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Efficient Biodiesel Conversion from Microalgae Oil of *Schizochytrium* sp.

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Abstract: Microalgae oil has been regarded as a promising feedstock for biodiesel production. However, microalgae oil usually contains some non-lipid components, such as pigments. Microalgae oil could be converted to biodiesel effectively with a two-step process to decrease the negative effect caused by by-product glycerol generated in traditional biodiesel production process. Firstly, microalgae oil was hydrolysed to free fatty acids (FFAs) and then FFAs were converted to methyl ester. In this study, the hydrolysis of microalgae oil from *Schizochytrium* sp. was systematically investigated and microalgae oil could be hydrolysed effectively to FFAs at both non-catalytic and acid-catalytic conditions. The hydrolysis degree of 97.5% was obtained under non-catalytic conditions of 220 °C and a water to oil ratio of 10:1 (w:w). The hydrolysis degree of 97.1% was obtained with the optimized sulphuric acid catalytic conditions of 95 °C, and a ratio of water to oil 3:1. The lipase Novozym435-mediated esterification with the hydrolysed FFAs was explored and a FAME (Fatty Acids Methyl Ester) yield of 95.1% was achieved. The conversion of different FFAs also was compared and the results indicated that lipase Novozym435-mediated methanolysis was effective for the preparation of biodiesel as well as poly unsaturated fatty acids (PUFAs).

Keywords: microalgae oil; lipase; methanolysis; biodiesel; esterification

1. Introduction

Microalgae has been regarded as a promising feedstock for biodiesel production because of its high growing rates, short life cycle, and diversified cultivation conditions [1–4]. It has been demonstrated that both chemical ways and enzymatic ways can be used for biodiesel conversion with microalgae oil [5–7]. Compared with the conventional chemical catalysis, enzymatic ways have shown distinctive features of mild reaction conditions, a low ratio of alcohol to oil and waste-free during the process [8–11]. Among different forms of biocatalysts, immobilized lipase has been widely investigated for biodiesel preparation, due to the fact that the immobilized lipase can be easily reused and has a good thermal as well as pH stability [12,13].

However, it has been demonstrated that during immobilized lipase-mediated alcoholysis for biodiesel production process, the by-product glycerol would be adsorbed onto the surface of the immobilized lipase. Since the by-product glycerol has a poor solubility in oils [14–16], glycerol would lead to a negative effect on lipase's activity [17,18]. Organic solvents were proposed to be introduced in the system to avoid inactivation [12,19,20].

To decrease the negative effects caused by the by-product glycerol generated in the traditional biodiesel production process, hydrolysis is adopted before the enzymatic conversion. The microalgae oil is hydrolysed to free fatty acids firstly, and then the oil phase (mainly free fatty acids) is used further

as the substrate for the second-step esterification catalysed by lipase. In this two-step process, the glycerol generated in the hydrolysis step remains in the water phase, and no glycerol is produced during the second-step esterification process. There are several processes for the hydrolysis of triglyceride (TG) to produce free fatty acids (FFAs), and these processes can be classified into catalytic hydrolysis process and non-catalytic hydrolysis process [21–24].

In this work, the hydrolysis process with microalgae oil was systematically investigated. Non-catalytic hydrolysis and catalytic hydrolysis processes with microalgae oil were compared, and the related reaction conditions were optimized. After the hydrolysis, the oil phase mainly containing FFAs from microalgae oil was further adopted, and the lipase-mediated esterification for biodiesel production was explored. Since some poly-unsaturated fatty acids (PUFAs) were found in the microalgae oil [25–27], the conversion of PUFAs also was discussed during this process. This study has established the proper hydrolysis and esterification process for biodiesel production from microalgae oil. It is also useful for the further optimization of biodiesel production with microalgae oil.

2. Results and Discussion

2.1. Analysis of FFAs Constitution of *Schizochytrium* sp. Oil

Different from other vegetable oils, microalgae oil usually has complicated components [28]. Different components affect the following transesterification. Consequently, the oil constitution of *Schizochytrium* sp. was analysed. The results are shown as Figure 1. The acid value and saponification value of *Schizochytrium* sp. oil are 20 mg KOH/g and 182 mg KOH/g, respectively.

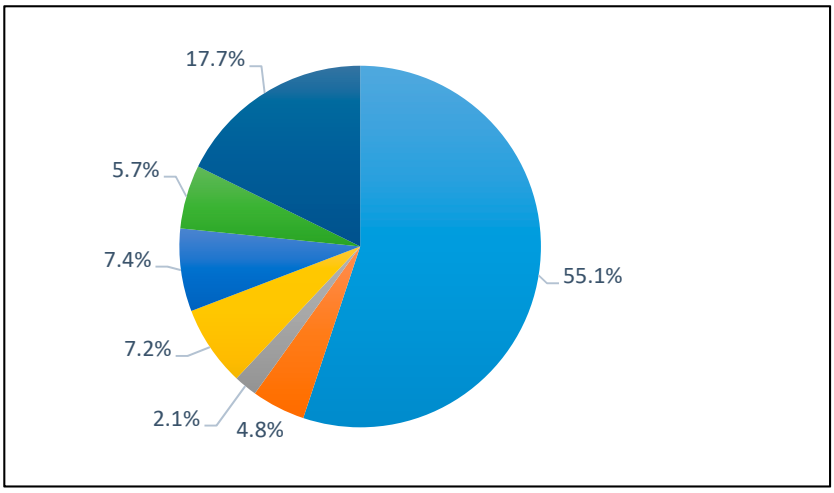

Figure 1. Fatty acids constitution of *Schizochytrium* sp. oil.

