Appl Biochem Biotechnol (2011) 164:1215–1224
DOI 10.1007/s12010-011-9207-1

Disruption of *Chlorella vulgaris* Cells for the Release of Biodiesel-Producing Lipids: A Comparison of Grinding, Ultrasonication, Bead Milling, Enzymatic Lysis, and Microwaves

Hongli Zheng · Jilong Yin · Zhen Gao · He Huang · Xiaojun Ji · Chang Dou

Received: 3 November 2010 / Accepted: 15 February 2011 /
Published online: 24 February 2011

Abstract A comparative evaluation of different cell disruption methods for the release of lipids from marine *Chlorella vulgaris* cells was investigated. The cell growth of *C. vulgaris* was observed. Lipid concentrations from different disruption methods were determined, and the fatty acid composition of the extracted lipids was analyzed. The results showed that average productivity of *C. vulgaris* biomass was 208 mg L^{-1} day^{-1}. The lipid concentrations of *C. vulgaris* were 5%, 6%, 29%, 15%, 10%, 7%, 22%, 24%, and 18% when using grinding with quartz sand under wet condition, grinding with quartz sand under dehydrated condition, grinding in liquid nitrogen, ultrasonication, bead milling, enzymatic lysis by snailase, enzymatic lysis by lysozyme, enzymatic lysis by cellulose, and microwaves, respectively. The shortest disruption time was 2 min by grinding in liquid nitrogen. The unsaturated and saturated fatty acid contents of *C. vulgaris* were 71.76% and 28.24%, respectively. The extracted lipids displayed a suitable fatty acid profile for biodiesel [C16:0 (~23%), C16:1 (~23%), and C18:1 (~45%)]. Overall, grinding in liquid nitrogen was identified as the most effective method in terms of disruption efficiency and time.

Keywords Microalga · Lipid concentration · Cell disruption

Introduction

The depletion of fossil fuels and the effect of greenhouse gas emissions on global warming are creating much interest in microalgal biodiesel. Microalgae are the most primitive form

H. Zheng · J. Yin · H. Huang · X. Ji · C. Dou
State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, No. 5 Xinmofan Road, Nanjing 210009, People's Republic of China

Z. Gao (✉)
College of Food Science and Light Industry, Nanjing University of Technology, No. 5 Xinmofan Road, Nanjing 210009, People's Republic of China
e-mail: zhhl2981@126.com

of plant able to use CO_2 and the energy from the sun to produce lipids for biodiesel. They have been proposed as very good candidates for developing biodiesel because of their high photosynthetic efficiencies, high biomass production and rapid growth compared to other energy crops, and ability to fix CO_2 efficiently from different sources [1, 2]. The main metabolites of oleaginous microalgae are intracellular lipids, which are used as feedstock for biodiesel production [3].

Of all steps of biodiesel production from microalgae, including species selection, cultivation, harvest, cell disruption, etc., the cell disruption is a particularly important step because the cells' walls are generally thick. The cell wall of *Chlorella vulgaris* is composed predominantly of hemicellulose and saccharides which hinders the release of intracellular lipids [4]. The derived lipid concentration of *C. vulgaris* is dependent on the disruption method and device, and lipid oxidation may be caused during the disruption process. Moreover, the cell disruption creates a huge operational cost during lipid extraction, thereby, rendering algae-based biodiesel less economically attractive. A range of alternative processes have been suggested for breaking algal cells. Belarbi et al. describe ultrasonication as a method for recovery of high purity eicosapentaenoic acid esters from microalgae [5]. During ultrasonication process, high frequency sonic waves bring about implosive collapse of the gas-filled cavitation bubbles and generate intense local shock waves equivalent to thousands of atmosphere pressure, which ultimately cause cell wall disruption. A POTTER glass homogenizer is applied to break microalgal cells for chlorophyll extraction by Schumann et al., which involves the application of high shear forces to deform and rupture the cell wall [6]. Fu et al. report that immobilized cellulase may be applied as enzymatic hydrolysis of microalgal cell walls for lipid extraction [7].

