Combination of cell disruption method and pH variation as pre-treatment for lipid extraction of *Nannochloropsis* sp.

S Melanie* and D Fithriani

Research and Development Center for Marine and Fisheries Product Processing and Biotechnology, Ministry of Marine Affairs and Fisheries, Jakarta, Indonesia

*E-mail: susianam@yahoo.com

Abstract. The quantity of fossil-based fuel also has depleted due to over-exploitation. Therefore, environmentally friendly and more sustainable energy resources are being searched to fill in the energy supplies in the future. Microalgae have been considered by the researcher as alternative biofuel feedstock due to their high lipid content. Lipid extraction is one of the crucial steps in biofuel production from microalgae. The aim of this study was to analyze the best combination of pre-treatments to obtain microalgae lipid for biodiesel. The utilization of different cell disruption methods, including sonication and microwave, combined with pH variation (3, 5, 7) was implemented on microalgae *Nannochloropsis* sp. The experiment shows cell disruption with sonication and combined with low pH 3 of oil extraction from *Nannochloropsis* sp. resulted in the highest yield of oil content (1.15% gr/gr dry weight). The lipid extracted from each pH pre-treatment were analyzed using GCMS for its fatty acid profile. The best pretreatments combination was obtained from lipid extraction from pH 5 and sonication-assisted method.

Keywords: lipid extraction, microalgae, microwave, *Nannochloropsis* sp., sonication

1. Introduction

Currently, global warming has raised awareness of energy resources. The global warming mainly caused by fossil fuel which has depleted each year due to its non-renewable resources. The increase in energy demand and decrease in fossil fuel resources cause exploration to find renewable energy resources (Cai *et al* 2013, Zhu *et al* 2014). Microalgae biomass get noticed for as one of the renewable energy sources for biodiesel production due to its advantages, such as high oil content, high growth rate, maximum utilization of solar energy, low land use, and easy to culture (Lee *et al* 2010, Gullberg *et al* 2014, Cheng *et al* 2014a). Microalgae have been considered as a promising biofuel feedstock, biopharmaceutical, and nutraceutical industries. Microalgae have superiority compared with other traditional feedstocks. Microalgae are renewable, environmentally friendly, and also sustainable (Khan *et al* 2018, Deng *et al* 2009). However, all of those advantages sometimes failed to meet the expectation such as low biomass production due to contamination of the microalgae culture by other organisms. Thus, the lipid produced from the extraction of microalgae is decreased following the reduction of microalgae biomass production.
Various methods have been studied previously to improve the lipid production of the microalgae cells. A US Patent presents two-stages of culturing microalgae: a first stage for increasing the biomass, followed by the second stage to increase the lipid content by limitation of nutrients (Demaris et al. 2011). Two-stages of culturing microalgae for producing lipid also describes by Oyler (2008) by applying autotrophic conditions for increasing the biomass and heterotrophic stage to increase the lipid production of microalgae.

Nowadays, the microalgae harvesting particular methods are needed to be applied i.e. chemical, mechanical, biological, electrical, and sometimes a combination of two or several of those methods. Microalgae harvesting microalgae mainly consists of two major processes which are thickening of the biomass and dewatering the biomass before followed with the drying process. Adding chemical or biological flocculant/coagulant into the biomass are some methods in the thickening stage, which could increase the effectiveness of harvesting the biomass and reduce the cost incurred (Barros et al. 2015). *Nannochloropsis* sp. cells have a very small particle size. Flocculant needs to be added into *Nannochloropsis* sp. culture to increase the effective particle size. Hereafter, the dewatering stage by gravitational sedimentation can be easier (Grima et al. 2003). Chemical that used to trigger coagulation and flocculation in this study was sodium hydroxide (NaOH). According to Brady et al. (2014), increasing pH can stimulate auto flocculation. Therefore, by adding NaOH into the algal culture will make the particle size of the algal cell enlarge and aggregate, thus easier to be filtered. After filtration, the process can be followed by drying the biomass using sun drying.