For some species, a high concentration of polyunsaturated fatty acids (PUFAs), in particular, *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA, C22:6) and *cis*-5,8,11,14,17-eicosapentaeonic acid (EPA, C20:5) are contained in microalgae oils [25,29–31]. As shown in Figure 1, the Palmitic acid (C16:0) took up 55.1% of the *Schizochytrium* sp. oil, and there was more than 20% PUFAs (C22:5 and C22:6) in *Schizochytrium* sp oil, that was different from the plant oil, such as soybean oil, rapeseed oil, and so on. The PUFAs was reported favourably modulate many diseases [32–34]. Herein, it is significant to concentrate such PUFAs during the conversion of microalgae oils for biodiesel production.

Although hydrolysis of conventional renewable oils has been reported at high temperature and pressure [18], the research on microalgae oil, especially on microalgae oil containing a high content of PUFAs, needs to be investigated. In the following study, hydrolysis of microalgae oil from *Schizochytrium* sp. was carried out in a non-catalytic process, and the effect of different parameters were studied systematically.

2.2. Hydrolysis of Microalgae Oil from Schizochytrium sp. at Non-Catalytic Condition

For the hydrolysis of renewable oils at non-catalytic conditions, usually the ratio of water to oil, temperature as well as reaction time may have a profound influence on the reaction process. Herein, the effect of ratio of water to oil, temperature, and reaction time on the hydrolysis of *Schizochytrium* sp. oil was investigated.

2.2.1. The Effect of Water to Oil Ratio on the Hydrolysis of *Schizochytrium* sp. Oil

Water plays an important role in the hydrolysis process, since hydrolysis is a reversible reaction. The ratio of water to oil varying from 2:1 to 12:1 was adopted and the results are indicated in Figure 2.

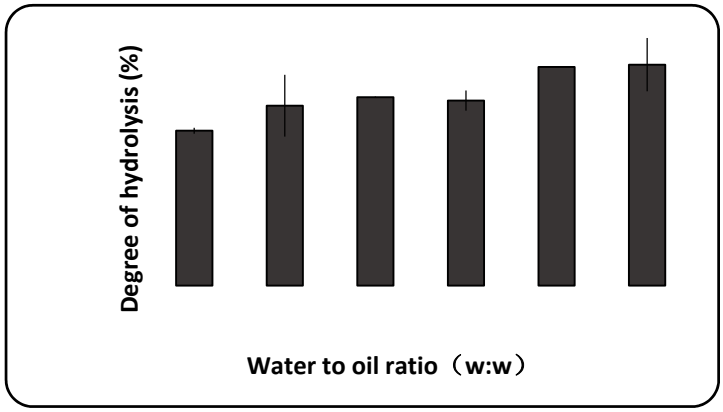

Figure 2. The effect of the water to oil ratio on the hydrolysis of *Schizochytrium* sp. oil. Conditions: Temperature 200 °C, pressure 2.0 Mpa, 5 hours, 150 rpm.

Figure 2 indicates that by increasing the water to oil ratio from 2:1 to 10:1, the degree of hydrolysis was visibly enhanced. A further increase of the water to oil ratio from 10:1 to 12:1 resulted in little improvement of the hydrolysis degree. Consequently, the optimized water to oil ratio was 10:1 and a maximum of 96% degree of hydrolysis was obtained.

2.2.2. The Effect of Temperature on the Hydrolysis of *Schizochytrium* sp. Oil

The effect of temperatures ranging from 200–260 °C on the hydrolysis of *Schizochytrium* sp. oil was studied and the results are suggested in Figure 3.

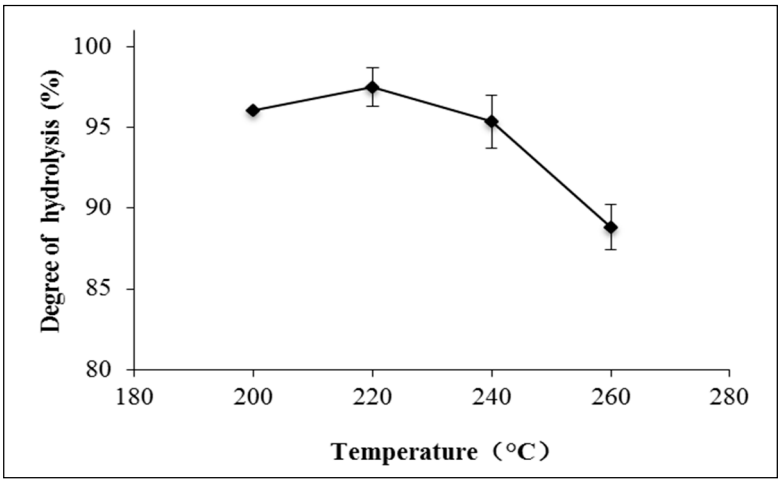

Figure 3. The effect of temperature on the hydrolysis of *Schizochytrium* sp. oil. Conditions: Water to oil ratio 10:1 (w:w), reaction time 5 hours, 150 rpm.

