Evaluating of Several Parameters in Marine *Chlorella* sp. Flocculation Process, and Biodiesel Production via Chlorophyll-Extracted Microalgal Biomass (CEMB)

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ABSTRACT

The screening for a high efficient method in harvesting microalgae is an important step to a large scale microalgal biodiesel production. Magnesium salt ($MgSO_4 \cdot 7H_2O$) concentration of 0.0083 gram per litter of marine *Chlorella* sp. culture with biomass concentration of 3.78 g/L showed the highest flocculation efficiency (FE) of 94.63% at pH of 11 after only 10 minutes of flocculation time. There was no any difference of FE between two different volumes of culture, after 10 minutes of flocculation.

Keywords: Chu 13, flocculation efficiency, magnesium salt, marine *Chlorella* sp., photoautotroph, rapid harvesting

Introduction

Microalgae are more promising biofuel feedstock compared to the previous two generations of energy sources of biofuel, due to the prospect of these organisms which show high biomass yields without requiring any arable land (Chiaramonti, 2007; John *et al*., 2011; Trent, 2012). However, harvesting of microalgal biomass consume large amount of energy. It jeopardizes the massive interests of algal biomass from the result of energy analyses and the life-cycle assessment (Zheng *et al*., 2012).

Low final concentration of microalgal biomass and small size of microalgae make harvesting of the algal biomass is challenging. Flocculation of microalgal cultures by addition of inorganic compounds under acidic or alkaline conditions was known as the most promising method for harvesting of

microalgal biomass. It was because flocculation could be easily scale-up and could be applied for various species of microalgae (Uduman *et al*., 2010). Therefore, in the view of economic and technological feasibility, flocculation can be a convenient and an effective method for harvesting microalgae from large scale of microalgae cultures (Wu *et al*., 2012).

Since Cheirsilp and Torpee (2012) reported that marine *Chlorella* sp. under photoautotrophic cultivation could accumulate lipid to about 30% based on dry weight and showed promising potentials as biodiesel feedstock. Then this study aimed to investigate several parameters that related to marine *Chlorella* sp.'s harvesting process such as the effects of magnesium salt addition, time of flocculation after the magnesium salt addition, pH before the magnesium salt addition, and volume of marine *Chlorella* sp. photoautotrophic cultures, to the flocculation efficiency (%). Biodiesel produced from chlorophylls-extracted microalgal biomass (CEMB) of marine *Chlorella* sp. was also studied.

EXPERIMENTAL

Microalgae strain and growth medium.

Marine *Chlorella* sp. was obtained from the National Institute of Coastal Aquaculture, Thailand. The mediums used in this study were modified Chu 13 medium (Largeau et al., 1980). One liter of Chu 13 medium contains 0.4 g KNO_3, 0.08 g K_2HPO_4, 0.107 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g Fe citrate, 0.1 g citric acid, 0.00002 g $CoCl_2$, 0.00572 g H_3BO_3, 0.00362 g $MnCl_2 \cdot 4H_2O$, 0.00044 g $ZnSO_4 \cdot 7H_2O$, 0.00016 g $CuSO_4 \cdot 5H_2O$, 0.000084 g Na_2MoO_4, 1 drop of 0.072 N H_2SO_4 and 1 mL of trace metal solution. The pH was adjusted to 7.8. One liter of trace metal solution contains H_3BO_3 2.85 g, $MnCl_2 \cdot 4H_2O$ 1.8 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $CuSO_4 \cdot 5H_2O$ 0.08 g, $CoCl_2 \cdot 6H_2O$ 0.08 g and $Na_2MoO_4 \cdot 2H_2O$ 0.05 g.

