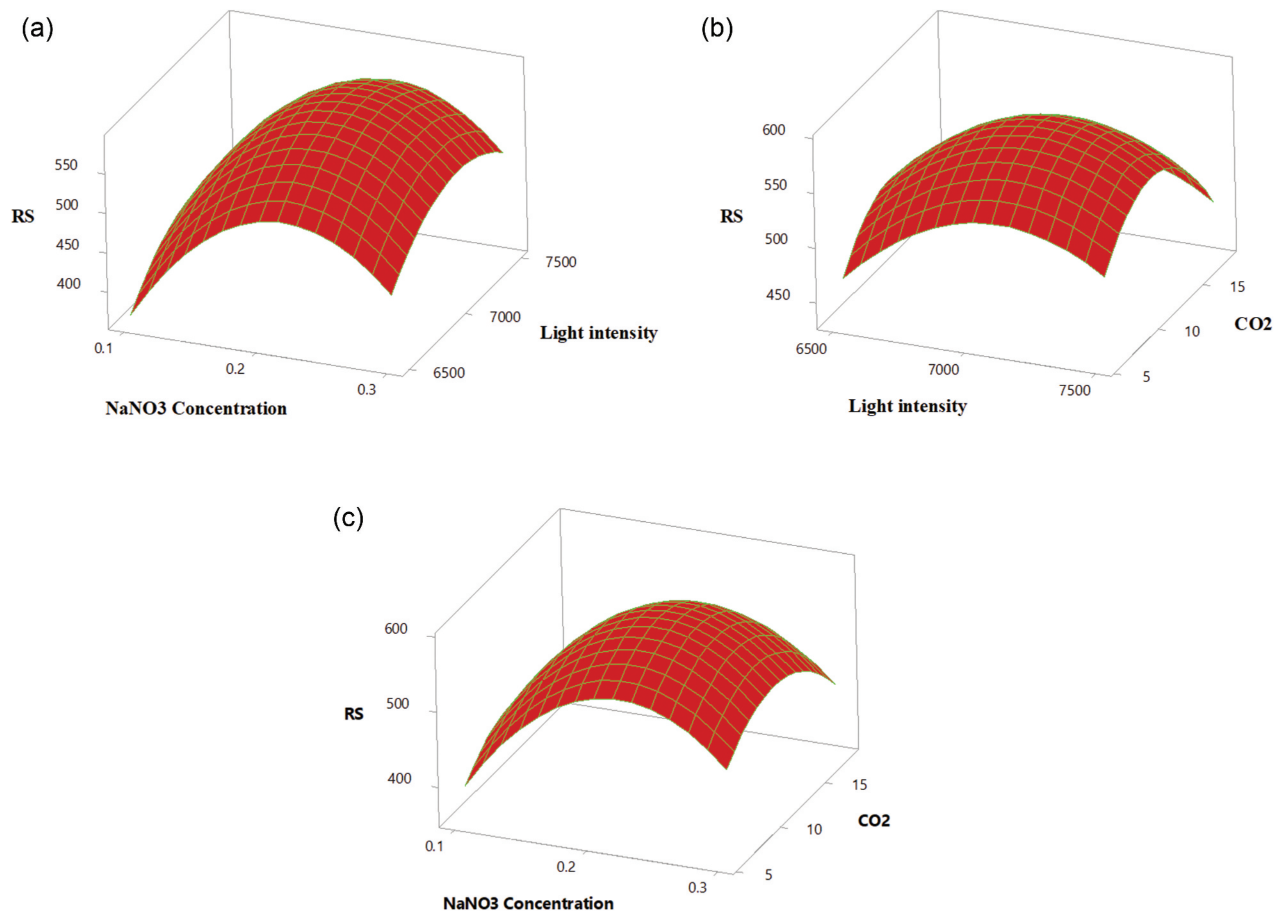

Fig. 2. Response surface plots for the effects of (a) $NaNO_3$ concentration and light intensity; (b) CO_2 and light intensity; (c) $NaNO_3$ concentration and CO_2 concentration on RS.

Effects of combined high hydrostatic pressure extraction techniques and enzymatic saccharification for leaching of reducing sugars

The RS content of untreated *C. vulgaris* was 120 mg g^{-1} dry biomass. To enhance the leaching of RS of *C. vulgaris*, conventional acid and alternative pretreatment methods such as HHP and US were performed (Fig. 3). As shown in Fig. 3, the combined HHP and enzymatic treatment gave significantly higher RS content than that of the HHP treatment alone ($p < 0.05$). The highest RS content was obtained from the combined conventional acid and enzymatic treatments (Fig. 3).