As shown in Figure 3, when the temperature was below 220 °C, with the temperature increasing, the hydrolysis degree was enhanced, and the highest hydrolysis degree of 97.5% was obtained at 220 °C. However, further increasing the temperature from 220 °C to 260 °C lead to an obvious decrease of the hydrolysis degree.

The microalgae oil contains a significant amount of PUFAs, and the polymerization or cleavage reaction might occur, subsequently resulting in a lower degree of hydrolysis at higher temperature. The different FFAs constitution variation in the hydrolysis process was further compared at different temperatures (Figure 4).

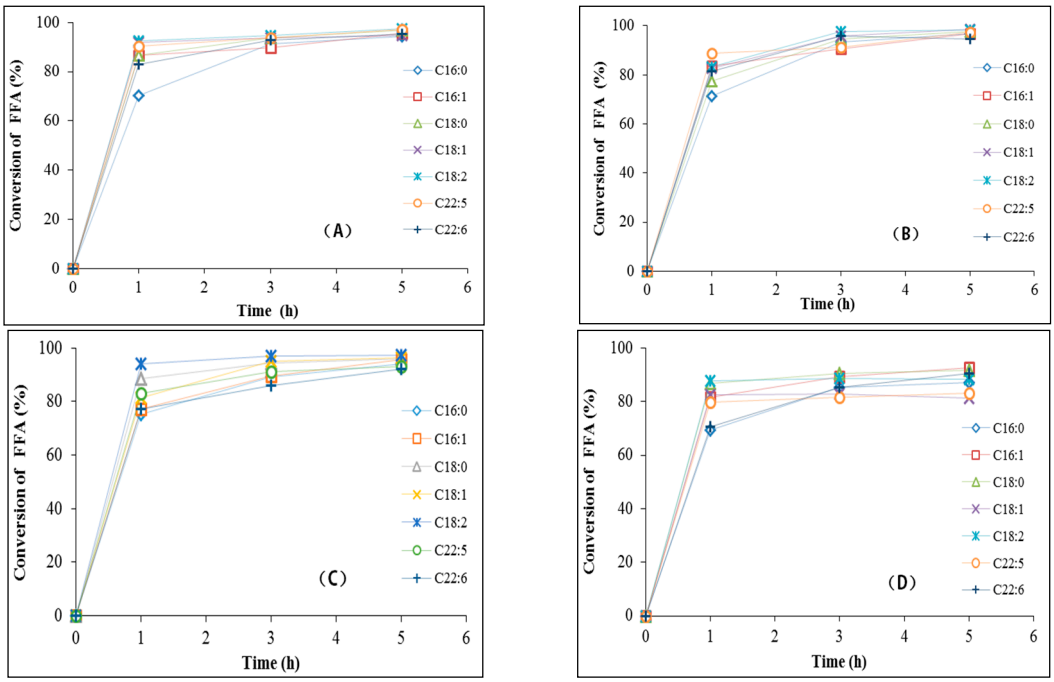

Figure 4. Free fatty acids (FFAs) change profile at different temperature. (**A**) 200 °C; (**B**) 220 °C; (**C**) 240 °C; (**D**) 260 °C.

From Figure 4, it can be noticed that there was no observable difference in the conversion of different FFAs from hydrolysis process, which indicates that high temperature and pressure hydrolysis was effective for the preparation of PUFAs.

2.2.3. The Effect of Reaction Time on the Hydrolysis of *Schizochytrium* sp. Oil

Effect of reaction time varying from 1 to 5 h on the hydrolysis was studied as Figure 5. The degree of hydrolysis reached 78.8% within 1 h, and then the degree of hydrolysis was improved slowly. The highest degree of hydrolysis of 97.5% was obtained at 5 h

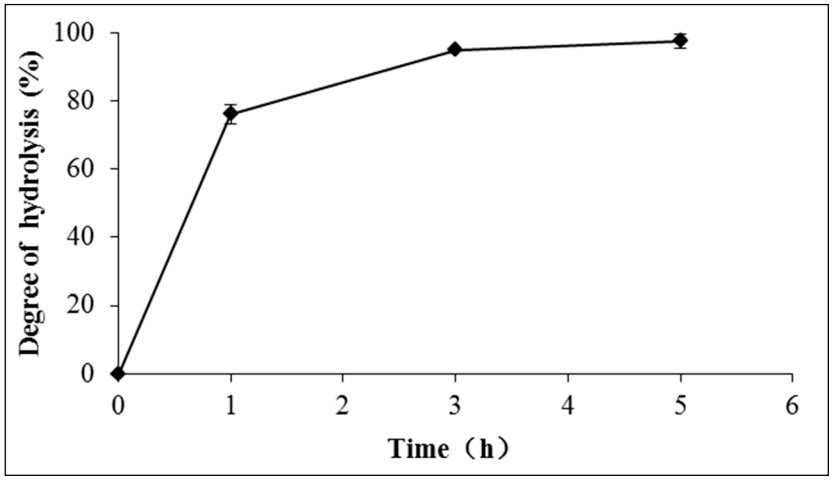

Figure 5. The effect of the reaction time on the hydrolysis of *Schizochytrium* sp. oil. Conditions: Temperature 220 °C, pressure 2.8 Mpa, water to oil ratio = 10:1 (w:w), 150 rpm.

The constitution variation of triglycerides, diglycerides, and monoglycerides was further analysed in Figure 6.

