Effective production of bioenergy from marine *Chlorella* sp. by high-pressure homogenization

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**ABSTRACT**

This study investigated the use of a high-pressure homogenization process for the production of high shear stress on *Chlorella* sp. cells in order to effectively degrade their cell walls. The high-pressure homogenization process was conducted by using various pressure conditions in the range of 68.94–275.78 MPa with different numbers of repeated cycles. The optimal high-pressure homogenization pretreatment conditions were found to be two cycles at a pressure of 206.84 MPa, which provided an extraction yield of 20.35% (w/w) total cellular lipids. In addition, based on the confocal microscopic images of *Chlorella* sp. cells stained by using nile red, the walls of *Chlorella* sp. cells were disrupted more effectively using this process when compared with the disruption achieved by conventional lipid-extraction processes. By using the by-product of *Chlorella* sp., 47.3% ethanol was obtained from *Saccharomyces cerevisiae* cultures. These results showed that the high-pressure homogenization process efficiently hydrolysed this marine resource for subsequent bioethanol production by using only water.

**KEYWORDS**

Shear stress; high-pressure homogenization process; *Chlorella* sp.; biofuel

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on the shape of cultured bovine pulmonary artery endothelial cells via transformation of the cellular cytoskeleton. Therefore, this work introduced a simple high-pressure homogenization process for the economical production of bioenergy from microalgae without causing any environmental issues.

Materials and methods

Materials

Chlorella sp. cells (KMMCC-329, obtained from the Marine Microalgae Culture Center, Korea) were grown in F/2 culture medium (29.23 g NaCl, 1.10 g KCl, 11.09 g MgSO₄·7H₂O, 1.21 g Tris-base, 1.83 g CaCl₂·2H₂O, 0.25 g NaHCO₃ and 3.0 mL trace metal solution that was composed of 281.3 mg NaNO₃, 21.2 mg NaH₂PO₄·H₂O, 16.35 mg Na₂ ethylenediaminetetraacetic acid(EDTA), 11.8 mg FeCl₃·6H₂O, 675 µg MnCl₂·4H₂O, 37.5 µg CoCl₂·6H₂O, 37.5 µg ZnSO₄·7H₂O, 22.5 µg Na₂MoO₄·0.375 mg Vitamin B1 and 0.188 µg biotin) at a light intensity of 20 µmol photons/m²/s—25 µmol photons/m²/s, with CO₂ supplied at a rate of 250 mL/min, in a 5-L photobioreactor.[18] Then, the algal biomass was harvested by centrifugation (Combi 514R Model, Hanil Science Industrial Co., Korea) at 3000 rpm for 10 min. After removing the supernatant, the biomass was freeze-dried to produce a powder for more precise measurement of the extraction yields from every pretreatment process, even though the use of wet biomass obtained from the centrifugation would be more practical for future industrial applications. The powdered sample was sealed and refrigerated at 4 °C until further use.

Pretreatment of Chlorella sp. cells

The pretreatment protocols for high-pressure homogenization (high-pressure processor; MN400BF; PiCOMAX, Korea) or ultrasonification (50 cm × 50 cm × 65 cm, Hankkuk Ind., Seoul, Korea) of the cells were as follows: for the high-pressure homogenization process, dry powder was added to 1 L of distilled water to a final concentration of 10% and this material was ground for 20 min at 20,000 rpm by using a high-speed grinder (HC-1500Y2, HUANGCHENG, China) for downsizing the biomass enough to be passed through the small orifice of the high-pressure homogenizer. Afterwards, 68.94–275.78 MPa of pressure was applied to the ground powder in the range of 0–4 cycles for a total operating time of 30 min through the orifice of 70 µm diameter, during which the low-quality material was broken down into particles less than 5 µm in size. The regimen used for the ultrasonification process was application of 120 W of input power at 130 kHz and 25 °C for 60 min. Then, the particles and powder were used in the experiments. The control sample was non-pretreated. The optimal pretreatment conditions were determined by using a 3D surface plot with a total lipid content as function of the applied pressure and number of cycles.