To make it more economically attractive, a feasible cell disruption method should be established to ensure a low operating cost, high product recovery, and high quality of the recovered lipids. The purpose of this study was to compare and evaluate a range of different physical and chemical treatments on the disruption of cells of marine *C. vulgaris* for lipid recovery. Finding the most appropriate method of cell disruption for *C. vulgaris* would maximize the lipid concentration and improve the quality of the extracted lipids. Chlorophyll is an essential compound of autotrophic microalgae and often extracted together with lipids during lipid extraction. A pretreatment method was used to decompose microalgal chlorophyll before lipid extraction. Measurements of lipid concentrations were obtained to indicate cell disruption efficiency as a correlating variable.

Materials and Methods

Microalgal Strain and Cultivation Conditions

The microalgal *C. vulgaris* (strain CCTCC M 209256) was obtained from the China Center for Type Culture Collection, Wuhan, China. The strain was preserved in 20% (*v*/*v*) glycerol at −80 °C. The culture medium was the same as described in a previous work by Fan et al. [8] and was composed of (in mg L^{-1}): instant ocean synthetic sea salt (Aquarium Systems, Inc., USA), 34,000; KNO_3, 100; KH_2PO_4, 10; Na_2EDTA, 10; $FeSO_4{\cdot}7H_2O$, 2.5; $MnSO_4$, 0.25; vitamin B_1, 0.006; and vitamin B_{12}, 0.00005. The culture system used was a 10-L jacketed bubble column photobioreactor (50.0 cm in height, 16.0 cm in diameter, a closed system) with a working volume of 8 L. The culture temperature was 25 °C, and it was regulated by water recycled in the out layer of photobioreactor. Ten fluorescent lamps were arranged around the photobioreactor to supply continuous illumination of 80 μmol

photons m^{-2} s^{-1} with a 12/12 h light/dark cycle. At the bottom of the reactor, there was a gas sparger. CO_2 of 3.0% was prepared with the combination of room air and pure CO_2 from a compressor, and aeration rate was carried out at 200 mL min^{-1}. The cultivation cycle was 10 days. One identical 500-mL cell suspension sample was applied for each disruption method.

Cell Growth

The dry weight of *C. vulgaris* (DW, g L^{-1}) and the optical density at 680 nm wavelength by a UV/VIS spectrometer (Lambda 25, PerkinElmer, Inc., USA) in a quartz cuvette with a 1-cm light path were linearly correlated by the equation:

$$\mathrm{DW} = 0.560 \times \mathrm{OD}_{680}\left(r^2 = 0.986\right) \tag{1}$$

The optical density measurements were periodically checked by gravimetry.

The KNO_3 concentration in broth measured by the spectrometric method is reported by Collos et al. [9]. Cells were centrifuged at 1,600×*g* for 5 min at room temperature. The absorbance of supernatant was measured at 220 nm. A standard curve was determined from authentic potassium nitrate at concentrations from 0 to 0.44 mM.

The concentration of CO_2 in the outlet gas of the 10-L photobioreactor was measured online using Tandem Gas Analyzers (Magellan Instruments Ltd, UK). The volume of residual CO_2 (V_R) could thus be calculated via integration of the volume percentage of CO_2 of the outlet gas as follows,

$$\mathrm{V}_R = \frac{\mathrm{Q} \cdot \int_0^{\mathrm{t}} \mathrm{C_t dt}}{\mathrm{V}} \tag{2}$$

where, Q is the CO_2 gas flow, C_t is the CO_2 concentration measured at the outlet gas of the photobioreactor, V is the volume of the culture medium, and t is the culture time. The constant of the concentration of CO_2 in the inlet gas, the volume of the culture medium, and the CO_2 gas flow and well-mixing of the gas phase have been assumed.

Methods for Cell Disruption of *C. vulgaris*

Manual Grinding

Three kinds of manual grinding methods were tested in this experiment. Method 1: A sample of harvested microalgae from 500 mL cell suspension was put into a ceramic mortar. About 10 mL liquid nitrogen was added, and the sample was allowed to thaw and ground with a pestle for 1 min. Method 2: Quartz sand of 0.1 g was added to a second identical sample and ground directly for 10 min. Method 3: A third identical sample was dried at 60 °C for 8 h in an oven, and 0.1 g quartz sand was added before being ground as the second sample. After grinding, the samples were washed with distilled water before lipid extraction.

