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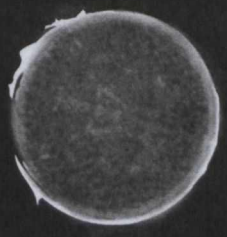

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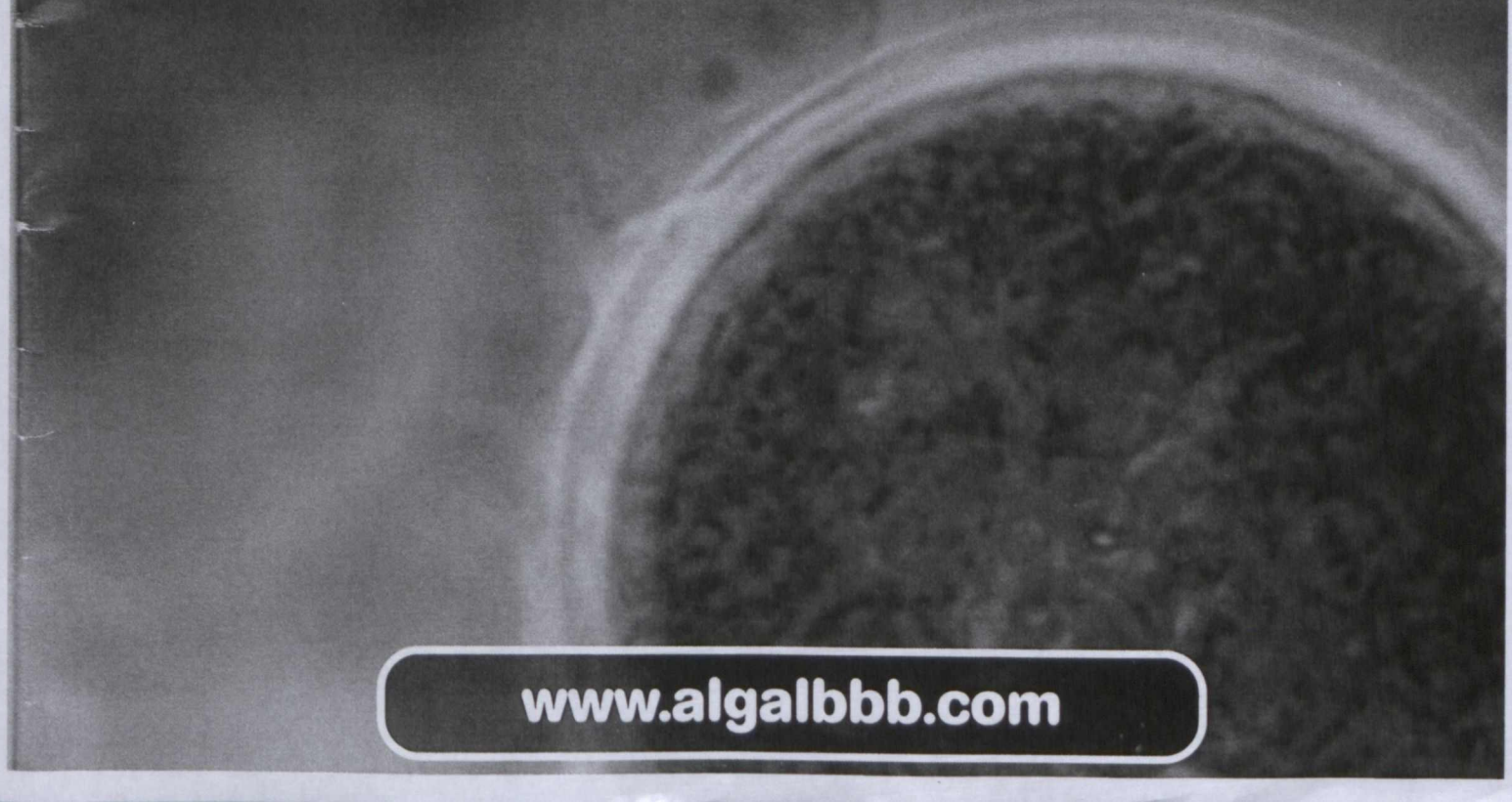