The RS content of microalga ranged from 339.38 ± 22.79 to 419.44 ± 11.57 mg g^{-1} under the different HHP conditions (Table 6). Compared with the untreated control samples, RS concentration increased by 71.4% with 1% acid concentration at 500 MPa for 12.5 min treatment. The highest RS content (419.44 ± 11.57 mg g^{-1}) of *C. vulgaris* after 48 h enzymatic saccharification was obtained at 1% (w/w) acid concentration and 500 MPa for 12.5 min. In contrast, the lowest RS content (339.38 ± 22.79 mg g^{-1}) after 48 h enzymatic saccharification was obtained at 2% (w/w) acid concentration and 500 MPa for 5 min (Table 6). RS content was significantly affected only by the interaction between acid concentration and time ($p < 0.05$) (data not shown). The RS were also low compared with the conventional techniques followed by enzymatic saccharification during 48 h (Table 6).

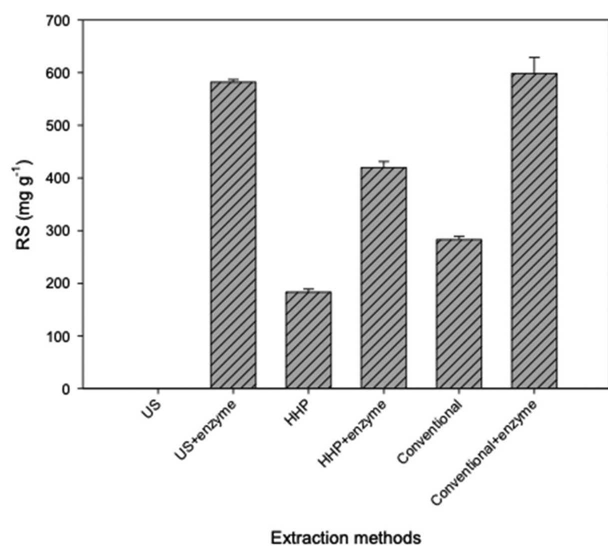

Fig. 3. Reducing sugar content (mg g^{-1}) under the different extraction methods.

Table 6. Leaching of reducing sugars (RS) (mg g^{-1}) using combination of HHP+ES, US+ES, and conventional technique +ES after 48 h.

	HHP+ES*				US+ES**				Conventional+ES
Run	D (%)	E (MPa)	F (min)	RS (mg g^{-1})	G (%)	H (%)	J (min)	RS (mg g^{-1})	RS (mg g^{-1})
1	3	350	5	360.41 ± 11.01^{bcd}	90	1	22.5	543.40 ± 18.48^{bc}	587.34 ± 4.05^{a}
2	3	350	20	383.10 ± 17.32^{abcd}	70	1	22.5	456.22 ± 3.08^{g}	388.12 ± 6.95^{ef}
3	1	200	12.5	362.42 ± 5.66^{bcd}	80	2	22.5	515.03 ± 14.84^{cde}	331.52 ± 15.86^{f}
4	2	350	12.5	393.50 ± 17.44^{ab}	90	3	22.5	478.39 ± 4.86^{efg}	385.49 ± 7.18^{ef}
5	1	350	5	386.48 ± 10.12^{abc}	90	2	15	497.64 ± 4.24^{def}	398.83 ± 0.00^{def}
6	1	350	20	341.07 ± 0.92^{cd}	70	3	22.5	523.06 ± 6.90^{bcd}	465.13 ± 13.29^{cd}
7	1	500	12.5	**419.44 ± 11.57^{a}**	80	3	15	**581.90 ± 4.96^{a}**	**598.23 ± 30.55^{a}**
8	2	350	12.5	348.38 ± 9.41^{bcd}	80	2	22.5	509.78 ± 7.42^{cde}	436.62 ± 20.86^{cde}
9	2	350	12.5	384.20 ± 15.70^{abcd}	80	1	15	557.85 ± 10.78^{ab}	466.86 ± 12.14 ^{cd}
10	2	200	5	379.57 ± 5.72^{abcd}	70	2	30	535.25 ± 6.71^{bc}	454.32 ± 40.20^{cde}
11	2	500	5	339.38 ± 22.79^{d}	70	2	15	538.16 ± 7.43^{bc}	417.41 ± 17.44^{cde}
12	3	500	12.5	360.56 ± 0.93^{bcd}	80	2	22.5	520.28 ± 7.42^{cd}	482.74 ± 23.00^{bc}
13	2	200	20	370.53 ± 6.64^{bcd}	80	1	30	**583.86 ± 13.23^{a}**	459.73 ± 11.50^{cd}
14	3	200	12.5	358.49 ± 6.38^{bcd}	90	2	30	449.87 ± 9.86 ^{g}	546.62 ± 1.34^{ab}
15	2	500	20	373.59 ± 1.79^{bcd}	80	3	30	460.54 ± 4.95^{fg}	555.25 ± 10.39^{a}