Ultrasonication

The cell suspension was ultrasounded in a 50-mL plastic centrifuge tube with an ultrasonic cell disintegrator (GA92-IID, Wuxi Shangjia Biotechnology Co., Ltd, Jiangsu Province, China) of 600 W ultrasonic power, 5 s interval time, 30 s ultrasonic time and 20 min total

working time. The samples were kept in an ice bath during the ultrasonic process to prevent overheating.

Bead Milling

The microalgal suspension was poured into the bead-beater chamber (240 mL; LS10; GA92-IID, Jiangying Jingcheng Chemical Machinery Co., Ltd, Jiangsu Province, China). And 160 mL of glass beads (size ranging from 0.40 to 0.60 mm) were added, and the chamber was cooled on ice for 10 min before disruption. The disruption time was 20 min with a rotational speed of 1,500 rpm. The inlet suspension was introduced at the bottom of the grinding chamber. A filter screen at the outlet in the upper part of the chamber prevented the glass beads from flowing out.

Enzymatic Lysis

The microalgal suspension was disrupted with cellulase (provided by Zesheng Bioengineering Technology Co., Ltd, Shandong Province, China; microalgal suspension pH adjusted to 4.8 by acetic acid before disruption), lysozyme, and snailase (both provided by Nanjing Genetime Biotechnology Co., Ltd, Jiangsu Province, China). The concentrations of the above three enzymes were 5.00 mg L^{-1}. The cells were broken by snailase at 37 °C with water bath for 2 h. The other two enzymes disrupted cells at 55 °C with water bath for 10 h.

Microwaves

The cell suspension was disrupted in a 250-mL glass flask with microwaves using a microwave oven (EG823MF4-NA, Midea Co., Ltd, Guangdong Province, China) at a high temperature (about 100 °C and 2,450 MHz) for 5 min.

Lipid Extraction

With the exception of enzymatic cell lysis, cell slurries from grinding, ultrasonication, bead milling, and microwaves were put into a 500-mL cylindrical glass beaker and shined in the sun for 10 min between 10 A.M. and 2 P.M. at any sunny day on the ground before lipid extraction for chlorophyll destruction. The cell slurries were extracted with a mixture of chloroform–methanol (1:1 *v*/*v*) with the samples in a proportion of 1:2 using a slightly modified version of Bligh and Dyer's method [10]. The mixtures were shaken for 5 min in a separatory funnel. The organic phases were then separated from the separatory funnel and evaporated with a rotary evaporator at 30 °C. Then the lipids were measured using an electronic scale. The lipid concentration was defined as dry weight ratio of extracted lipids to biomass. Finally, the lipids from all the disruption methods were applied to fatty acid composition analysis.

Fatty Acid Composition Analysis

A fatty acid composition analysis was performed using gas chromatography and mass spectrometry (GC–MS; Thermo/Finnigan Trace GC/MS, USA). Fatty acids were

methylated by BF_3 in methanol [11]. The method of GC–MS was similar to that described in our previous study [12]. Fatty acids were identified by comparison to external standards (Sigma, USA) and MS structure database.

Statistical Analysis

The experiment was designed and carried out at random. All the treatments were repeated three times, and data are reported as mean±SD values.

Results and Discussion

Cell Growth

To study the growth of *C. vulgaris*, dry weight, KNO_3 concentration, the volume of residual CO_2, and lipid yield as a function of culture time were each investigated. Dry weight, KNO_3 concentration, the volume of residual CO_2, and lipid yield varied during the whole batch culture process. Figure 1 shows the time course of the investigated parameters of *C. vulgaris* at different growth phases. As shown in Fig. 1, the lipid yield and dry weight increased slightly and the volume of residual CO_2 increased significantly and KNO_3 concentration decreased slightly during the first 2 days of inoculation. Upon the time of inoculation, cells began to adapt themselves to the new environment and assimilated almost no nutrients, which might suppress cell division; but cells still survived by supplied CO_2.