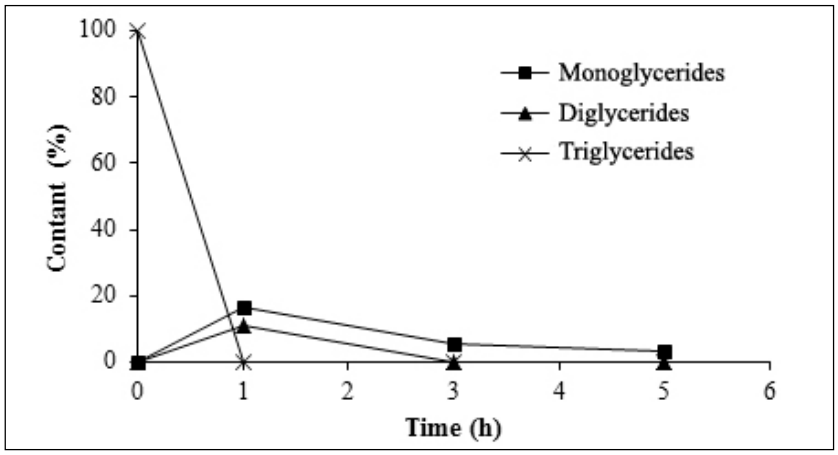

Figure 6. Change of triglycerids, diglycerides and monoglycerides during the process. Conditions: Temperature 220 °C, pressure 2.8 Mpa, water to oil ratio = 10:1 (w:w), 150 rpm.

As shown in Figure 6, the content of the triglycerides decreased rather quickly at 1 h in the reaction and then remained at a rather low level, while the content of the diglycerides and monoglcyerides increased from 0 to 1 h and decreased gradually from 1 to 5 h. The Final degree of hydrolysis reached 97.5% at 5 h, and the little unconvertable oils are mainly monoglcyerides.

2.2.4. The Effect of Water Recycle on the Hydrolysis of *Schizochytrium* sp. Oil

To save resources and decrease the cost of the whole process, the reuse of water for hydrolysis was also investigated. At the end of the reaction, two phases were formed. The water phase containing the by-product glycerol was recycled without any further treatment for the next hydrolysis reaction. The degree of hydrolysis was calculated with three cycles, and the results are suggested as Figure 7.

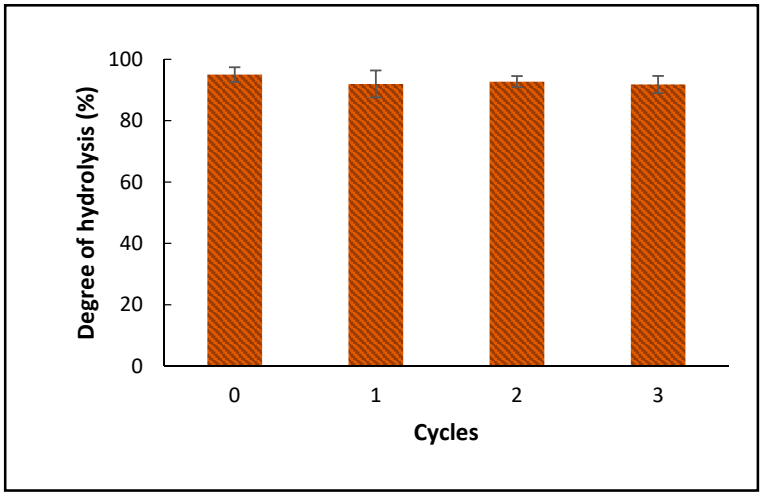

Figure 7. The effect of water recycling on the hydrolysis of *Schizochytrium* sp. oil. Conditions: Temperature 220 °C, pressure 2.8 Mpa, water to oil ratio 10:1 (w:w), 150 rpm.

Figure 7 shows that water can be reused effectively, even though a light decrease in the degree of hydrolysis was observed during the process. With the water recycled three times, a degree of hydrolysis of 91.8% still could be obtained. This little decrease might be caused by the accumulation of glycerol in the process, causing it to somewhat influence the hydrolysis.

2.3. Hydrolysis of Microalgae Oil from Schizochytrium sp. with Acid Catalysis

The hydrolysis process with a catalyst is one of the most efficient oil hydrolysis processes under ordinary pressures. The hydrolysis of microalgae oil from *Schizochytrium* sp. with sulphuric acid catalytic was compared.

2.3.1. The Effect of the Acid Catalysis—Sulphuric Acid on the Hydrolysis of *Schizochytrium* sp. Oil

Catalyst plays a significant role in the oil hydrolysis under ordinary pressure and can affect reaction rate and reaction time to reach an equilibrium. Different sulphuric acid dosages were compared in the hydrolysis (Figure 8).