*D: Acid concentration (%), E: Pressure (MPa), F: Treatment time (min).
**G: Amplitude, H: Acid concentration, J: Treatment time (min).

Effects of combined ultrasonication extraction and enzymatic saccharification for leaching of reducing sugar

Figure 3 indicates that combined US and enzymatic treatment gave significantly higher RS content than that of the US and HHP alone and the combination of HHP and enzymatic treatment ($p < 0.05$). The RS of microalga ranged from 449.87 ± 9.86 to 583.86 ± 13.23 mg g^{-1} under the different US conditions (Table 6). The highest RS of *C. vulgaris* was 583.86 ± 13.23 mg g^{-1} under 80% amplitude, and 1% (w/w) acid concentration for 30 min. When amplitude was increased from 80 to 90%, RS was increased from 515.03 ± 14.84 to 543.40 ± 18.48 mg g^{-1} under the same acid concentration and time (Table 6, Run 3 and 1). Park & Jeong (2021) reported that raising the ultrasonic amplitude resulted in an increase in glucose production. Moreover, when acid concentration was increased from 1 to 3%, RS was increased from 557.85 ± 10.78 to 581.90 ± 4.96 mg g^{-1} under the same amplitude (80%) and time (15 min) (Table 6, Runs 9 and 7).

The enzymatic conversion was found as 79.3% at 80% amplitude treated with 3% (w/w) acid concentration for 15 min. No significant differences in enzymatic conversion and RS were observed using either runs 7 and 13 ($p > 0.05$) (Table 6). Moreover, the lowest RS was 449.87 ± 9.86 mg g^{-1} under 90% amplitude and 2% (w/w) acid concentration for 30 min after 48 h enzymatic saccharification (Table 6).

Only the treatment time as a primary factor and the interaction between acid concentration and amplitude in addition to acid concentration and time affected RS content significantly ($p < 0.05$) (data not shown). There were no significant differences for the RS content between the conventional and US treatments followed by enzymatic saccharification during 48 h (Table 6).

Discussion

Light intensity, CO_2 concentration and nitrogen limitation as optimization key parameters significantly affected the biomass accumulation, RS and protein content helped to understand how these parameters act individually and in combination. Rosli *et al.* (2020) reported that biomass for *C. vulgaris* was 0.692 g l^{-1} at 216 μmol photons m^{-2} s^{-1} (9391 Lux) light intensity and 9% CO_2 concentration. Hulatt & Thomas (2011) declared that a maximal biomass concentration of *C. vulgaris* was 3.79 0.05 g l^{-1} at 350 μmol photons m^{-2} s^{-1} (ca. 15 217 Lux) light and 4% (v/v) CO_2. Moreover, Chiu *et al.* (2008) showed that *C. vulgaris* cultivated at 2% (v/v) CO_2 and 300 μmol photons m^{-2} s^{-1} (ca. 13 043 Lux) light intensity resulted in a maximum biomass concentration (1.4 g l^{-1}). The *C. vulgaris* biomass of this study (0.8192 g l^{-1}) was found to be higher than the study of Rosli *et al.* (2020). The high light intensity has led to photoinhibition of the culture resulting in low biomass growth (Chiu *et al.*, 2008).

The protein content of microalga ranged from 0.0157 to 0.0683 mg g^{-1} under the different culture conditions. Nitrogen starvation caused a decrease in the protein content. Agirman & Cetin (2015) also reported that protein content decreased when the *C. vulgaris* culture was subjected to nitrogen starvation. Nitrogen starvation decreases protein synthesis and cell division which was also reported in previous studies (Heraud *et al.*, 2005; Guccione *et al.*, 2014; Wu & Miao, 2014). It was also reported that nitrogen starvation limited cell growth of *C. vulgaris* (Lv *et al.*, 2010).

Some carbohydrates such as starch, hemicellulose and cellulose are entrapped within the cell walls of algae (Hernández *et al.*, 2015). Thus, some pretreatment methods and enzymatic hydrolysis should be needed to release these carbohydrates from the cell.