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Investigation of extraction and transesterification of algae by immobilized lipase as biofuel

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Abstract

In this study, three different methods were investigated to study the disruption of algae cells walls such as ultrasonication, soxhlet and hexane (solvent) extraction. *Chlorella* sp. and *Spirulina* sp. were two selected algae species to analyze the effect of each method for biodiesel production. The oil yield is calculated to determine the best effective method for oil extraction. The highest oil yield obtained by ultrasonication followed by Soxhlet and hexane extraction. Moreover, acid value for *Chlorella* is the lowest for all extraction method. Then, the crude algae oil was transesterified by Novozyme 435, an immobilized enzyme, at 60°C, 300 rpm for 24 hours to hydrolyze triglycerides into free fatty acids. The ester content was analyzed by FTIR and found at 1735, 1731, 1214, 1162, 1161, 1064, 1060, 1059 cm^{-1} frequency. Fatty acid derivatives were measured by gas chromatography and palmitic acid was among of the major component in microalgae biodiesel.

1.0 Introduction

Depletion of oil supply, energy security and climate change due to global warming are amongst of problems arise when discussed about biofuel (Noraini et al., 2014). Biofuel is one the alternative energy to replace fossil fuel today with special characteristics such as

environmental friendly, reduces net carbon dioxide emissions by 78% (Gerpen, 2005), renewable, non-toxic, non-flammable and biodegradable (Ahmad et al., 2011; Shahid and Jamal, 2011). Microalgae have attracted much attention as one the potential feedstock for biofuel. A lot of benefits are associated with microalgae such as high oil content (Borugadda and Goud, 2012; Wang et al., 2013; Yan et al., 2014), high biomass production (Demirbas 2011; Wang et al., 2013; Nautiyal et al., 2014), reduce gas emission through carbon dioxide fixation (Demirbas, 2011; Wang et al., 2013), non-toxic, no sulphur and highly biodegradable (Demirbas, 2011).

The conventional process for biodiesel production is via transesterification of oil and alcohol catalyzed by catalysts, or supercritical conditions with or without the presence of catalyst. Soap generation and dewatering are the challenges faced when operated under homogeneous catalyst or alkaline-based solid catalyst (Tran et al., 2013; Noraini et al., 2014). Other drawbacks for conventional process are high energy intensive, difficulty in recovering the glycerol and significant produce amount of alkaline waste water (Tan et al., 2010; Tran et al., 2013). Applying enzyme in transesterification catalyzed by lipase has many advantages due to its mild reaction, less energy intensive, does not promote side reactions and more environmental friendly (Borugadda and Goud, 2012; Rahmath and Ravindra, 2013; Tran et al., 2013).

Novozyme 435 is an immobilized enzyme and used extensively in enzymatic transesterification converting triglycerides into fatty acid methyl ester (FAME). Novozyme 435 has been successfully converting triglycerides into FAME for several feedstocks with more than 90% conversion such as sunflower oil (Ranganathan et al., 2008), cotton seed oil (Royon et al., 2007), palm oil (Zhang et al., 2010) and microalgae; *Chlorella* sp. (Lai et al., 2012; Tran et al.; 2012). Infrared Spectroscopy (IR) is a well-established alternative nondestructive analytical technique which allows reliable, direct, and fast determination of

several properties without pre-treatment. It was been applied in almost every aspect of biodiesel quality control and good indicator to find potential feedstocks for biofuel production. Fourier Transform Infrared Spectroscopy (FTIR) especially was used widely to study lipid composition, quality of biodiesel as mentioned in previous studies (Stehfest et al., 2005; Dean et al., 2010; Zhang, 2012; Mayers et al., 2013).