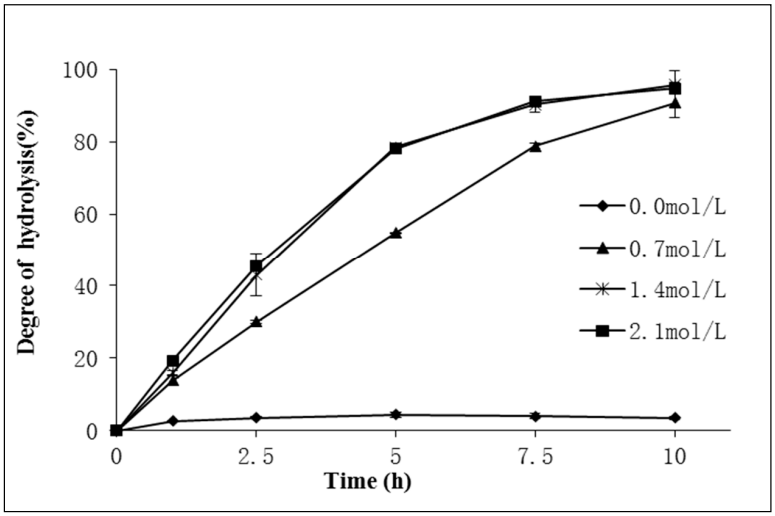

Figure 8. The effect of the sulphuric acid dosage on the hydrolysis of *Schizochytrium* sp. oil. (Reaction temperature = 95 °C, Water to oil ratio = 3:1 (w:w), 1% Sodiumdodecyl-sulfate based on oil weight, 750 rpm).

The results showed that hydrolysis could not be processed without the sulphuric acid added as the catalyst. With the sulphuric acid concentration increasing, the reaction rate enhanced. However, when the sulphuric acid concentration was higher than 1.4 mol/l, the degree of hydrolysis was almost the same.

2.3.2. The Effect of Sodium Dodecylsulfate Addition on the Hydrolysis of *Schizochytrium* sp. Oil

Because oil hydrolysis is an interface chemical reaction, the hydrolysis process is difficult to perform under ordinary pressure conditions if there are no surfactants. On the other hand, many surfactant additions could cause emulsification. The effect of sodium dodecyl sulfate on the hydrolysis process was explored. The sodium dodecyl sulfate concentration was in the range of 0.5% to 1.5% (based on oil weight), and the hydrolysis without a sodium dodecyl sulfate addition was the control. The results are shown as Figure 9.

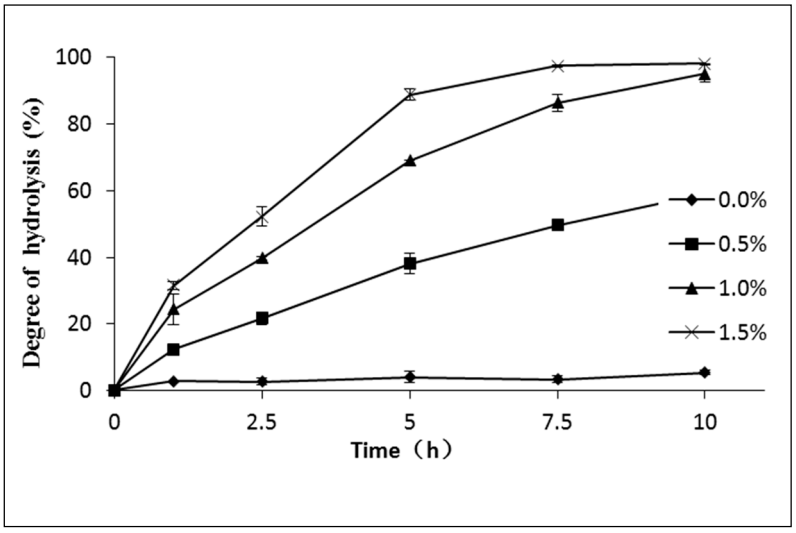

Figure 9. The effect of sodium dodecylsulfate dosage (based on oil weight) to the hydrolysis of *Schizochytrium* sp. oil. (Reaction temperature = 95 °C, Water to oil ratio = 3:1 (w:w), 1.4 mol/L sulphuric acid, 750 rpm).

Figure 9 showed that the hydrolysis could not be processed without sodium dodecyl sulfate additions, and with the sodium dodecylsulfate concentration increasing, the hydrolysis reaction rate was also enhanced. However, when the sodium dodecylsulfate concentration was higher than 1%, the final degree of hydrolysis was slightly improved.

2.3.3. The Effect of Water to Oil Ratio on *Schizochytrium* sp. Oil Hydrolysis

The ratio of water to oil also affects the degree of hydrolysis. The different ratios of water to oil were set as 1:1, 1:3, and 1:5. The results are indicated in Figure 10.

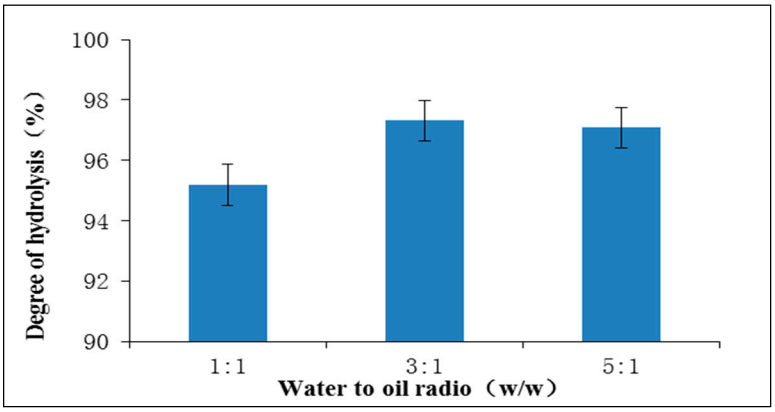

Figure 10. The effect of water dosage on the hydrolysis of *Schizochytrium* sp. oil. (95 °C, 1% Sodiumdodecyl-sulfate based on oil weight, 1.4 mol/L sulphuric acid, 750 rpm).