Cultivation of microalgae

Microalgae strain was pre-cultured in 400 mL of Chu 13 medium in a 500 mL bottle. The pre-cultures were incubated at 30 ^oC and air-aerated at a flow rate of 0.01 mL/min under a 3,000 lux light intensity with a 16:8 h light and dark cycle for 7 days (Cheirsilp and Torpee, 2012). This was used as a seed culture. The batch cultivation of the microalgae was performed by inoculating 10% (v/v) seed culture into each 3 L of Chu 13 medium in a 3.78 L (1 US gallon) glass bottle. Cultures were incubated at 30 ^oC and air-aerated at a flow rate of 0.01 mL/min. The cultures then were illuminated with a 3,000 lux light intensity with a 16:8 h light and dark cycle for 5 days. During microalgae cultivation, ten mililitres of sample was

taken every day. The optical density at 660nm (OD_{660}) of cultivated microalgae were measured by using spectrophotometer (Libra S22 Biochrom). The pH of the culture was measured every day by using pH meter (Mettler Toledo). The dry mass of microalgae, and the specific growth rate were determined.

Flocculation of the microalgal biomass

Microalgal biomass were harvested by flocculation method using flocculent ($MgSO_4 \cdot 7H_2O$). Flocculation experiments were performed by varying pH of microalgal culture (9.5-12), time of flocculation (10-60min), flocculent's concentration in microalgal culture (1.5-8.3 mg/L), and volume of algal culture (0.02-3.78 L). Flocculation efficiency was calculated using the following equation (Wu et al., 2012):

Flocculation efficiency (%) = (1-A/B) x 100. A is the OD_{660} of supernatant from half the height of the clarified layer after flocculation and B is the initial OD_{660} of the algal culture suspension. Meanwhile, the photos of microalgae before and after flocculation were taken by using light microscope (Nikon) equipped with Improved Neubauer Bright Line (BOECO, Germany), and digital camera (Samsung).

Investigating the Effects of Several Related Parameters to the Flocculation Efficiency

The initial biomass concentrations (g/L) of the culture were determined by taking 100 mL of culture, measuring its OD_{660} and weighing its dry mass. Ten mililiter of microalgal culture were placed to each of reaction tube. The pH (9.5-12) of microalgal culture were adjusted by adding 1 M HCl or 1 M NaOH to each of harvested culture. After that the $MgSO_4 \cdot 7H_2O$ salt was subsequently added to each of reaction tube for investigating the effect of several parameters to the flocculation efficiency (%). The parameters of flocculation process including, concentrations of flocculent ($MgSO_4 \cdot 7H_2O$) in medium (varied from 1.5 to 8.3 mg/L) and the flocculation time (varied from 10 min to 60 min) while the volume of microalgal cultures were prepared varied from 0.02 to 3.78 L. The flocculent was added to the culture medium then the reaction tubes were vortex for 5 seconds. The microalgal suspensions were left to settle for certain time of flocculation without agitation. Subsequently, the optical density of the supernatant from half the height of the clarified layer and the sludge were measured at 660nm. The flocculation efficiency was then calculated. The experiments were conducted in triplicates.

Biodiesel production from CEMB of marine Chlorella sp.

The modified method of Archanaa, *et al.* (2012) was performed to extract chlorophyll from dry microalgal biomass as follows, acetone used in lipid extraction from 100 mg of dried microalgal biomas, the tube was sealed then centrifuged at 4500 rpm for 5 min at room temperature, afterward supernatant was withdrawn and the same extraction procedure repeated to the pellet until supernatant was colorless. The first collected supernatant was analyzed for its chlorophyll presence by fluorescence spectrophotometer. The pellet obtained after separating colorless supernatant was chlorophyll-extracted microalgal biomass (CEMB). This CEMB then washed by 1 mL of DI water centrifuged at 4500 rpm for 5 min. Afterwards, the clean CEMB was dried overnight in 60^{o}C oven and weighed. Furthermore, modified method of Bligh and Dyer (1968) then used to extract lipid from 0.0625 g of dried CEMB and the extracted lipid was transesterified by modified sonication-assisted. Small amount of the extracted lipids from modified method of Bligh and Dyer then re-suspended in DI water. Afterward, 3 mL of the suspension was analized by spectrofluorometer (FP-8200 JASCO, Japan) for its chlorophylls. The fluorescence spectrum was recorded with an excitation wavelength of 435nm.