Up to 0.17 g sugars g^{-1} biomass and 0.14 g sugars g^{-1} biomass were found at 75°C after organosolv and acid hydrolysis treatment of *C. vulgaris* biomass, respectively (Barajas-Solana *et al.*, 2014). The RS of this study (284.14 mg g^{-1}) was found to be higher than a previous study of Barajas-Solana *et al.* (2014) carried out with acid hydrolysis treatment.

The digestion of microalgae cell walls by different extraction techniques to release the RSs used hydrolysis by cellulase and Viscozyme L enzyme cocktail containing cellulase and xylanase enzymes. In this study, simultaneous enzymatic saccharification and the two different novel process techniques (HHP and US) were used to evaluate their efficiency for RS extraction from the microalga *C. vulgaris*. The highest RS of *C. vulgaris* were 583.86 ± 13.23 mg g^{-1} under 80% amplitude and 1% (w/w) acid concentration for 30 min, which revealed 79.4% theoretical RS extraction. Combined US assisted dilute acid pretreatment and enzymatic hydrolysis were also found to be more effective than HHP assisted dilute acid pretreatment and enzymatic saccharification.

Ma *et al.* (2020) investigated the reducing sugar production from *C. vulgaris* residual assisted by radio frequency heating and enzymatic saccharification. A yield of RSs from microalga residual was obtained as 54.5% after 72 h saccharification. Aswathy *et al.* (2010) reported that the highest RS (639.42 mg g^{-1}) with 71% efficiency was obtained by the combination of acid and enzymatic hydrolysis of water hyacinth biomass. The differences in yield values of this and previous studies were related to the differences in the raw material, enzyme type and processing conditions.

Park & Jeong (2021) investigated production of RS from *Gracilaria verrucosa* using US assisted acid catalyst and subsequent enzymatic hydrolysis. The RS yield was obtained as 60.38% with 100 mM sulphuric acid and 60% amplitude for 60 min. After enzymatic hydrolysis, the yield of RS was increased to 76.26%. RS production from microalgae by radio frequency heating resulted in 59.66% yield after 72 h saccharification (Ma *et al.*, 2020). Nasirpour *et al.* (2022) suggested using sulphuric acid and ionic liquid (IL) on the carbohydrate conversion from microalgae by ultrasonication as a pretreatment method. The maximum carbohydrate conversion of *Chlorella* by ultrasonic-assisted extraction and by IL method was 98.3 and 98%, respectively (Nasirpour *et al.*, 2022). Total carbohydrate content of defatted biomass of *C. sorokimiana* NITTS3 was estimated as 26.32 ± 0.14% after 20 min ultrasonic pretreatment with ultrasonic intensity of 0.35 W ml^{-1} (Dhandayuthapani *et al.*, 2021). According to Asada *et al.* (2012), the amount of starch extracted from the microalga using ultrasonic homogenizer was 43.5% for 39 min treatment. The conversion ratio of glucose from microalga (*Chlamydomonas fasciata* Ettl NIES 437) by ultrasonic homogenizer was low. This means that the ultrasonic pretreatment did not degrade starch to glucose directly, thus the enzymatic saccharification step should be added to break down α-1,4, and β-1,6, glucoside linkages (Asada *et al.*, 2012).

However, Uzuner (2018) determined the effect of HHP combined with dilute acid and enzymatic saccharification for the production of RS from hazelnut shells which is lignocellulosic biomass. The optimal RS was found as 473.4 mg g^{-1} with 88.4% RS yield after applying dilute acid assisted with HHP and enzymatic saccharification. US assisted dilute acid pretreatment and enzymatic saccharification of microalgal biomass was more promoted to enhance the RS production than that of HHP. Zhao *et al.* (2013) reported that ultrasonic power had a positive effect on microalgae hydrolysis for glucose yield during ultrasound assisted extraction.

The results of this investigation demonstrate that the HHP pretreatment with enzymatic saccharification can be used as an appropriate strategy for RS extraction from microalga biomass. The results also show that microalgal biomass could provide a cheap and sustainable resource for high-value-added products. It is also a comparably more sustainable resource than the lignocellulosic biomass. Furthermore, since HHP with enzymatic saccharification was effective for the cell wall degradation of microalgae, this might be applied on an industrial scale to increase extraction efficacy of cellulose and hemicellulose present in microalgae.