The objectives of this study are to determine the best algae lipid extraction method and investigate the potential of biofuel conversion from microalgae. In this work, microalgae from *Chlorella* sp. and *Spirulina* sp. were extracted by three different methods to disrupt the algae cells for instance soxhlet extraction, ultrasonication and solvent extraction. Then, the immobilized lipase (Novozyme 435) was used to catalyze the enzymatic transesterification under constant operating condition. The ester content was further analyzed by FTIR and gas chromatography.

2.0 Materials and Methods

2.1 Raw materials, chemicals and reagents

Dried algae (*Chlorella* sp. and *Spirulina* sp.) were supplied by local supplier. Immobilized lipase (Novozyme 435) was obtained from Sigma-Aldrich. All organic solvents (*n*-hexane, methanol, ethanol, chloroform) were analytical grade obtained from Fisher Scientific.

2.2 Extraction of lipid from microalgae

2.2.1 Soxhlet extraction

Thirty grams (30g) of dried algae lipids were subjected to Soxhlet extraction with hexane as the solvent with a ratio of 1:6 (w/v). The extraction was carried out for 8 hours and the mixture was filtered several times to remove the residue. Remaining solvent was fully distillate by rotary evaporator to obtain crude algae oil. The crude algae oil was kept for

further transesterification process.

2.2.2 Ultrasonication

The disruption of algae cells by ultrasonication was carried out as described in Tran et al. (2012) with minor modifications. Thirty grams (30g) of dried algae was mixed with distilled water and processed with a Vibra-cell ultrasonicator (Model VCX500, Sonics & Materials, Inc USA) at 70% amplitude for 15 minutes to disrupt the algae cells. Then, the mixture was mixed with bi-phase solvent of chloroform and methanol (1:1; v/v) for 10 minutes. Centrifugation process (Rotofix 46, Hettich Zentrifugen) is necessary to separate algae residue and solvent at 2000 rpm for 15 minutes. Bottom layer consist of chloroform and crude oil was dried under hood to obtain slurry oil. Hexane was added and evaporated to remove remaining solvent. This step was repeated several times to obtain crude algae oil. The crude algae oil was kept for further transesterification process.

2.2.3 Solvent extraction

Thirty grams (30g) of dried algae was mixed with hexane and agitated at 300 rpm for 8 hours at 60°C. The mixture was centrifuged at 2000 rpm for 20 minutes to remove algae residue. The top layer mixture was filtered several times and evaporated to remove remaining solvent. The crude algae oil was kept for further transesterification process.

2.3 Conversion of algae oil to biodiesel by immobilized lipase

Microalgae oil was reacted with methanol as the acyl acceptor via lipase-catalyzed transesterification to produce fatty acid methyl ester (FAME). Fresh mixture containing cell wall-disrupted microalgal biomass was transferred into 50 ml cylindrical glass bottle in the presence of 5% (w/w) immobilized lipase, Novozyme 435. Desired amounts of hexane and

methanol were then added into the bottle to carry out the transesterification process. Methanol was added in stepwise addition as described in Shimada et al. (2002), Dizge et al. (2009) and Rahmath et al. (2013). The reaction took place for 24 hours at 300 rpm agitation at 60°C. Samples were withdrawn and kept for further analysis.

2.4 Analysis

The extracted crude oil was collected and kept for further analysis. The oil yield for each extraction was calculated as below:

$$\text{Oil yield (\%)} = \frac{\text{Mass of oil}}{\text{Mass of algae}} \times 100$$

The acid value of oil was determined according to ASTM D664 by acid value tester (Mettler Toledo). All assays were carried out in triplicate for the calculation of the mean value.

After transesterification, the product or FAME was analyzed by FTIR (Model: Spectrum 400, Perkin Elmer) according to ASTM D7371. Drops of oil were placed on the glass plate to be analyzed by system. Ester content was observed between 1750-1735 cm^{-1} and 1320-1000 cm^{-1} frequency at room temperature.