The method of Cheng *et al*. (2013) was used in transesterification by adding a fresh mixture of chloroform (0.575mL): methanol (0.425mL) (volumetric ratio of 1.35) in the presence of 50µL of sulfuric acid (98%) and mixed 5 seconds using vortex then subsequently sonicated (Transsonic model 460/H, Elma, Singen, Germany) at 50^{o}C for 40 min. Afterward, 1 mL of DI water was added to the mixture then centrifuged at 4500 rpm for 10 min. There were 3 layers produced, upper layer was aqueous phase, middle thin layer was a little amount of sludge, and lower layer was the organic phase containing produced biodiesel. The organic phase was then separated well using micro pipette, dried overnight in 60^{o}C oven. Then the produced microalgal biodiesel was used in GC analysis to evaluate its fatty acid composition and percent of FAME.

The lipid was converted to fatty acid methyl esters (FAME) before using in analysis of fatty acid composition. The first step was conducted with 1 mL of KOH/MeOH (0.5 M) at 100^{o}C for 5 min. In the second step, 400µL of aq. HCl/MeOH (4:1, v/v) was added to the mixture from the first step and then the mixture was heated in an oil bath for 15 min at 100^{o}C. Furthermore, the tube was cooled and 2 mL of water was added. After that, the mixture was extracted with 2x3 mL of petroleum ether. The organic layer

was dried quickly over anhydrous Na_2SO_4, evaporated and redissolved in 500 µL of $CHCl_3$. The analysis of FAME was conducted using a Gas Chromatograph (HP6850) equipped with a cross-linked capillary FFAP column (length 30 m, 0.32 mm I.D., 0.25 µm film thickness) and a flame ionization detector (FID). The operating conditions were as following: inlet temperature 290°C, oven initial temperature 210°C hold 12 min ramp to 250°C at 20°C/min hold 8 min and detector temperature 300°C. Fatty acids were identified by comparing their retention times with those of standard ones and calculated as percentage based on their respective peak area using a standard mixture of FAME (Jham et al., 1982).

RESULTS AND DISCUSSION

Flocculation efficiency

Flocculation efficiency was measured in condition of without flocculent addition and with flocculent addition by varying the pH of culture (9.5, 10.0, 10.5, 11.0, 11.5, 12.0) and time of flocculation (10, 30, and 60 min). Culture volume used for flocculation was 20 mL with microalgal concentration of 3.78 g/L, and flocculent ($MgSO_4 \cdot 7H_2O$) concentration of 0.0083 g/L culture suspension which was found as the selected concentration of flocculent. The highest flocculation efficiency (FE) achieved with flocculent addition was 99.7% at pH 12 after 60 minutes while the highest FE achieved without flocculent addition was 20.7% at the pH of 10.5 after 60 minutes (Fig. 1).

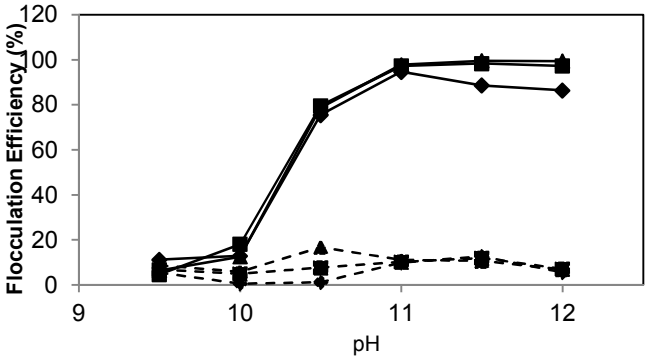

Fig. 1. Flocculation efficiency (%) at various pH and time of flocculations (min) of marine *Chlorella* sp. (solid line represent flocculation with $MgSO_4 \cdot 7H_2O$ of 0.0083 g/L culture; culture volume of 0.02 L; dash line represent flocculation without $MgSO_4 \cdot 7H_2O$; ◆ 10 min, ■ 30 min, ▲ 60 min).