Determination of the total lipid content and the fatty acid profile

The total lipid content (%, w/w) of the cells was determined by using the Folch method.[19] A 1-g specimen of dried cells was added to a 20-fold volume of a CHCl₃: methanol solvent (2:1, v/v), after which the solution was agitated and centrifuged for 15 min at 500 rpm. Then, the supernatant was added to a 0.9% (w/v) NaCl solution. This mixture was vortexed for several seconds and then centrifuged for 15 min at 2000 rpm. Afterwards, the supernatant was discarded, the lipid-containing pellet was dried and weighed, and its lipid content was evaluated.[19,20] Gas chromatography (GC) (HP 6890 series) was performed to determine the fatty acid profiles of the three kinds of lipids — from the non-treated cells (control), from the high-pressure homogenization and the ultrasonification processes. Before the GC analysis was performed, the obtained lipids were transesterified by using the methyl-esterification method. For methylation, a NaOH/MeOH (0.5 mol/L NaOH) solution was added to the lipids. Then, methanol (1.5 mL) was added, and the mixture was heated at 95 °C for 60 min to convert the lipids into their methyl ester derivatives. Afterwards, hexane (1 mL) was added and mixed well before the hexane-containing supernatant layer was removed and analysed via GC. The GC was equipped with a flame ionization detector (FID) and an SP-2560 (100 m × 0.25 mm × 0.2 µm, #24056, Supelco, USA) column. The oven temperature was increased from 100 °C to 240 °C with a rate of 4 °C/min. The injection temperature and the detector temperature were set to 250 °C and 280 °C, respectively. The flow rate was 1 mL/min, with a split ratio of 50:1, and the injection volume was 1 µL.

Nile red fluorescence-based determination of the neutral and polar lipid contents

Before and after the lipids were extracted, dried Chlorella sp. cells were re-suspended in a physiological saline by using agitation (400 rpm, 12 h), and then the cells were centrifuged (1500 × g, 10 min) and the pellet was washed three times using physiological saline. The cells were resuspended again using the saline solution and were treated for 10 min with a
Using a solution of 4% NaOH and was brought to 100 °C for 5 min in a water bath, and then its temperature was reduced to room temperature. The absorbance of the cooled solution at 557 nm was measured using a microplate reader (Tecan Sunrise, Switzerland) to obtain the light-absorbance value, which was compared with the light absorbance values of glucose standard solutions to determine the total sugar content (g/L).

**Analysis of the fermentable sugar content**
For the analysis of various sugar contents of *Chlorella* sp. extracts, glucose, xylose and galactose were purchased from Sigma Aldrich and were used as standards. The sugar components of *Chlorella* sp. were analysed, and the total sugar content (g/L) was determined using high-performance liquid chromatography (HPLC) (Waters 510 system, Waters 410 RI-detector; Waters, Milford, MA, USA). A carbohydrate analysis column (300 mm × 7.8 mm, Aminex HPX-87P, Bio-Rad, USA) was used. The mobile phase analysis was performed using distilled water (100%) at a flow rate of 0.6 mL/min at 70 °C for 30 min.[23]

**Ethanol production by the pretreated hydrolysates**
To evaluate the ethanol production of the hydrolysates, 100-mL aliquots of the obtained liquids, by using both processes, were fermented by *Saccharomyces cerevisiae* (ATCC 24858) in a modified yeast extract-peptone-dextrose medium (1% yeast extract and 2% peptone, pH 5.5) in a 500-mL flask at 30 °C, with agitation at 150 rpm, for 45 h. The ethanol concentration (g/L) in the culture broth was determined by GC (HP-5890II, Agilent, USA) using a FID, at an oven temperature of 150 °C and injector and FID temperature of 250 °C. N2 was used as a carrier gas, supplied at a flow rate of 50 mL/min, and an INNO-Wax column (30 m × 0.32 mm, Agilent, USA) was used for the analysis. n-Butanol was used as the internal standard.[24] The first process (A) was conducted with hydrolytic conditions for untreated *Chlorella* sp. cells with 1% sulphuric acid at 121 °C for 15 min reaction period (control), and the second process (B) was conducted with hydrolytic conditions for *Chlorella* sp. cells pretreated with high-pressure homogenization with 1% acetic acid at 121 °C for 15 min reaction period.