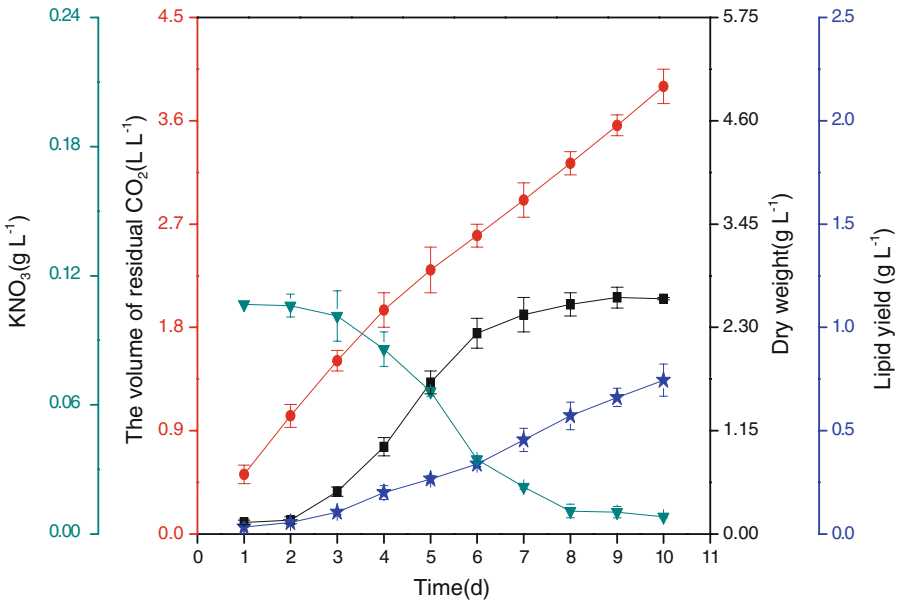

Fig. 1 The variation of the volume of residual CO_2, KNO_3 concentration, dry weight, and lipid yield at different growth phases. *Black square* dry weight, *black circle* the volume of residual CO_2, *black down-pointing triangle* KNO_3 concentration, *black star* lipid yield

From the stationary phase, i.e., 7 days after the inoculation, dry weight and KNO_3 had almost no change; while the increase of lipid yield and CO_2 consumption (difference of the volume of CO_2 of inlet gas and that of residual CO_2) was obvious. Similar phenomenon has also been observed by Valenzuela-Espinoza et al. [13]. The possible reason was that cells stop growing, but lipid synthesis of the cells was continuing since CO_2 was the substrate of lipid synthesis. Carbon is the dominant nutrient (45–50% of the dry weight) of *C. vulgaris*. The biosynthesis of 1 g (dry weight) *C. vulgaris* biomass consumed 2.88 g CO_2. Theoretically, 1.65–1.83 g CO_2 is needed for the biosynthesis of 1 g (dry weight) of algal biomass and the difference between the result in our study and the theoretical value is caused by the CO_2 loss during *C. vulgaris* growth [14].

Effect of Different Methods on Cell Disruption of *C. vulgaris*

In order to disrupt *C. vulgaris* cells, different disruption methods were tested (Fig. 2). The lipid concentration readings of different cell disruption methods were used to indicate the extent of disruption in this study. A higher degree of disruption causes increased breakdown of the cells and more released intracellular materials [15]. It also reduces the relative amount of intact cells in the disruptate, thus resulting in higher lipid concentration in our study. All disruption methods applied in this study were confirmed to be able to disrupt *C. vulgaris* cells. Grinding in liquid nitrogen had the highest lipid concentration of 29%. The lipid concentration of this method was found to be 9.7-, 5.8-, 4.8-, 1.9-, 2.9-, 4.1-, 1.3-, 1.2-, and 1.6-fold higher than that of control, grinding with quartz sand under wet condition, grinding with quartz sand under dehydrated condition, ultrasonication, bead milling, enzymatic lysis by snailase, enzymatic lysis by lysozyme, enzymatic lysis by cellulose, and microwaves, respectively. The lowest lipid concentration of only 5% was obtained by grinding with quartz sand under wet condition. Of all the enzymatic treatments, cellulase and lysozyme at the dosage of 5.00 mg L^{-1} gave lipid concentrations of 24% and 22%, respectively.