The results indicated that with the ratio of water to oil increasing, the degree of hydrolysis was also higher. However, the degree of hydrolysis could not increase with the higher ratio of 5:1, and the highest degree of hydrolysis, 97.1%, was obtained at a water to oil ratio of 3:1.

2.3.4. The Effect of Temperature on the Hydrolysis of *Schizochytrium* sp. Oil

Temperature visibly affects the hydrolysis of *Schizochytrium* sp. oil. Different temperatures (65 °C, 75 °C, 85 °C, 95 °C, and 100 °C) were set in the hydrolysis process and the results are suggested in Figure 11.

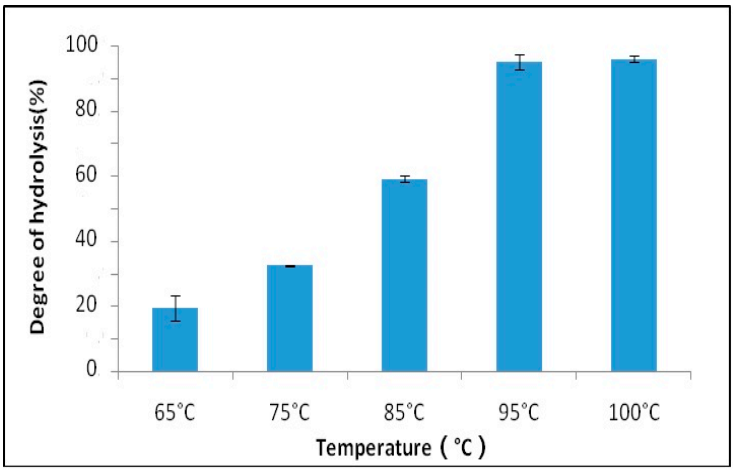

Figure 11. Effect of temperature on the hydrolysis of *Schizochytrium* sp. oil. (Water to oil ratio = 3:1 (w:w), 1% Sodiumdodecyl-sulfate based on oil weight, 1.4 mol/L sulphuric acid, 750 rpm).

As Figure 11 indicated, degree of hydrolysis was quite different at different temperature. With the increasing of the reaction temperature, the degree of hydrolysis increased, while it did not change when the temperature was higher than 95 °C. The degree of hydrolysis of 95.2% was obtained at 95 °C.

2.3.5. The Effect of Stirring Speed on the Hydrolysis of *Schizochytrium* sp. Oil

Stirring speed is one of the most considerable factors, because oil hydrolysis is an interface chemical reaction and the stirring speed might affect the mass transfer during the reaction [22]. The stirring speeds of 200 rpm, 700 rpm, and 1200 rpm were studied in the hydrolysis (Figure 12). The results suggested that the stirring speeds tested had no obvious effect on the acid catalytic hydrolysis on *Schizochytrium* sp. oil, and similar reaction rates and degree of hydrolysis (DH) were observed in all cases.

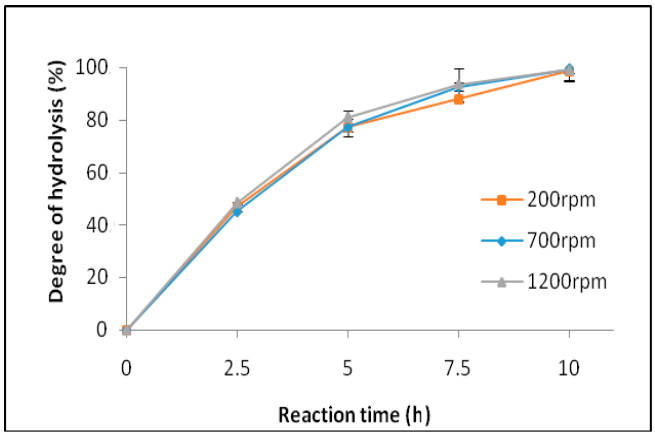

Figure 12. Effect of the stirring speed to the hydrolysis on *Schizochytrium* sp. oil. (Reaction temperature = 95 °C, Water to oil ratio = 3:1 (w:w), 1% Sodiumdodecyl-sulfate based on oil weight, 1.4 mol/L sulphuric acid).

2.3.6. The Effect of Water Recycle on the Hydrolysis of *Schizochytrium* sp. Oil

The effect of water recycling on the soybean oil and *Schizochytrium* sp. oil after the hydrolysis process is compared in Figure 13.

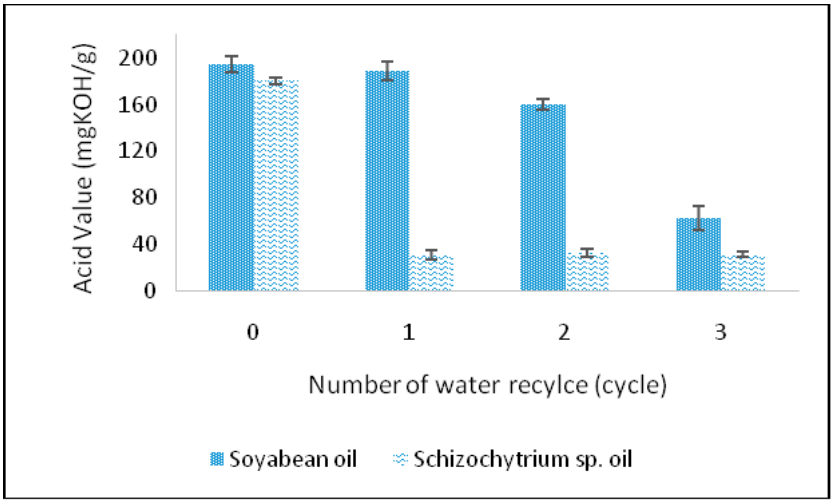

Figure 13. Effect of water recycle to the hydrolysis on soybean oil and *Schizochytrium* sp. oil (Reaction temperature = 95 °C, Water to oil ratio = 10:1 (w:w), 1% Sodiumdodecyl-sulfate based on oil weight, 1.4 mol/L sulphuric acid).