**Statistical analysis**
The data were expressed as the mean values ± standard deviation and the mean value was the average of five test results per experiment. The data were analysed using student’s *t*-test (SAS 9.1, SAS, Cary, NC, USA). The experiments were repeated at least three times to confirm the results.

**Results and discussion**
**Observation of the structure of pretreated *Chlorella* sp. cells**
Figure 1 is a schematic diagram of the high-pressure homogenization equipment interior, in which the highest shear stress occurred when the cells passed through tubes with a diameter of approximately 70 μm. Thus, the cells crashed in the collision zone, reducing their sizes. [25] In general, the intensity of the shear stress was calculated according to the diameter of the pipe and the existing pressure. However, this equipment provided a high level of shear stress because the diameter of the pipe was very small. Figure 2 shows the overall morphology of *Chlorella* sp. cell walls following simple water
extraction and ultrasonic pretreatment, as observed by using SEM. Compared to the untreated Chlorella sp. cells (Figure 2(A)) as a control, in which the cell wall was slightly damaged, the cell wall of the cells treated with high-pressure homogenization (Figure 2(B)) or ultrasonification (Figure 2(C)) process was found to be more disrupted. However, the ultrasonification process did not seem to be effective, because only small parts of the cell walls were broken, whereas the high-pressure homogenization process completely demolished the hard cell membranes. This result demonstrated that the high-pressure homogenization process could play an important role in increasing the lipid-extraction yields and the quality of the produced biodiesel. Also, this process could lessen the extent of damage or degradation of lipids caused by the conventional lipid extraction process that employs large amounts of solvents and harsh conditions.[26,27] These mild lipid-extraction conditions should also positively affect the application of the lipid extractions’ by-products.

Optimal high-pressure homogenization conditions for Chlorella sp. lipids extraction.

Figure 3 shows the optimal conditions for pretreating Chlorella sp. cells, determined by changing the applied pressure within the range of 68.94–275.78 MPa and the number of high-pressure homogenization cycles within the range of 0–4. When a homogenization process was not applied, (the control Figure 2(A)), the total lipid yield was approximately 14.64% (w/w), whereas a lipid yield of approximately 20.27% was obtained when 206.84 MPa of pressure was applied in only one cycle. By using the same conditions of only one cycle of homogenization, when the highest pressure of 275.78 MPa was applied, the lipid yield was 19.87%, which was lower than the one obtained through applying 206.84 MPa. However, when the lipid contents were analysed after applying different number of cycles with 137.89 MPa, the highest lipid content of approximately 18.01% was obtained after three cycles. These results demonstrated
that the applied pressure had a greater impact on the
degree of cell wall damage than the number of cycles
had. Increasing the pressure and number of cycles had a
positive effect on the extent of the cell wall damage;
however, repeating the cycle for more than three times,
or applying more than 275.78 MPa of pressure, did not
increase the effect. Thus, we confirmed that the amount
of the total lipid was 20.35% (w/v) from the optimal pre-
treatment conditions — 206.84 MPa of pressure and two
cycles. Therefore, from the contour plot of Figure 3, it
could be seen that the optimal process conditions were
two cycles at a pressure of 206.84 MPa, when consider-
ing the lipid content, as well as the quality of the lipids
for the biodiesel shown in Figure 4. Anyway, there was
not much difference in the content and quality of the lip-
ids when applying one or two cycles. The results also
proved that the lipid content, obtained in this condition,
was also approximately 4% higher than that from the
ultrasonification process. In addition, as shown in
Figure 4, *Chlorella* sp. cells were stained using nile red,
which stains neutral lipids, to confirm the lipid extraction
effects on the quality of biodiesel from the three tested
pretreatment processes. The lipids of nile red stained
microalgae fluoresced at different wavelengths, depend-
ing on their polarity, with neutral lipids appearing yellow
and polar lipids appearing red.[21] As calculated in
Table 1 and by using a picture taken from the micro-
scope, the numbers of the lipid droplets (neutral lipids
(yellow) and the polar lipids (red)) were counted and cal-
culated in percentages. Part of the neutral lipids
(72.62%) remained in the treated by the ultrasonification
process cells, whereas approximately 85% of the neutral

