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RESEARCH ARTICLE

# BIODIESEL PRODUCTION FROM *CHLORELLA VULGARIS* WITH SPECIAL EMPHASIS ON IMMOBILIZED LIPASE CATALYZED TRANSESTERIFICATION

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**ABSTRACT**

The production of biodiesel by transesterification employing acid or base catalyst has been industrially accepted for its high conversion and reaction rates. Downstream processing costs and environmental problems associated with biodiesel production and byproducts recovery have led to the search for alternative production methods. Recently, enzymatic transesterification involving lipases has attracted attention for biodiesel production as it produces high purity product and enables easy separation from the byproduct, glycerol. An indigenous microalgae *Chlorella vulgaris* grown in open pond cultivation obtained a high oil content of up to 14.72%. The microalgal oil was then converted to biodiesel by enzymatic transesterification directly using disrupted microalgal biomass was catalyzed by immobilized lipase, and the conversion rate reached up to 97.25%. In a word, the approach including high-density fermentation of Chlorella and enzymatic transesterification process were set up and proved to be a promising alternative for enzymatic biodiesel production.

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## INTRODUCTION

Limited supplies of fossil fuels have lead to a growing interest in developing new sources of alternative fuels, with biodiesel being a promising candidate for widespread adoption. Biodiesel is now mainly produced from vegetable oil (Demirbas, 2002), soybean oil (Cao *et al.,* 2005), sunflower oil (Granados *et al.,* 2007), palm oil (Mekhilef *et al.,* 2011), rapeseed oil (Georgogianni *et al.,* 2009), and waste cooking oil (Zhang *et al.,* 2003). The cost of biodiesel is based on the raw materials and the necessary processing, and at present the overall cost is two-fold that of commercial diesel, because of the expense of raw materials, which account for 60–70% of the total amount (Wright, 2006). Identification of alternative oil sources for biodiesel synthesis has thus attracted much interest, and among the various candidates, oleaginous microalgae appear to be the most promising feedstock for biodiesel production (Chisti, 2007). Microalgae have been highlighted as potential candidates for fuel production since they have a high photosynthesis efficiency, biomass productivity, and growth rate when compared with other energy crops (Miao and Wu, 2006; Gouveia and Oliveira, 2009; Posten and Schaub, 2009; Mata *et al.,* 2010). In addition, the lipid content of oil-rich microalgae (e.g., Chlorella vulgaris) could reach 50–60% of total dry cell weight (Yeh *et al.,* 2010; Yeh and Chang, 2011).

Sonication techniques, which apply a high-intensity ultrasonic wave to physically disrupt the cell wall, were shown to be effective in destroying the cell walls of microalgae (Wiltshire *et al.,* 2000; Pernet and Tremblay, 2003). The ultrasound method has been reported to extract over 90% of the different substances in one step and conserves the relationships of pigments and fatty acids (Wiltshire *et al.,* 2000). In addition, no alteration or breakdown products were observed with the ultrasonication method (Wiltshire *et al.,* 2000), allowing accurate quantitative extraction of both pigments and fatty acids from *Scenedesmus obliquus* and other algae (Wiltshire *et al.,* 2000). The ultrasonication method was also been found to be as effective for *Cryptomonas erosa* (Cryptophyceae), *Cyclotella meneghiniana* (Bacillariophyceae), *Microcystis aeruginosa* (Cyanophyceae), and *Staurastrum paradoxum* (Chlorophyceae, Desmidiaceae) and is thus applicable to a wide spectrum of algae (Wiltshire *et al.,* 2000). Therefore, this work used an ultrasonicator as a tool to disrupt cell wall of *Chlorella vulgaris* in order to make the microalgal oil available for the enzymatic transesterification.