2.5 Gas chromatography analysis of FAMEs

The composition of FAMEs was analyzed using a gas chromatograph (GC) (7890A, Agilent Technologies, USA) equipped with HP-INNOWax column (30 m length x 0.32 mm diameter) and a flame-ionization detector with nitrogen as the carrier gas. Samples were injected at a column temperature 60°C. The column temperature was maintained at 200°C for 2 minutes gradually increased to 240°C at a rate of 10°C/min, and then maintained for 9 minutes. Detector and injection temperatures were 300°C.

3.0 Results and discussion

3.1 Extraction of algae lipid

In this work, three different methods of algae lipid disruption were carried out and compared to determine the highest oil yield extracted from two different species of algae. A common method of Soxhlet extraction was also studied and compared with solvent (hexane) and ultrasonication. Fig.1 shows the percentage oil yield for three different methods of extraction. Ultrasonication process is the most efficient methods to extract the algae cells for both species of microalgae, while the mass of dried algae was kept constant. Ultrasonication is one of the efficient methods to disrupt the microalgae cells which have been mentioned in several studies in the literature (Adam et al., 2012; Tran et al., 2012; Natarajan et al., 2014; Safi et al., 2014). During ultrasonication process, sonicwaves were transmitted to the microalgae culture and created series of microbubble cavitations which imparted kinetic energy into the surface of the cells and eventually ruptured the cells (Halim et al., 2012). As the cells are disrupted, the cell concentration decreases. The ultrasonication process becomes less energy effective at low cell concentrations (Adam et al., 2012; Natarajan et al., 2014).

Fifteen minutes of ultrasonication process achieved a higher crude oil yield compared to eight hours Soxhlet and solvent extraction (oil yield for ultrasonication after 15 minutes = 2.83 g for *Chlorella* sp. and 1.98 g for *Spirulina* sp., oil yield for Soxhlet extraction after 8 hours = 1.05 g for *Chlorella* sp. and 1.25 g for *Spirulina* sp., oil yield for solvent extraction after 8 hours = 0.68 g for *Chlorella* sp. and 0.77 g for *Spirulina* sp. Higher percentage of oil yield for *Chlorella* sp. (9.4%) and *Spirulina* sp. (6.6%) in this study can be compared to *Chloroccum* sp. from other study which only obtained 4.5% of extraction (Halim et al., 2012). Similar study has been carried out for Soxhlet extraction in Halim et al (2011) using *Chlorococcum* sp. and the lipid yield was much lower, which 0.032 gram obtained from 4 g

dried algae. Hexane is the most popular and economic solvent which commonly used in the extraction study. However, hexane was not work well for *Chlorella* sp. as it only can dissolve non-polar compounds in the algae. This can be observed in Fig 1, two methods of extraction which using hexane as the solvent, giving lower oil yield compared to *Spirulina* sp.

Table 1 shows the comparison study of oil yield based on different extraction method. Lee et al (2010) studied the effects of five different cell disruption method on three different species including *Chlorella vulgaris*. Based on the investigation, the best method to disrupt the *Chlorella vulgaris* cells were microwave oven (10% lipid yield) and autoclaving (10% lipid yield) (Table 1). However, sonication only resulted for 5% lipid yield for 5 minutes reaction time. As a comparison, our finding shows for 15 minutes sonication process, maximum of 9% lipid yield was achieved for *Chlorella* sp. The results show that longer reaction time was needed to disrupt the thick cell walls of *Chlorella* sp. to increase the lipid yield in sonication process. It is noted that different species of microalgae have different disruption capacity. For instance, *Botryococcus* species achieve 16% of lipid yield by using ultrasonication (Lee et al., 1998). After all, it depends on the thickness and diameter of the microalgae cells walls.