![Figure 3. 3D surface plot of the total lipid content of *Chlorella* sp.
cells after a high-pressure homogenization process treatment with
different values of applied pressure and different number of cycles.](image1)

![Figure 4. Remaining lipid content and confocal images of *Chlorella* sp.
cells stained with nile red following various treatments. (A) Control sample,
non-treated; (B) ultrasonification treated cells; (C) high-pressure homogenization
processed cells. Note: scale bar = 10 μm.](image2)
lipids remained in the control cells. Notably, the high-pressure homogenization process extracted almost all of the neutral lipids. This result also strongly supported that the optimal pretreatment conditions should play an important role in improving both quantity and quality of biodiesel from microalgae.

Comparison of the fatty acid composition

We compared the fatty acid compositions of Chlorella sp. cells treated with the ultrasonification or with the high-pressure homogenization processes, with the fatty acid composition of the non-treated cells (control). The levels of C14–C18 saturated and unsaturated fatty acids in the cells of Chlorella sp. were determined and are shown in Table 2. The results showed that the fatty acid contents of cells treated with ultrasonification and high-pressure homogenization processes were higher than that of the non-treated sample. In particular, the fatty acids contents after the high-pressure homogenization process were found to be approximately 10.0% of C16:1 and 6.3% of C18:0, showing that the monounsaturated fatty acid content, compared to that of the control, was also increased by the treatment. It was also confirmed that the amount of oleic acid (C18:1) was increased by up to 1.65 times after the high-pressure homogenization process, when compared with that from the non-treated sample (control). The percentages were 5.7% vs. 9.4%, respectively. However, the biodiesel quality depends on its fatty acids composition. Biodiesel with a high content of saturated fatty acids, such as palmitic acid (C16) and stearic acid (C18), has worse fluidity at low temperatures due to its relatively high melting point. The optimal level of unsaturated fatty acids for a biodiesel with low melting point has been evaluated.[28] Therefore, a high-quality biodiesel should have a high content of monounsaturated fatty acids, such as palmitoleic acid (C16:1) and oleic acid (C18:1).[29] This result was confirmed by similar results from other studies.[30,31]

Determination of the sugar content of the lipid-extraction by-products

The quantitative analysis results of the reducing sugars and fermentable sugars contents, obtained from the by-products of lipid extraction using the DNS method and HPLC are shown in Table 3.[32] The by-product of the non-treated Chlorella sp. cells, hydrolysed by using 1% sulphuric acid, had a total reducing sugar content of approximately 1.03 g/L. The by-product of high-pressure homogenized cells, hydrolysed using 1% acetic acid, had a total reducing sugar content of 1.19 g/L, which was similar to the sugar content of cells treated with sulphuric acid. These results showed that the hydrolysis of cells with high-pressure homogenization was more effective than that from conventional hydrolysis using strong acid.

### Table 1. Comparison of the proportions of the neutral lipids in cells treated with two different processes.

| Pretreatment                        | Numbers of the neutral lipid droplets* | Numbers of the total lipid droplets** | Neutral lipids (%) |
|------------------------------------|----------------------------------------|---------------------------------------|-------------------|
| Control                            | 628                                    | 736                                   | 85.33             |
| High-pressure homogenization       | 3                                      | 64                                    | 4.68              |
| Ultrasonification                  | 244                                    | 336                                   | 72.62             |

Note: *numbers of yellow coloured lipid droplets in each picture of Figure 4; **total number of red and yellow lipid droplets in each picture of Figure 4.