The algal lipid extraction needs to be enhanced by wetting the dried biomass. According to a research conducted by Halim et al. (2011), a wet paste of microalgae had higher lipid yield (0.071 g lipid content/g dried microalgae) compared to dried microalgae (0.058 g lipid content/g dried microalgae). The lipid will be easier to exit the cells if the microalgae were in a wet form since the polar solvent can diffuse with a polar layer of the biomass (i.e. proteins, water, nutrients) (Yoo et al. 2012, Steriti et al. 2014). González-Delgado et al. (2017) reported that acid hydrolysis improves the lipid extraction of microalgae *Amphiprora* sp. by rupturing the algae cell walls and provoke the solvent to have higher contact with the lipid content inside the cells. The acid solution added into the biomass can act both as wetting agent and acid hydrolysis solution.

Cheng et al. (2014) conducted research where wet microalgae were extracted with hexane after microwave-assisted cell disruption. According to another study by Byreddy et al. (2015), a combination of chloroform and hexane gave better results compared to hexane alone on lipid extraction of microalgae *Scenedesmus dimorphus* and *Chlorella protochloroides*. However, due to the properties of chloroform that dangerous to humans, it was not visible to use chloroform in large scale algal lipid production. Moreover, hexane still gave the best total lipid production compared to other solvents alone.

Fatty acid methyl esters originating from vegetable oils and animal fats are known as biodiesel. A previous study by Ma et al. (2016) reported that fatty acid profiles of several *Nannochloropsis* strains can be affected by various components during cultures, such as light intensity, temperature, pH, and other environmental stresses. Lipid from microalgae should have appropriate fatty acid profiles to be considered as biodiesel, not only based on the high content of lipid (Ma et al. 2016). Fatty acid profiles influence the properties of biodiesel, i.e. cetane number, kinematic viscosity, oxidative stability, cloud point, cold filter plugging point, and cold soak (Knothe 2009). The greater the number of the cetane, the fastest the ignition start.

Many previous studies reported various pre-treatments for extracting lipid from microalgae. However, there were rarely reported about the combination of those pre-treatments. Therefore, this study was...
tried to figure out the best combination of cell disruption methods and pH variation as pre-treatments for lipid extraction of *Nannochloropsis* sp.

2. Methods

2.1. Culture of *Nannochloropsis* sp.
Culture of *Nannochloropsis* sp. was conducted in a laboratory-scale for inoculum culture in 5 L flasks. Afterward, the inoculum culture was expanded into a pilot scale with outdoor culture open ponds with bigger capacity (≥100 L). Christenson and Sims (2011) said that open ponds are the most common system used in large scale microalgae cultivation. Seawater with a salinity of 15 ppt was used as culture medium. The culture ponds were exposed to direct sunlight for illumination. Combination of fertilizers urea 120 ppm, TSP 60 ppm, and ZA as much as 60 ppm were added as nutrients for the *Nannochloropsis* sp. culture. There are several methods for harvesting the microalgae, i.e. chemical-based, mechanical-based, electrical-based, and biological-based (Christenson and Sims 2011). One of the methods used in this study was chemical based harvesting by adding flocculant into the algal culture. The culture was harvested after 10-15 days using NaOH solution as a flocculant agent until pH 9.0 was reached. Flocculant agent was needed to enlarge the size of *Nannochloropsis* sp. cells so that the microalgae cells will be coagulated and easier to be filtered. After being coagulated, the microalgae were filtered using filter bag and followed with drying the microalgae cells under the sunlight until reached constant weights.