The results showed that acid value visibly decreases with every cycle of water reuse. However, the acid value of soybean oil decreases slowly. The reason is considered to be that the recovery of sulphuric acid or sodiumdodecyl-sulfate was incomplete, and another possible reason is the inhibition of glycerol, the by-product of the hydrolysis.

2.4. Lipase-Mediate Reaction of Hydrolysed Microalgae Oil for Biodiesel Production

After the hydrolysis, the oil phase (mainly FFAs) was collected and lipase Novozym435-mediated methanolysis of the hydrolysed microalgae oil was further conducted.

As Figure 14 suggested, the hydrolysed microalgae oil could be effectively converted to methyl esters and a fatty acid methyl yield (FAME) of 95.1% could be achieved at a molar ratio of methanol to oil of 2:1. During the reaction process, 3Å molecular sieves (1.2:1, *w*/*w*, based on oil weight) were used to remove the water that could cause the reverse of esterification [35,36].

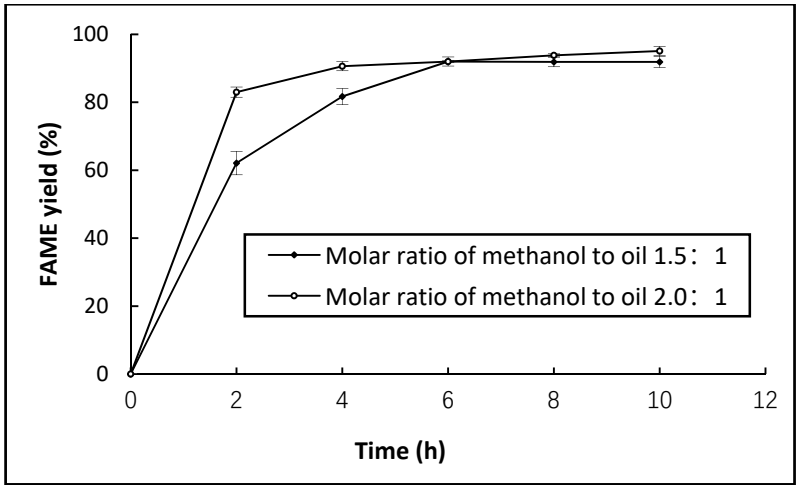

Figure 14. Lipase-mediated methanolysis of hydrolysed microalgae oil for biodiesel production.

Considering that lipase might show a substrate specificity towards different FFAs, the conversion of different FFAs, including PUFAs, was further compared, as shown in Figure 15.

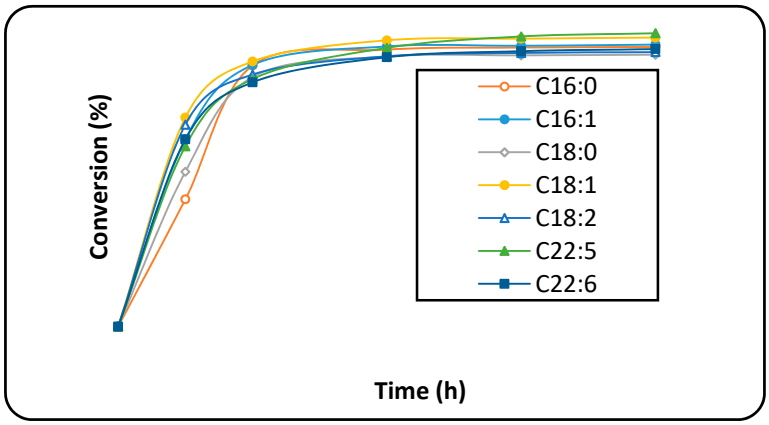

Figure 15. Conversion of different FFAs for corresponding methyl ester synthesis. Conditions: 1.5% Novozym435, 250 rpm, 45 °C, 3Å molecular sieves (1.2:1, *w*/*w*, based on oil weight), Molar ratio of methanol to oil 2:1.

Figure 15 showed that there was no observable difference in the conversion of different FFAs towards their corresponding methyl esters, which indicated that lipase Novozym435-mediated methanolysis was effective for the preparation of biodiesel as well as the PUFAs.

3. Materials and Methods

3.1. Materials

Schizochytrium sp. microalgae oil, with an acid value of 20 mgKOH/g and a saponification value of 182 mg KOH/g, was kindly supplied by ENN Group Co., Ltd. (Langfang, China). Immobilized lipase Novozym435 (*Candida antarctica* B lipase immobilized on a macroporous acrylic resin, activity 10000 PLU/g) was purchased from Novozymes (Copenhagen, Denmark). Chromatography reagents, such as heptadecanoic acid methyl ester and heptadecanoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents of analytical grade, such as methanol and ethanol were obtained commercially.