Transesterification of oils with alcohol to produce biodiesel is usually accelerated by using catalysts, which are the major cost of the biodiesel processing (30–40% total cost). Transesterification is conventionally catalyzed by chemical catalysts, such as acid (Ma and Hanna, 1999), alkali (Vyas *et al.,* 2010), solid catalyst (Jothiramalingam and Wang, 2009), and lipase enzymes as biocatalysts (Ranganathan *et al.,* 2008; Jothiramalingam and Wang, 2009). While using acid, alkali, and free lipase increases the cost of post-processing treatment for purification of biodiesel, the same is not true for solid catalysts and immobilized lipase, since generate a lower amount of by-products, allowing easy isolation and purification of biodiesel, as well as the easy recovery of the catalysts for repeated use. However, the remaining activity of these catalysts during repeated operations is also a major consideration. Therefore, developing transesterification catalysts with the characteristics of high activity and high reusability is an urgent requirement for commercial

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applications in biodiesel production. As a biocatalyst, lipase is able to catalyze transesterification reactions at mild temperatures (25–65 °C) and in the presence of water and free fatty acids (Fukuda *et al.,* 2001; Ranganathan *et al.,* 2008). Moreover, lipase can be immobilized on supporters, enabling easy recovery and reuse of the biocatalyst. These unique advantages make lipase a better catalyst for applications in converting microalgal oil to biodiesel.

In this work, Biodiesel was produced using microalgal oil extracted from *Chlorella* sp, in this method transesterification was conducted directly using the cell-wall-disrupted biomass of the Chlorella strain. In addition, this process was also performed using wet microalgal biomass for enzymatic transesterification. The effects of reaction parameters (i.e., catalyst loading, temperature, water content, methanol to oil molar ratio, and hexane content) on the performance of lipase-catalyzed transesterification of microalgal oil with methanol were also examined.

## MATERIALS AND METHODS

### *Microalgae production, Strain and culture medium*

The *C. vulgaris* was isolated from a pond located in Annamalai nagar, Chidambaram. Basal medium was used for the pre-culturing (Sorokin and Krauss, 1958; Shi *et al.,* 1997), which consisted of (g $l^{-1}$): $KNO_3$, 1.25; $KH_2PO_4$, 1.25; $MgSO_4.7H_2O$, 1; $CaCl_2$, 0.0835; $H_3BO_3$, 0.1142; $FeSO_4.7H_2O$, 0.0498; $ZnSO_4.7H_2O$, 0.0882; $MnCl_2.4H_2O$, 0.0144; $MoO_3$, 0.0071; $CuSO_4.5H_2O$, 0.0157; $Co(NO_3)_2.6H_2O$, 0.0049; and EDTA.2Na, 0.5. The microalga was precultured at 25 °C for 4–5 days. The *C. vulgaris* strain has been reported as high potential in producing lipid for biodiesel production (Yeh *et al.,* 2010).

#### *Cultivation of Microalgae*

Chlorella was cultivated in 2L flask containing basal medium. During microalgae growth, the liquid sample was collected from the sealed glass vessel at various times to determine the microalgae biomass concentration and lipid content of the microalgal biomass.

Microalgae biomass was harvested from culture broth by centrifugation at 10,000g for 10 min. To produce dried microalgae powder, the microalgal cells were washed with deionized water twice, lyophilized, and weighed. Wet microalgae were also prepared by the above procedures, but without lyophilization. The water content of the wet microalgae was pre-determined before being used for biodiesel synthesis.

#### *Oil extraction*

In brief, 0.5–1 g dried microalgae were mixed with 15 ml deionized water. The mixture was subjected to sonication at 70 amplitude for 10 min using a sonicator to the disrupt cell walls of the microalga. The mixture containing the disrupted cells was then vigorously mixed with bi-phase solvent solution (chloroform– methanol, 10 ml: 10 ml) for 5 min. The resulting mixture was centrifuged at 6700g for 10 min and formed two layers. The lower layer contained a mixture of chloroform and microalgal oil, which were separated by evaporation in a hood under ambient condition to obtain slurry (oil portion). The slurry was repeatedly mixed with hexane and evaporated several times to completely remove chloroform and obtain crude microalgal oil.