Of all the above methods, grinding in liquid nitrogen was the quickest method with 2 min (Table 1). The processing time of grinding with quartz sand under wet condition,

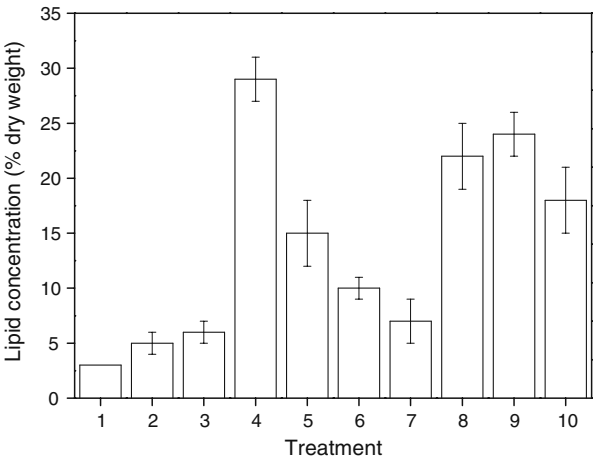

Fig. 2 Effect of different methods on cell disruption of *Chlorella vulgaris*. *1* Control (intact), *2* grinding with quartz sand under wet condition, *3* grinding with quartz sand under dehydrated condition, *4* grinding in liquid nitrogen, *5* ultrasonication, *6* bead milling, *7* snailase, *8* lysozyme, *9* cellulase, *10* microwaves

Table 1 Performance of various cell disruption methods for the release of lipids

Cell disruption method	Processing volume per single operation (mL)	Processing time (min)	Illuminated time in the sun (min)
Grinding in liquid nitrogen	500	2	10
Grinding with quartz sand under wet condition	500	10	10
Grinding with quartz sand under dehydrated condition	500	10	10
Ultrasonication	30	540	10
Bead milling	60	300	10
Enzymatic lysis by snailase	500	120	None
Enzymatic lysis by lysozyme	500	600	None
Enzymatic lysis by cellulase	500	600	None
Microwaves	100	25	10

grinding with quartz sand under dehydrated condition, ultrasonication, bead milling, enzymatic lysis by snailase, enzymatic lysis by lysozyme, enzymatic lysis by cellulose, and microwaves was 5, 5, 270, 150, 60, 300, 300, and 12.5 times, respectively, higher than that of grinding in liquid nitrogen. Processing volume per single operation of ultrasonication, bead milling, and microwaves was 30, 60, and 100 mL, respectively, and that of the other methods was 500 mL (Table 1). Before disruption, microalgae harvesting was necessary for grinding, but the cells for other disruption methods were not necessary to be harvested. Before lipid extraction, with the exception of enzymatic lysis, cell slurries from disruption in this study were illuminated in the sun for 10 min for chlorophyll decomposition. Chlorophyll can be degraded into hydrophilic acids by solar illumination and cannot be extracted together with lipids during lipid extraction [16].

In this study, we found out that cells disrupted by different methods affected lipid concentrations significantly. Similar result has been obtained by Pernet and Tremblay [17] that different disruption methods affect TAG level extracted from *Chaetoceros gracilis* significantly. Grinding in liquid nitrogen was an efficient method. The rupture of cells by the principle of grinding in liquid nitrogen is cryo-impacting. The basic idea is that individual cells or masses of cells when frozen at very low temperatures (−196 °C) will crack easily under low-impact force. The liquid nitrogen used in the disruption process will evaporate and will not affect the downstream lipid extraction. The low temperature it offers also prevents the oxidation of lipids, thus improving their quality. Ultrasonication and bead milling methods were simple methods for disrupting *C. vulgaris*. However, lipid concentrations from ultrasonication and bead milling were low. Pernet and Tremblay's [17] study also shows that grinding and ultrasonication can disrupt the cell wall of *C. gracilis* for lipid extraction and the TAG level is significantly influenced by treatment in relation to either storage time or sampling volume; the grinding and ultrasonication methods give the highest TAG level of 3.42 $\mu g/10^6$ cells at 1 day storage time with 15 mL sampling volume and 3.51 $\mu g/10^6$ cells at 0 day storage time with 15 mL sampling volume, respectively. Similar results are also found in Bermejo et al. [18] study that ultrasonication of suspended microalgal cells can be used as a simple method to disrupt small amounts of biomass. Enzymatic lysis has the advantage of being specific and gentle, and enzymes have the specific effect on hemicellulose and

saccharides of cell wall [19]. Our study found out that two of the three foreign enzymes with an appropriate dosage disrupted the cell wall of *C. vulgaris* effectively. But the processing time was a bit long, and a relatively stable reaction temperature was required. A similar result is found in a previous study that microalgal lipid extraction efficiency increases dramatically to around 56% from 32% after cell wall hydrolysis by cellulose [7]. This cell disruption method is also easy to scale-up. Enzymes that do not demand strict reaction conditions should be used. The performance of the microwave oven method was not as good as grinding in liquid nitrogen. Most of the extracted lipids (triglyceride) from microalgae were non-polar [7]. The efficiency of microwaves can be poor when either the target compounds or the solvents are non-polar or when they are volatile during disruption and extraction process [20]. In a previous report, the concentration of recombinant hepatitis B core antigen is applied to evaluate cell disruption efficiency [12]. In this study, we used lipid concentration extracted from *C. vulgaris* to assess the cell disruption efficiency. The results showed that lipid concentration represented the disruption efficiency well.