3.2. Experimental Procedure

3.2.1. Non-Catalytic Hydrolysis Process

The non-catalytic hydrolysis process was carried out in a 300 mL batch reactor with a mechanical stirrer and temperature control (± 0.1 °C). The hydrolysis reaction with the microalgae oil and deionized water was stopped at 5 hours. Then the oil phase (mainly containing FFAs) was cooled to room temperature.

3.2.2. Catalytic Hydrolysis Process with Sulphuric Acid

The sulphuric acid-mediated hydrolysis of microalgae oil was carried out with 40 g microalgae oil, sulphuric acid (1.8 mol/L), deionized water, and 0.4 g sodium dodecylsulfate in a 500 mL mechanical stirring flask performed at 95 °C for 10 hours with an oil bath.

3.2.3. Enzymatic Esterification

The immobilized lipase-catalysed process was carried out in a 150 mL Erlenmeyer flask at 45 °C and 250 rpm. The reaction mixture contained FFAs collected from the above hydrolysis process, molecular sieves of type 3Å, and Novozym435. Samples were taken from the reaction mixture and centrifuged at 60 °C, then vacuum distilled with the vacuum degree 0.1 MPa to evaporate the methanol and collect the oil layer for GC analysis.

3.3. Analytical Methods

3.3.1. Determination of Acid Value and Degree of Hydrolysis

The acid value was measured according to the traditional method [37]. The saponification value was determined by ASTM (American Society for Testing and Materials) D558.95 [38]. The degree of hydrolysis (DH) was determined as the following formula:

$$\text{Degree of hydrolysis}\,(\%) = \frac{AV_t - AV_{t0}}{SV - AV_0}$$

where SV is the saponification value, AV_0 is the acid value of the oil sample, AV_{t0} is the initial acid value of the sample before the hydrolysis, and AV_t is the acid value in time t.

3.3.2. Fatty Acid Methyl Ester (FAME) Analysis

The maximum FAME content of oil is measured by the standard procedure AOAC 991.39 (Association of Analytical Communities) [39]. FAMEs were analysed by a 7890 A gas chromatography (Agilent Technologies, Santa Clara, CA, USA) with a CP-FFAP capillary column (0.32 mm × 25 m). The initial column temperature was set at 160 °C and maintained for 0.5 min, then heated to 250 °C at the rate of 10 °C/min and held for 6 min. Detector and injector temperature were set at 250 °C and 245 °C, respectively [39].

The analysis procedure is described as follows: 6 mg product and 0.6 mL heptadecanoic acid methyl ester (0.8 g/L, internal standard) ethanol solution were mixed and then 1 µl sample was taken and injected for GC analysis. FAME yield was calculated as the following formula:

$$\text{FAME yield} = \frac{\text{FAME content}}{\text{The maximum FAME content}} \times 100\%.$$

3.3.3. Analysis of Glyceride

Glyceride analysis is carried out by a Shimadzu 20A HPLC system (Shimadzu, Kyoto, Japan) with a C18 column (5µm, 250 mm × 4.6 mm) (Dikma Technology, Beijing, China) and ELSD-LT(Evaporative Light Scattering Detector) II low temperature-evaporative light scattering detector. The mobile phase

was dichloromethane and acetonitrile with 0.15% acetic acid mixed with the gradient elution program (as in Table 1). The temperature of the column and detector was set to 45 °C and 70 °C, respectively, with the hydrogen pressure kept at 320 KPa. For analysis, 5 μL heptadecanoic acid (internal standard) and 1 mL acetone were mixed thoroughly, and then a 20 μL sample was taken for HPLC analysis [40].

Table 1. Gradient elution program.

Time (min)	Flow Rate (mL/min)	Dichloromethane (V/V, %)	Acetonitrile with 0.15% Acetic Acid (V/V, %)
0	1.50	0	100
4	1.50	0	100
12	1.50	10	90
25	1.50	10	90
30	1.50	30	70
35	1.50	30	70
45	1.50	80	20
55	1.50	80	20
60	1.50	0	100
65	1.50	0	100

4. Conclusions

Microalgae oil can be converted to biodiesel efficiently with the two-step process and a FAME yield of 95.1% could be achieved through lipase-mediated methanolysis. For the first step, microalgae oil was hydrolysed to free fatty acids (FFAs) and then FFAs were converted to methyl ester. Both non-catalytic hydrolysis and acid-catalytic hydrolysis of microalgae oil were studied systematically, and the high hydrolysis degree could be reached under both conditions. The optimized condition of non-catalytic hydrolysis was 220 °C, and the water to oil ratio of 10:1 (w:w) and the hydrolysis degree of 97.5% were obtained. For the acid catalytic process with sulphuric acid as the catalyst, the final hydrolysis degree reached 97.1% at the condition of 95 °C, the ratio of water to oil 3:1, and the sodium dodecylsulfate addition ratio 1%. A stirring speed from 200–1200 rpm has no obvious effect on the hydrolysis. With the hydrolysed microalgae oil, a final FAME yield of 95.1% could be achieved through lipase Novozym435-mediated esterification. GC analysis indicated that PUFAs can also be transformed efficiently through this two-step process. Consequently, this process is very promising for biodiesel production derived from microalgae oil feedstock.

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