### *Lipase production*

The lipase-producing strain was isolated from the soil. The strain was identified *Pseudomonas* sp. by biochemical characters. The strain was stored in the 1 ml mixture of 0.5 ml glycerol (30%) and 0.5 ml LB (25 g $l^{-1}$) medium under -80 °C as a stock source. The stock microorganism was precultured in 4 ml LB (25 g $l^{-1}$) medium at 30 °C and a shaking speed of 200 rpm for 12 h. The pre-culture was transferred to a fermentor containing 2 l of optimal sterilized medium which consisted of 2 g $l^{-1}$ yeast extract, 0.2 g $l^{-1}$ $MgCl_2.6H_2O$, 9.9 g $l^{-1}$ KCl, 6 g $l^{-1}$ $(NH_4)_2SO_4$, 5.4 ml $l^{-1}$ olive oil, and 5 ml $l^{-1}$ hexadecane (Wang, 2010). The fermentative production of lipase with *Pseudomonas* sp. was conducted at a controlled pH of 6.5, a fixed temperature of 30 °C, an aeration rate of 1 vvm, and a stirring speed of 400 rpm for 30 h (Wang, 2010). The fermentation broth was centrifuged at 9000g for 10 min and the supernatant part was harvested. The supernatant (containing crude lipase) was used to determine lipase activity and protein concentration, and was also used as lipase source for immobilization onto sodium alginate.

### *Lipase immobilization*

The seed culture of *Pseudomonas* sp was grown and the cells were harvested by centrifuging at 1000 rpm for 10 minutes and the cells were discarded and collect supernatant suspended in 0.1%(W/V) NaCl. Then, 2 % (W/V) of sodium alginate was added to the cells (2% W/V) biomass suspension and mixed thoroughly without forming any air bubbles in the slurry. The slurry containing the cells was extended as drops through a small tube into 4% $CaCl_2$ solution. The gel beads were kept in 4% $CaCl_2$ solution at $5^oC$ for about an hour for complete gelatin. The immobilized lipase was lyophilized at -50 °C for 24 h. The lyophilized lipase powder was used to determine the enzyme activity and to catalyze transesterification of microalgal oil with methanol.

#### *Analysis*

#### *Lipase activity assay*

Lipase activity of the samples was determined with the pH stat titrimetry method. The assay was carried out by adding 1 ml or 0.05 g sample into the reaction mixture containing 5% gum arabic and 10% olive oil (Liu *et al.,* 2007; Wang, 2010) under the condition of pH 9.0 and 55 °C for 5 min (Liu *et al.,* 2007; Wang, 2010). One unit (U) of lipase activity was defined as the amount of lipase liberating 1 μmol equivalent of fatty acid from olive oil per min. Total protein concentration in the solution was determined by Bradford assay using a commercialized Bio- Rad dye. The protein standard used was bovine serum albumin (BSA).

#### *Fatty acid methyl ester production*

The immobilized lipase catalyzed transesterification was performed in shaking flasks and heated to the reaction temperature on a constant temperature shaker, with the reaction rate of 180 rpm. The reaction conditions were 30% immobilized lipase (w/w, 12,000), 10% water content (w/w)

based on lipids quantity and 3:1 molar ratio of methanol to oil, at the temperature of 38°C and pH value of 7.0 (Stevenson *et al.,* 1994, Shimada *et al.,* 2002, Soumanou and Bornscheuer 2003). To determine the reaction rate and conversion from heterotrophic lipids to biodiesel, the reaction mixture was sampled every 2 h and analyzed by gas chromatograph (GC), through which the concentration of triglycerides, diglycerides, monoglycerides and fatty acid methyl esters could be determined. The conversion rate was calculated by the proportion of fatty acid methyl esters in the mixture.

### *Determination of biodiesel conversion efficiency*

The biodiesel conversion efficiency from microalgae was expressed by weight percent of FAMEs converted against the weight of oil content (wt% oil) and against the weight of dry microalgal biomass (wt% biomass). Calculations of the biodiesel conversion are according to the following equations.
Biodiesel conversion (wt% biomass)

$$= \frac{\text{Weight of FAMEs}}{\text{Weight of dry microalgal biomass}} \times 100\%$$

## RESULTS AND DISCUSSION

### *Lipase production and immobilization*

The lipase used in this work was produced from *Pseudomonas* sp., which was previously isolated and purified from crude oil contaminated soil. The *Pseudomonas* strain was found to produce an alkaline thermo active lipase with optimal activity at pH 9.0 and a temperature of 50 °C (Liu *et al.,* 2007; Wang, 2010). The lipase was produced in optimal medium composition at an optimal temperature of 30 °C, pH 6.5, agitation rate of 400 rpm, and aeration rate of 1 vvm (Wang, 2010). The activity of the free lipase thus obtained was measured to be 17.23 U $ml^{-1}$. The lipase loading efficiency (LLE) of the free lipase immobilized on sodium alginate was estimated as 92%, which corresponds to a protein loading of 19.78 mg $g^{-1}$. The specific activity of the immobilized lipase was estimated at 14.08 U $g^{-1}$.