In addition to using lipid concentration as a means of assessing the effectiveness of disruption methods on the algal biomass, the direct effect that disruption methods had on the cell wall of the alga can be observed by scanning electron microscopy (Fig. 3a–c). The

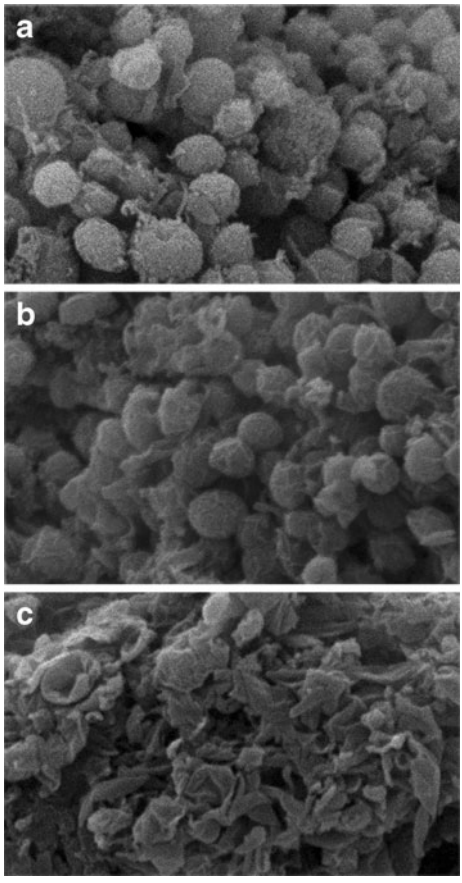

Fig. 3 Scanning electron micrographs of *Chlorella vulgaris* (×5,000), *bar size* 10 μm. **a** Control (intact) cells. **b** Cells disrupted by microwaves. **c** Cells disrupted by grinding in liquid nitrogen

undisrupted cells of *C. vulgaris* (Fig. 3a) were fully intact with no signs of pitting or damage to the cell wall. Most of the cells were intact after being disrupted by grinding with quartz sand under wet/dehydrated condition and enzymatic lysis by snailase (data not shown). In contrast, the cells subjected to microwaves (Fig. 3b), ultrasonic disruption, and bead milling (data not shown) resulted in some distortion and collapse of some cells. The algal cells were clearly disrupted into pieces (Fig. 3c) by grinding in liquid nitrogen. Enzymatic treatments with lysozyme and cellulose, respectively, had visible effect on the morphology of most cells (data not shown).

Fatty Acid Composition

The major fatty acid composition of the extracted lipids from the different cell disruption methods was determined using GC–MS system (Table 2). Different cell disruption methods had no obvious effect on the major fatty acid composition of *C. vulgaris*. In the tested *C. vulgaris*, palmitic acid (C16:0), physetoleic acid (C16:1), and oleic acid (C18:1) were commonly dominant. The unsaturated and saturated fatty acid contents of *C. vulgaris* were 71.76% and 28.24%, respectively. In particular, lipids with high content of unsaturated fatty acids have been reported to have a reasonable balance of fuel properties [21]. The chain length of fatty acids was between C14 and C20 in this study. In a previous report [22], fatty acids with chain length C14–C22 were recognized as the most common fatty acids contained in biodiesel. Therefore, fatty acids from *C. vulgaris* were suitable for the production of good quality biodiesel.

Conclusions

Of all the methods studied, grinding in liquid nitrogen showed the best disruption efficiency with the least time. Cellulase and lysozyme exhibited good performance in our study and disrupted cells gently with the longest time. The results manifested that grinding in liquid nitrogen had the best marine *C. vulgaris* cell disruption performance and could be scaled-up for potential industrial application. Lipids extracted from different cell disruption methods were determined using GC–MS system, and the results showed that the fatty acids of *C. vulgaris* were good for the production of biodiesel.