[TABLE 1]

[FIGURE 1]

3.2 Conversion of microalgae oil into FAME by lipase-catalyzed transesterification

Conversion of the extracted microalgae oil to biodiesel (FAME) was determined by an immobilized enzyme, Novozyme 435. According to Table 2, higher acid values were recorded for both species of microalgae compared to other researchers. Meanwhile, *Chlorella* sp. recorded lower acid value compared to *Spirulina* sp in all three types of extraction method. Acid value of microalgae oil extracted from *Chlorella pyrenoidosa* was very low

with value of 2.52 mg KOH/g by Soxhlet extraction method (Lai et al., 2012). Meanwhile, other findings recorded value from 8.8-9.42 mg KOH/g by Soxhlet extraction method (Li et al., 2007; Nautiyal et al., 2014) from different species of algae.

[TABLE 2]

Methanol was added into a mixture of fresh microalgae oil and immobilized lipase in a stepwise manner to complete the transesterification process. In this study, 1/3 molar methanol was added at initial stage, 2/3 molar methanol at 4 hour and 3/3 molar methanol was added at 8 hour. Methanol can inhibit lipase reaction and should be used in an amount that is an excess amount of the stoichiometric ratio (Tran et al., 2012). High concentrations of methanol are toxic to lipase which will cause the decreasing enzyme activity and affect its stability (Li et al., 2007; You et al., 2013). Thus, the methanol stepwise addition is the common choice since it not only avoids the methanol toxic effect, but also expand the contact area of oil and methanol to achieve a high yield (Shimada et al., 2002; Yan et al., 2014).

Moreover, high content of phospholipids in oil feedstock also will affect yield of biodiesel or FAME. Phospholipids content of microbial oils are much higher if compared with vegetable oils. Li et al. (2014) investigated the effect of phospholipids on free lipase-mediated biodiesel production. High content of phospholipids, which more than 5% appeared to have negative effects on biodiesel production. Further investigation revealed that co-existence of phospholipids and methanol exhibited negative effect on biodiesel production. Another researcher Du et al. (2004), also found out lipids in crude oil sources itself as the enzyme inhibitor.

3.3 Analysis of FTIR result for the conversion of algae oil into FAME

The peaks or bands in the FTIR spectrum are due to the functional groups present in a particular sample. In the present study, mid FTIR region was selected to identify the functional groups in the particular sample. FTIR spectra for both species showed several distinct absorption bands, over the wavenumber range 4000-400 cm^{-1} frequency. The bands were assigned to specific molecular groups on the basis of biochemical standards and published studies, as describes previously (Stehfest et al., 2005; Dean et al., 2010; Zhang, 2012). The bands of interest to locate esters bonds in the compounds can be found in two regions; 1750-1735 and 1320-1000 cm^{-1} frequency. Summary of infrared spectroscopy bands for each microalgae species for different method of extraction after lipase-catalyzed transesterification were described in Table 3.

Fig. 2 shows FTIR results for both species of microalgae after transesterification for Soxhlet extraction method. There were six peaks identified for specific bonds in the FAME from *Chlorella* sp. microalgae. Bands were attributed to: i) alkanes, methyl and methylene groups (2922 cm^{-1} and 2855 cm^{-1}), ii) aldehydes, C=O stretch (1731cm^{-1}), iii) alkanes, C-H bend (1459cm^{-1}) and iv) esters, C-O stretch (1214cm^{-1} and 1064cm^{-1}). Highest peak was spotted at 1064cm^{-1} frequency indicates the ester bond. Meanwhile, there were five peaks identified for FAME from *Spirulina* sp. after transesterification. The bands were recognized as: i) alkanes, methyl and methylene groups (2924 cm^{-1} and 2855 cm^{-1}), ii) aldehydes, C=O stretch (1733cm^{-1}), iii) alkanes, C-H bend (1458cm^{-1}) and iv) esters, C-O stretch (1214cm^{-1}).