### *Production of oil-rich microalgal biomass*

The biomass and lipid productivities of the strain were estimated as 0.0475 and 0.030 g $l^{-1}$ $d^{-1}$, respectively. The total lipid content was estimated as 63.17%. The fatty acid profile of the microalgal lipid includes C16:0, C18:0, C18:1, C18:2, C18:3, and other, accounting for 28.7%, 4.96%, 26.7%, 19.6%, 8.71%, and 11.3% of total fatty acids, respectively. The molecular weight of the microalgae oil was estimated that 789.3 g $mol^{-1}$ based on this lipid composition.

### *FAMEs production from extracted microalgal oil via enzymatic transesterification*

Since microalgae culture is in the form of dilute solution containing a solid content of less than 1%, the harvesting, dewatering, and oil extraction of oleaginous microalgae contribute significantly to the overall cost of microalgal biodiesel production (Miao and Wu, 2006; Mata *et al.,* 2010). As a result, it would be preferable and cheaper if microalgal biodiesel could be produced directly from wet microalgal biomass without significant dewatering and oil extraction. In this process, the microalgal biomass was directly used as the oil source for enzymatic transesterification. As shown in Tables 1–3, biodiesel production performance with this appllication was much better than that obtained instead of dried biomass. Under this catalyst loading, this method attained a biodiesel conversion of 95.17 wt% oil (Figure 1). The optimal temperature for that 40 °C, reaching a biodiesel conversion of 93.26 wt% oil (or 55.95 wt% biomass).

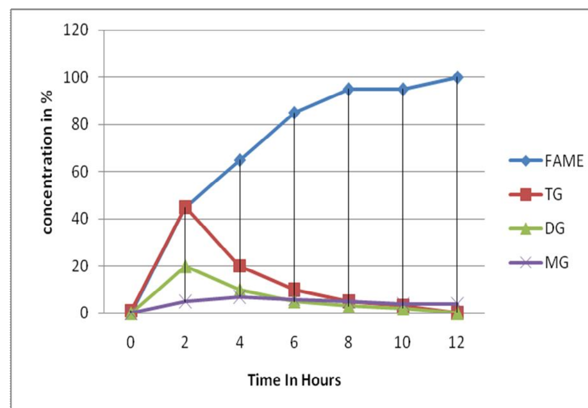


**Fig.1** Product composition for transesterification during the reaction time

In this process, the optimal water content, molar ratio of methanol to oil, and hexane content in the transesterification were higher, due to the absorption/adsorption and mass transfer barrier caused by the microalgal cell walls, despite the destruction of these walls by sonication prior to the reaction. The optimal water content in the approach was 61.39 wt%, leading to the maximum conversion of 94.21 wt% oil (or 56.52 wt% biomass) (Table 1). Similarly, the optimal molar ratio of methanol to oil went up to 67.93, achieving the highest conversion of 96.32 wt% oil (or 57.79 wt% biomass) (Table 2). Moreover, this process required up to 80.57 wt% hexane to obtain the maximum conversion of 92.15 wt% oil (or 55.29 wt% biomass) (Table 3).