Table 2 Fatty acids composition of *Chlorella vulgaris*

Fatty acids	Fatty acid composition (percent of total fatty acids)
C14:0	0.92±0.1
C16:0	22.50±0.5
C16:1	23.31±0.3
C16:2	1.95±0.4
C18:0	2.18±0.2
C18:1	45.36±1.8
C18:2	1.14±0.1
C20:0	2.64±0.3
Unsaturated fatty acids	71.76±2.5
Saturated fatty acids	28.24±0.7

Acknowledgments The authors would like to thank Prof. Walter K. Johnson from Brigham Young University for the assistance in polishing the English language of this study. This work was funded by the Key Program of National Natural Science Foundation of China (no. 20936002), the National Basic Research Program of China (nos. 2007CB707805, 2009CB724700, and 2011CB200906), the Fifth of Six Projects Sponsoring Talent Summits of Jiangsu Province (no. 2008-D-63), the College Industrialization Project of Jiangsu Province (no. JH09-30), the Fok Ying Tung Education Foundation (no. 123014), and the Program for New Century Excellent Talents in University from the Ministry of Education of China (no. NCET-09-0157).

References

1. Chisti, Y. (2007). *Biotechnology Advances, 25*, 294–306.
2. Reijnders, L. (2008). *Trends in Biotechnology, 26*, 349–350.
3. Silva, T. L., Reis, A., Medeiros, R., Oliveira, A. C., & Gouveia, L. (2009). *Applied Biochemistry and Biotechnology, 159*(2), 568–578.
4. Am, A. S., & Ya, M. T. L. (1993). *Biologia Plantarum, 35*, 629–632.
5. Belarbi, E. H., Molina, E., & Chisti, Y. (2000). *Enzyme and Microbial Technology, 26*, 516–529.
6. Schumann, R., Häubner, N., Klausch, S., & Karsten, U. (2005). *International Biodeterioration & Biodegradation, 55*, 213–222.
7. Fu, C. C., Hung, T. C., Chen, J. Y., Su, C. H., & Wu, W. T. (2010). *Bioresource Technology, 101*, 8750–8754.
8. Fan, L. H., Zhang, Y. T., Zhang, L., & Chen, H. L. (2008). *Journal of Membrane Science, 325*, 336–345.
9. Collos, Y., Mornet, F., Sciandra, A., Waser, A. N., Larson, A., & Harrison, P. J. (1999). *Journal of Applied Phycology, 11*, 179–184.
10. Bligh, E. G., & Dyer, W. M. (1959). *Canadian Journal of Biochemistry and Physiology, 37*, 911–917.
11. Metchalfe, L. D., & Schmitz, A. A. (1961). *Analytical Chemistry, 33*, 363–372.
12. Jin, M. J., Huang, H., Xiao, A. H., Zhang, K., Liu, X., Li, S., et al. (2008). *Biotechnological Letters, 30*, 1087–1091.
13. Enrique, V. E., Roberto, M. N., & Filiberto, N. C. (2002). *Aquacultural Engineering, 25*, 207–216.
14. Jiřĺ, D., František, S., & Karel, L. (2005). *Journal of Applied Phycology, 17*, 403–412.
15. Ho, C. W., Tan, W. S., Kamaruddin, S., Ling, T. C., & Tey, B. T. (2008). *Biotechnology and Applied Biochemistry, 50*, 49–59.
16. Maunders, M. J., & Brown, S. B. (1983). *Planta, 158*, 309–311.
17. Pernet, F., & Tremblay, R. (2003). *Lipids, 38*, 1191–1195.
18. Bermejo Román, R., Talavera, E. M., & Alvarez-Pez, J. M. (2001). *Journal of Chromatography A, 917*, 135–145.
19. Geciova, J., Bury, D., & Jelen, P. (2002). *International Dairy Journal, 12*, 541–553.
20. Wang, L. J., & Weller, C. L. (2006). *Trends in Food Science & Technology, 17*, 300–312.
21. Rashid, U., Anwar, F., Moser, B. R., & Knothe, G. (2008). *Bioresource Technology, 99*, 8175–8179.
22. Demirbas, A. (2009). *Energy Conversion and Management, 50*, 14–34.