Furthermore, based on t-test:paired two sample for means of FE (%) between two flocculation times (10 min and 30 min) respectively 94.63% and 97.30% with flocculent addition at pH 11. There was no significant difference of FE between those two flocculation times. Therefore the optimum FE (94.63%) after 10 min of flocculation process was more preferable. Moreover, based on t-test: paired two sample for means of FE (%) between two

different volume of culture (3.78 L and 0.02 L), at pH 11, flocculent ($MgSO_4 \cdot 7H_2O$) concentration is 0.0083 g/L culture, and after 10 minutes of flocculation. It was found that no difference of FE between those two volume of cultures.

Figure 3 shows the photos of marine *Chlorella* sp. before flocculation and after flocculation using $MgSO_4 \cdot 7H_2O$. A) Before flocculation. B) After flocculation using $MgSO_4 \cdot 7H_2O$

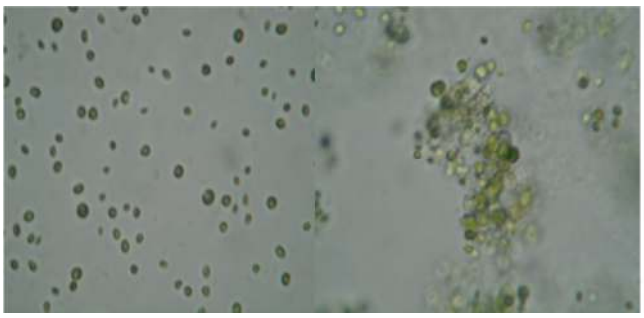

Fig. 3. Photos of marine *Chlorella* sp., were taken: A) Before flocculation, and B) After flocculation process using $MgSO_4 \cdot 7H_2O$ by light microscope (Nikon) equipped with Improved Neubauer Bright Line (BOECO, Germany), and digital camera (Samsung, Korea).

Tabel 4. Microalgea lipid extraction

No.	Dry tube (g)	Dry tube + Dry lipid (g)	Dry Lipid (g)	Dry lipid average mass (g)	stdev (g)	Percent of extracted lipid to dry algal biomass (% w/w)	Average Percent of extracted lipid (%)	stdev (%)
1	1.1601	1.1758	0.0157			25.12		
2	1.0638	1.0746	0.0108	0.0143	0.0030	17.28	22.8	4.827
3	1.1039	1.1202	0.0163			26.08		

The extracted lipid yield obtained was 22.8±4.8% while the percent of FAME was 3.66±0.56 %. Fatty acid profiles measured from the produced biodiesel of methods using marine *Chlorella* sp. biomass as the feedstock were summarized in Table 8.

Table 8. Fatty acid profiles measured from the produced biodiesel of 5 different methods.

Method	Fatty acid (%)									
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C22:1	C24:1	USFA	SFA
CEMB	71,06	5,35	2,26	16,27	5,07	0	0	0	28,94	71,06

Conclusions

Magnesium salt ($MgSO_4 \cdot 7H_2O$) concentration of 0.0083 gram per litter of marine *Chlorella* sp. culture with biomass concentration of 3.78 g/L showed the highest flocculation efficiency (FE) of 94.63% at pH of 11 after only 10 minutes of flocculation time. Meanwhile, without flocculent addition, the highest FE (20.7%) was achieved at the pH of 10.5 after 60 min. No significant difference of FE (%) was found between two different volumes of culture (3.78 L and 0.02 L) at pH 11, flocculent concentration of 0.0083 g/L culture, and after 10 minutes of flocculation. The $MgSO_4 \cdot 7H_2O$ has displayed great potentials as flocculent in both the efficient harvesting and the rapid harvesting of marine *Chlorella* sp. biomass.

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