2.2. Pretreatment for lipid extraction
The pretreatment for lipid extraction consisted of acid lysis (pH adjustment) and physical cell disruptions (sonication and microwave-assisted) (Surendhiran and Vijay 2014). The acetic acid solution was added into 25 g of dried *Nannochloropsis* sp. to obtain wet microalgae biomass with pH 3, 5, and 7. Lipid extraction can be increased by blending the polar and non-polar solvents (Naghdi et al. 2016). The polar solvent allowed the lipid released from the phospholipid bonds of the cell walls (Ryckebosch et al. 2011). Cell disruption methods were used to enhance the lipid extraction from within the cells. The sonication and microwave-assisted were conducted to break the cell wall so that the lipid content of the microalgae can be easily attracted by the extraction solvent. The first batch of *Nannochloropsis* sp. biomass was sonicated at 10kH for 15 minutes. The second batch of *Nannochloropsis* sp. slurry was microwaved at 90°C. All treatments were done in triplicate.

2.3. Lipid extraction
After pretreatment with acid lysis and physical cell disruptions (sonication and microwave-assisted), the wet microalgae were mixed with hexane. Hexane was used as a polar solvent to attract the lipid content inside the microalgae cells, with ratio hexane to wet microalgae was 1:1 and stirred until well-mixed. The mixture then was left for 24 hours until the polar and non-polar layers were clearly separated. The polar layers were collected and transferred into 100 mL glass beakers, and then left for 24 hours to evaporate the solvent.

2.4. Analysis
The dried microalgae were analyzed for its water content to ensure all the samples have the same water content condition. After vaporization of the solvent, the lipid obtained from the extraction process was analyzed for its yield. The remaining lipid content was analyzed using gravimetry to determine the yield of all combination of pre-treatment methods. The best yield from cell disruption pretreatment then was further analyzed with GC-MS for its fatty acid profiles.
3. Results and discussion

Figure 1 shows the average initial water content of microalgae *Nannochloropsis* sp. used in the lipid extraction before pretreatments, which gives a similar amount of water contents. The *Nannochloropsis* sp. which will be pre-treated in pH 3, pH 5, and pH 7 had the water contents of 7.85 ±0.22, 7.32±1.60, and 7.53±1.35% w/w respectively. These results indicate that the sodium hydroxide used for microalga harvesting did not affect the water content of the dried biomass. Thus, it can be said that all the samples were in similar conditions before the pre-treatments.

![Figure 1](image1.png)

*Figure 1*. The initial water contents of dried *Nannochloropsis* sp. biomass before pretreated for lipid extraction (% w/w).

![Figure 2](image2.png)

*Figure 2*. Total lipid content of *Nannochloropsis* sp. that extracted in pH 3, pH 5 and pH 7 with a sonicator (●) and microwave (○) cell disruption in gr/gr dry weight.
Figure 2 shows the lipid content of *Nannochloropsis* sp. extracted in various pH conditions (pH 3, pH 5 and pH 7) followed with physical cell disruption methods (sonication and microwave-assisted). Pretreatment with pH 3 in both sonication and microwave-assisted cell disruption methods gave the highest lipid content compared to pH 5 and pH 7. Meanwhile, the sonication disruption method gave higher lipid content compared to microwave-assisted, with the lipid content of 1.12 ± 0.07 gr/gr dry weight and 0.88 ± 0.13 gr/gr dry weight respectively. These results were comparable to that of a previous study using supercritical CO$_2$ method (SSCO$_2$), where the lipid extract obtained from wet paste microalgae was 0.07 g/g dry weight and from dried microalgae was 0.06 g/g dry weight.

**Table 1.** Saturated fatty acid and unsaturated fatty acid contents of the *Nannochloropsis* sp. lipid extracted with sonication-assisted disruption method after pretreatment with pH 3, 5, and 7.

| Variation | Saturated fatty acid (%) | Unsaturated fatty acid (%) |
|-----------|--------------------------|----------------------------|
| pH 3      | 3.93                     | 96.05                      |
| pH 5      | 62.547                   | 37.453                     |
| pH 7      | 50.649                   | 49.351                     |

**Table 2.** Fatty acid profiles of *Nannochloropsis* sp. extracted with sonication-assisted disruption method after pretreatment with pH 3, 5, and 7.