**Table 1** Effect of water content on conversion of biodiesel from oil of Chlorella vulgaris

| Runs | $H_2O$ Content (Wt%)[a] | FAMEs Conversion[b] Wt % biomass[c] | Wt % oil[d] |
|---|---|---|---|
| 1 | 0 | 50.38 | 83.96 |
| 2 | 23.77 | 52.89 | 88.15 |
| 3 | 48.34 | 55.47 | 92.45 |
| 4 | 65.27 | 56.09 | 93.49 |
| 5 | 71.39 | 56.62 | 94.21 |
| 6 | 75.72 | 55.25 | 92.09 |
| 7 | 82.39 | 54.98 | 91.63 |
| 8 | 86.18 | 51.08 | 85.01 |
| 9 | 88.63 | 48.22 | 80.36 |
| 10 | 90.34 | 45.62 | 76.03 |

[a] wt % $H_2O = (m_{H20})/ (m_{oil}) + m_{oil}) \times 100\%$
[b] Estimated at the 48th hour
[c] weight percent based on dry weight of the microalgal biomass
[d] weight percent based on oil content of the microalgal biomass

In summary, the best condition for this method is catalyst loading, 1203.11 U $g^{-1}$; reaction temperature, 40 °C; water

content, 71.39 wt%; molar ratio of methanol to oil, 67.93; and hexane content, 80.57 wt%. Under this optimal condition, the highest conversion of 97.25 wt% oil (or 58.35 wt% biomass) during 48 h of reaction. The reason for requiring more methanol, water, and hexane, because in the former most oil mainly remained inside the ultrasonically pretreated microalgae (containing up to 36.83 wt% biomass), which may adsorb some of these liquids during transesterification. Hexane in particular is important in these process, since much more of this was needed (over 80 wt%) to achieve the optimal conversion. This indicates that mass transfer could be the major factor affecting biodiesel conversion when directly using pretreated microalgal biomass as the oil source for transesterification. It is known that the lipids produced by Chlorella vulgaris contain a significant amount of polyunsaturated fatty acids, such as palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2) and a-linolenic acid (C18:3) (Yeh *et al.,* 2010; Yeh and Chang, 2011). These acids could be partially oxidized during the extraction process, thus resulting in the loosening of their original content before transesterification (Belarbi *et al.,* 2000).

**Table 2** Effect of molar ratio of methanol to oil on conversion of biodiesel from oil of Chlorella vulgaris

| Runs | Molar ratio of methanol to oil | FAMEs Conversion[a] Wt % biomass[b] | Wt % oil[c] |
|---|---|---|---|
| 1 | 0 | 0 | 0 |
| 2 | 18.53 | 46.28 | 77.14 |
| 3 | 37.05 | 50.38 | 83.96 |
| 4 | 67.93 | 57.79 | 96.32 |
| 5 | 98.81 | 56.87 | 94.78 |
| 6 | 123.51 | 46.71 | 77.84 |
| 7 | 185.26 | 45.16 | 75.27 |

[a] Estimated at the 48th hour
[b] Weight percent based on dry weight of the microalgal biomass
[c] Weight percent based on oil content of the microalgal biomass.

**Table 3** Effect of hexane content on conversion of biodiesel from oil of Chlorella vulgaris

| Runs | Hexane content (wt%)[a] | FAMEs conversion[b] (Wt% biomass)[c] | (Wt% oil)[d] |
|---|---|---|---|
| 1 | 0 | 0 | 0 |
| 2 | 50.9 | 0 | 0 |
| 3 | 67.46 | 38.59 | 64.31 |
| 4 | 75.68 | 51.23 | 85.39 |
| 5 | 80.57 | 55.29 | 92.15 |
| 6 | 83.83 | 53.49 | 89.15 |
| 7 | 86.15 | 52.19 | 86.86 |
| 8 | 89.24 | 51.19 | 85.32 |
| 9 | 91.2 | 43.83 | 73.05 |

[a] wt% Hexane = $(m_{Hexane})/(m_{Hexane} + m_{oil}) \times 100\%$.
[b] Estimated at the 48th hour.
[c] Weight percent based on dry weight of the microalgal biomass.
[d] Weight percent based on oil content of the microalgal biomass.

This work demonstrated that directly using disrupted microalgal biomass for transesterification can produce biodiesel efficiently. Using this process, the immobilized lipase used can achieve a high FAMEs conversion efficiency of 97.25 wt% oil (or 58.35 wt% biomass) and can function under high water content (>71.39 wt%) and high methanol to oil molar ratio (>67.93) conditions. The proposed biocatalyst and conversion process has thus shown good potential for application in the commercial production of microalgal biodiesel.

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