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Disclosure statement

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Author contributions

S. Uzuner: conducted a research and investigation process, specifically performed the experiments, or data/evidence collection, formal analysis (application of statistical or mathematical techniques to analyze or synthesize study data), wrote original draft (preparation and/or creation of the published work, specifically writing the initial draft); G. A. Evrendilek: specifically performed the experiments, or data/evidence collection, wrote review and edited

specifically critical review, commentary or revision-including pre- or post-publication stages; S. Kurhan: performed the experiments, or data/evidence collection.

References

Agirman, N. & Cetin, A.K. (2015). Effects of nitrogen starvations on cell growth, protein and lipid amount of *Chlorella vulgaris*. *Fresenius Environmental Bulletin*, **24**: 11.

Agirman, N. & Cetin, A.K. (2017). Effect of nitrogen limitation on growth, total lipid accumulation and protein amount in *Scenedesmus acutus* as biofuel reactor candidate. *Natural Science and Discovery*, **3**: 33–38.

Asada, C., Doi, K., Sasaki, C. & Nakamura, Y. (2012). Efficient extraction of starch from microalgae using ultrasonic homogenizer and its conversion into ethanol by simultaneous saccharification and fermentation. *Natural Resources*, **3**: 175–179.

Aswathy, U.S., Sukumaran, R.K., Devi, G.L., Rajasree, K.P., Singhania, R.R. & Pandey, A. (2010). Bioethanol from water hyacinth biomass: An evaluation of enzymatic saccharification strategy. *Bioresource Technology*, **101**: 925–930.

Barajas-Solana, A.F., Gonzalez-Delgado, A.D. & Kafarov, V. (2014). Effect of thermal pretreatment on fermentable sugar production of *Chlorella vulgaris*. *Chemical Engineering Transactions*, **37**: 655–660.

Benedetti, M., Vecchi, V., Barera, S. & Dall'Osto, L. (2018). Biomass from microalgae: The potential of domestication towards sustainable biofactories. *Microbial Cell Factories*, **17**: 173.

Brányiková, I., Maršálková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V. & Vítová, M. (2011). Microalgae-novel highly efficient starch producers. *Biotechnology and Bioengineering*, **108**: 766–776.

Brennan, L. & Owende, P. (2010). Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable Sustainable Energy Reviews*, **14**: 557–577.

Chiu, S.-Y.-Y., Kao, C.-Y., Chen, C.-H., Kuan, T.-C., Ong, S.-C. & Lin, C.-S. (2008). Reduction of CO_2 by a high-density culture of *Chlorella* sp. In a semi-continous photobioreactor. *Bioresource Technology*, **99**: 3389–3396.

Dhandayuthapani, K., Sarumathi, V., Selvakumar, P., Temesgen, T., Asaithambi, P. & Sivashanmugam, P. (2021). Study on the ethanol production from hydrolysate derived by ultrasonic pretreated defatted biomass of *Chlorella sorokiniana* NITTS3. *Chemical Data Collections*, **31**: 100641.

Dragone, G., Fernandes, B.D., Abreu, A.P., Vicente, A.A. & Teixeira, J.A. (2011). Nutrient limitation as a strategy for increasing starch accumulation in microalgae. *Applied Energy*, **88**: 3331–3335.

Guccione, A., Biondi, N. & Sampietro, G. (2014). *Chlorella* for protein and biofuels: From strain selection to outdoor cultivation in a Green Wall Panel photo bioreactor. *Biotechnology For Biofuels*, **7**: 84.

Halim, R., Harun, R., Danquah, M. & Webley, P. (2012). Microalgal cell disruption for biofuel development. *Applied Energy*, **91**: 116–121.

Heraud, P., Wood, B.R., Tobin, M.J., Beardall, J. & McNaughton, D. (2005). Mapping of nutrient-induced biochemical changes in living algal cells using synchrotron infrared microspectroscopy. *FEMS Microbiology Letters*, **249**: 219–225.

Hernández, D., Riaño, B., Coca, M. & García-González, M. (2015). Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production. *Chemical Engineering Journal*, **262**: 939–945.

Ho, S.H., Chen, C.Y. & Chang, J.S. (2012). Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. *Bioresource Technology*, **113**: 244–252.

Hulatt, C.J. & Thomas, D.N. (2011). Productivity, carbon dioxide uptake and net energy return of microalgal bubble column photobioreactors. *Bioresource Technology*, **102**: 5775–5787.