| Carbon chains | Fatty acid                        | Content (%)  |
|---------------|-----------------------------------|--------------|
|               |                                   | pH 3 | pH 5 | pH 7   |
| 6             | Hexanoic acid                     | 3.93 | 1.48 |        |
| 14            | Myristic acid                     |      | 7.79 |        |
| 14:1          | Myristoleic acid                  | 15.37 | 5.00 | 1.30   |
| 15:1          | cis-10-Pentadecenoic acid         | 2.16  |      |        |
| 16            | Palmitic acid                     | 61.06 | 11.69|        |
| 16:1          | Palmitoleic acid                  | 23.51 | 7.79 |        |
| 17:1          | cis-10-Heptadecanoic acid         | 1.41  |      |        |
| 18            | Stearic acid                      |      | 27.27|        |
| 18:1 n-9t     | cis-9-Oleic acid                  | 12.05 | 10.39|        |
| 18:1n9c       | trans-9-Elaic acid                | 8.51  | 3.12 |        |
| 18:2n6t       | Linoleic acid                     | 3.66  |      | 7.79   |
| 18:2n6c       | Linoleaidic acid                  | 2.41  | 0.10 |        |
| 18:3n3        | Linolenic acid                    | 35.70 |      | 2.60   |
| 20            | Eicosanoic acid                   |      | 2.60 |        |
| 20:4 n-6      | cis-8,11,14-Eicosatrenic acid     |      | 2.60 |        |
| 20:5n3        | cis-11,14,17-Eicosatrenic acid    | 14.80 | 5.72 | 5.20   |
| 22            | Behenic acid                      |      | 1.30 |        |
| 22:6 n-6      | Docosahexaenoic acid              |      | 11.69|        |

Table 1 shows saturated fatty acid and unsaturated fatty acid contents of the *Nannochloropsis* sp. lipid extracted on pH 3, 5, and 7 with sonication-assisted disruption method for the pretreatments. Microalgae that was pretreated in pH 3 gave highest unsaturated fatty acid compared to pH 5 and pH 7 with saturated fatty acid contents of 96.07 %, 37.45 %, and 49.35 %, respectively.

Table 2 shows the fatty acid profiles of *Nannochloropsis* sp. extracted on pH 3, 5, and 7 with sonication-assisted disruption method. The pH and cell disruption assisted pretreatments were needed.
to improve the lipid extraction from *Nannochloropsis* sp. It can be seen from figure 2 that lower pH (pH 3 and pH 5) gave a higher yield compared to neutral pH (pH 7). However, from table 1 it can be seen that pH 3 gave lower saturated fatty acid content, while pH 5 and pH 7 give higher saturated fatty acid content. Hu *et al* (2008) reported that saturated fatty acid did not affect biodiesel production, but affecting the fuel properties. It can be said that even though pH 3 gave higher lipid recovery, the fatty acid profiles of lipid produced from pH 3 and sonication pretreatments gave a low performance to be applied as biodiesel.

The higher cetane number of the fatty acid composition, the faster the ignition time start. The previous study reported that increasing the cetane number into around 60 can decreasing the NO\(_x\) gas emissions, where the saturated esters showed a high cetane number compared to unsaturated esters (Knothe 2009). Saturated esters resulted in low NO\(_x\) emissions compared to unsaturated esters. It is possible, therefore, that combination of pretreatment pH 5 or pH 7 with sonification-assisted cell disruption method more suitable to be applied in biodiesel production. Future research should be carried out to investigate the physical properties of biodiesel produced from a combination of these pretreatments such as combustion temperature, viscosity, ignition flashpoint, and other properties.

### 4. Conclusions

Low pH pretreatment gives a high yield of lipid content in *Nannochloropsis* sp. The sonication assisted cell disruption method gives a higher yield than microwave-assisted. The combination of pH 3 and sonication pretreatments resulted in the highest yield of lipid content, but with low saturated fatty acid content. The best pretreatments combination was obtained from lipid extraction from pH 5 and sonication-assisted method. It can be concluded that lipid extraction of *Nannochloropsis* sp. with the variation of pH 5 and pH 7 which improved with sonication-assisted cell disruption method has potential prospects to be developed as biodiesel.

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