### Table 2. Composition of major fatty acids in the lipids obtained from two different pretreatment processes.

| Fatty acids | Control | Ultrasonification | High-pressure homogenization |
|-------------|---------|-------------------|----------------------------|
| 14:0        | 2.0     | 4.5               | 3.7                        |
| 16:0        | 19.6    | 10.1              | 11.4                       |
| 16:1        | 6.2     | 7.9               | 10.0                       |
| 18:0        | 3.3     | 6.0               | 6.3                        |
| 18:1        | 5.7     | 6.8               | 9.4                        |
| 18:2        | 11.8    | 9.7               | 6.6                        |
| 18:3        | 22.3    | 18.3              | 17.4                       |

### Table 3. Comparison of the total reducing sugar content of Chlorella sp. cells subjected to the conventional acid-hydrolytic process or high-pressure homogenization followed by a milder acid hydrolytic process.

| Processes | Total reducing sugar content (g/L, w/v) | Glucose | Xylose | Galactose |
|-----------|----------------------------------------|---------|--------|-----------|
| A         | 1.03                                   | 0.54 ± 0.12 | 0.31 ± 0.012 | 0.03 ± 0.007 |
| B         | 1.19                                   | 0.60 ± 0.11 | 0.32 ± 0.029 | 0.02 ± 0.005 |

Note: Process A was conducted at hydrolytic conditions for untreated Chlorella sp. cells with 1% sulphuric acid (control); process B was conducted at hydrolytic conditions for Chlorella sp. cells pretreated with high-pressure homogenization with 1% acetic acid.
sulphuric acid, due to the high destruction of the cell walls by this process. Similar results were also reported in other studies.[26,33] The cell wall of Chlorella sp. cells is based on cellulose and contains starch. Therefore, an essential production of bioethanol was obtained in a large amount of glucose and xylose.[34,35] In addition, it was previously shown that a glucose conversion yield of 14.0% (w/w) was obtained using Chlorella vulgaris cells treated with sulphuric acid, which was similar to that obtained by cells treated using the high-pressure homogenization process.[36] These results confirmed that the high-pressure homogenization process had a positive effect on the extent of the cell wall damage.

**Bioethanol fermentation with by-products of lipid extraction**

The Chlorella sp. hydrolysate was used for ethanol fermentation, and the results are shown in Figure 5. The Chlorella sp. hydrolysate was rapidly consumed during the early stage of fermentation (approximately six hours) and the secondary metabolites of the bio-ethanol production were observed during the subsequent six hours. It was found that most of the glucose was consumed during the first 24 h, and thus the rate of bioethanol production gradually decreased thereafter. The bioethanol yield was approximately 47.3%, compared with the theoretical maximum yield of 50%.[23] This is because the by-product used to produce biodiesel can contain large amounts of fermentable sugars, which cannot be obtained using conventional lipid extraction methods. Moreover, the residue used for alcohol fermentation did not contain any harmful chemicals, such as hydroxymethylfurfural, which are inevitably generated using the normal extraction processes and even the acid treatments used for solubilizing the cellulosic material and agricultural bioresources. Therefore, the high-pressure homogenization process was effective for producing a microalgae by-product for utilization in the generation of bioethanol. In addition, the thick cell wall of microalgae was shown to be effectively disrupted by this process.

**Conclusions**

In this study, a simple high-pressure homogenization pretreatment process was utilized to effectively disrupt the hard cell wall of Chlorella sp. Cells without using harsh solvent-extraction processes, such as CHCl₃, methanol. Also, the lipids had high unsaturated fatty acid contents that can result in the production of a good quality biodiesel from Chlorella sp. It was also found that the pressure was more important in increasing the lipid-extraction yield than the applied number of cycles, because applying a 206.84 MPa pressure generated a shear stress sufficient enough to disrupt the cell wall. Once the cell wall was completely disrupted, a very weak and mild extraction process was enough for extracting most of the neutral lipids from the cellular material, which could result in the production of high-quality biodiesel. This mild, but effective extraction process would also definitely facilitate in obtaining a good alcohol-fermentation yield using the by-products from

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**Figure 5.** Comparative ethanol productivity and glucose content obtained through hydrolysate fermentation.
the lipid extraction process. These results strongly indicated the possibility of a simultaneous production of biodiesel and bioethanol from marine algae treated with one pretreatment process. This simple physical pretreatment process is more economical and environmentally friendly than the conventional chemical pretreatment processes. This method does not need a large-scale acid hydrolysis reaction tank and extra neutralization step for the transesterification reaction of the extract. It also takes less processing time, which can definitely reduce the production cost.

Disclosure statement

No potential conflict of interests was reported by the authors.

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