Izumo, A., Fujiwara, S., Oyama, Y., Satoh, A., Fujita, N., Nakamura, Y. & Tsuzuki, M. (2007). Physicochemical properties of starch in *Chlorella* change depending on the CO_2 concentration during growth: Comparison of structure and properties of pyrenoid and stroma starch. *Plant Science*, **172**: 1138–1147.

Khan, M.I., Shin, J.H. & Kim, J.D. (2018). The promising future of microalgae: Current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microbial Cell Factories*, **17**: 36.

Li, D.J., Wang, L., Zhao, Q.Y., Wei, W. & Sun, Y.H. (2015). Improving high carbon dioxide tolerance and carbon dioxide fixation capability of *Chlorella* sp. by adaptive laboratory evolution. *Bioresource Technology*, **185**: 269–275.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**: 265–275.

Lv, J.M., Cheng, L.H. & Xu, X.H. (2010). Enhanced lipid production of *Chlorella vulgaris* by adjustment of cultivation conditions. *Bioresource Technology*, **101**: 6797–6804.

Ma, Y., Wang, P., Wang, Y., Liu, S., Wang, Q. & Wang, Y. (2020). Fermentable sugar production from wet microalgae residual after biodiesel production assisted by radio frequency heating. *Renewable Energy*, **155**: 827–836.

Miao, X. & Wu, Q. (2006). Biodiesel production from heterotrophic microalgal oil. *Bioresource Technology*, **97**: 841–846.

Miller, G.L. (1959). Use of dinitrosalysilic acid reagent for determination of reducing sugar. *Analytical Chemistry*, **31**(**3**): 426–428.

Nasirpour, N., Ravanshad, O. & Mousavi, S.M. (2022). Ultrasonic-assisted acid and ionic liquid hydrolysis of microalgae for bioethanol production. *Biomass Conversion and Biorefinery*. doi:10.1007/s13399-021-02274-3

Park, M.-R. & Jeong, G.-T. (2021). Production of reducing sugar in *Gracilaria verrucosa* using physio-chemical pretreatment and subsequent enzymatic hydrolysis. *Algal Research*, **60**: 102531.

Rehman, Z.U. & Anal, A.K. (2018). Enhanced lipid and starch productivity of microalga (*Chlorococcum* sp. TISTR 8583) with nitrogen limitation following effective pretreatments for bioful production. *Biotechnology Reports*, **21**: e00298.

Rosli, S.S., Lim, J.W., Lam, M.K., Ho, Y.C., Yeong, Y.F., Mohd Zaid, H.F., Chew, T.L., Aljunid Merican, Z.M. & Mohamad, M. (2020). Cultivation of microalgae in fluidized bed reactor: Impacts of light intensity and

CO_2 concentration. *IOP Conference Series: Materials Science and Engineering*, **736**(**2**): 022018.

Subhadra, B. & Edwards, M. (2010). An integrated renewable energy park approach for algal biofuel production in United States. *Energy Policy*, **38**: 4897–4902.

Uzuner, S. (2018). Using high pressure processing for bioconversion of hazelnut shells to generate fermentable sugars. *Bioresources*, **13**: 8198–8208.

Wang, H.T., Yao, C.H., Ai, J.N., Cao, X.P., Xue, S. & Wang, W. L. (2014). Identification of carbohydrates as the major carbon sink of the marine microalga *Isochrysis zhangjiangensis* (Haptophyta) and optimization of its productivity by nitrogen manipulation. *Bioresource Technology*, **171**: 298–304.

Wu, H. & Miao, X. (2014). Biodiesel quality and biochemical changes of microalgae *Chlorella pyrenoidosa* and *Scenedesmus obliqus* in response to nitrate levels. *Bioresource Technology*, **170**: 421–427.

Yao, C.H., Ai, J., Cao, X., Xue, S. & Zhang, W. (2012). Enhancing starch production of a marine green microalga *Tetraselmis subcordiformis* through nutrient limitation. *Bioresource Technology*, **118**: 438–444.

Zhao, G., Chen, X., Wang, I., Zhou, S., Feng, H., Chen, W. N. & Lau, R. (2013). Ultrasound assisted extraction of carbohydrates from microalgae as feedstock for yeast fermentation. *Bioresource Technology*, **128**: 337–344.

Zhu, S.N., Wang, Y.J., Huang, W., Xu, J., Wang, Z.M., Xu, J.L. & Yuan, Z.H. (2014). Enhanced accumulation of carbohydrate and starch in *Chlorella zofingiensis* induced by nitrogen starvation. *Applied Biochemistry and Biotechnology*, **174**: 2435–2445.