[TABLE 3]

[FIGURE 2]

Transesterification also was carried out for crude oil obtained from both species by ult rasonication extraction process. As highlighted before, ultrasonication process gave highest p ercentage yield of oil extraction for both species. As a result, several distinct peaks were obse

rved in both species after each sample analysed by FTIR as shown in Fig 3. Six peaks identified for specific bonds in the FAME from *Chlorella* sp. microalgae. The bands were founds attributed to: i) alkanes, methyl and methylene groups (2923 cm^{-1} and 2856 cm^{-1}), ii) aldehydes, C=O stretch (1733cm^{-1}), iii) alkanes, C-H bend (1454 cm^{-1}) and iv) esters, C-O stretch (1161cm^{-1} and 1060 cm^{-1}). Highest peak was spotted at 1060 cm^{-1} frequency indicates the ester bond. In addition, six distinct peaks were observed in FAME from *Spirulina* sp. such as: i) alkanes, methyl and methylene groups (2922 cm^{-1} and 2854 cm^{-1}), ii) esters, C=O stretch (1735 cm^{-1}), iii) alkanes, C-H bend (1453 cm^{-1}) and iv) esters, alcohols, C-O stretch (1162 cm^{-1} and 1059 cm^{-1}). Highest peak was spotted at 1059 cm^{-1} frequency indicates the ester bond.

[FIGURE 3]

The patterns of bands distribution and peaks obtained were quite similar for both species of microalgae after transesterified by immobilized lipase. They showed similar functional groups were presence in the samples mixture such as alkanes, esters and aldehydes. Several bands were of particular interest found in both species, esters bond were found at 1735, 1214, 1161, 1162, 1064, 1060, 1059 cm^{-1} frequency showing the presence of esters in the FAME analyzed. The presence of peaks between 1733-1731, identified as C=O bonds in this study indicates the conversion of oil to biodiesel (Nautiyal et al., 2014).

Although the percentage of absorbance is quite low which below 51% absorbance, it is a good sign showing the transesterification reactions were successful for both species of microalgae. The presences of alkanes were due to mixture of hexane in the sample during transesterification reaction. The result from this study was agreed with Dean et al. (2010) at bands 1750-1735 cm^{-1} for ester groups and 1200-950 cm^{-1} associated with t(C-O-C) stretching of polysaccharides. Another study also has been carried out to determine the free fatty acids in fish oil for biodiesel production by using FTIR (Aryee et al., 2009). FTIR and

titrimetric method were compared to analyze the FFA content. However, FTIR method was more reproducible, accurate and simpler than the titrimetric method.

3.4 Analysis of ester content by gas chromatography

Gas chromatography analysis was used to study the chemical composition of algae biodiesel produced from *Chlorell*a sp. and *Spirulina* sp. by enzyme-catalyzed transesterification. The peaks in the chromatogram of biodiesel samples and the standards were compared and their respective retention time was used to identify and quantify the peaks. Table 4 shows composition of fatty acid methyl ester content obtained after enzyme-catalyzed transesterification reaction for each sample by different method of extraction. Palmitic acid, a saturated fatty acid was one of the major components for all *Chlorella* sp. and *Spirulina* sp. biodiesel in this study. *Chlorella* sp. biodiesel from Soxhlet extraction rich with linoleic acid, unsaturated fatty acids. Meanwhile, linolenic acid was the major component in *Spirulina* biodiesel for Soxhlet extraction. In addition, both *Chlorella* and *Spirulina* biodiesel by solvent extraction method have maximum component of palmitic acid, similar with *Spirulina* biodiesel by ultrasonication extraction method. Figure 4-6 explained in details the chromatogram being observed in gas chromatography.

[TABLE 4]

Another study also reported ester content in *Spirulina* by Nautiyal et al. (2014) which extracted by Soxhlet exraction in Table 5. Palmitic, linolenic and linoleic acids were the major fatty acids in the *Spirulina* biodiesel. Similar with our findings, linolenic acid was the major component in our *Spirulina* biodiesel by Soxhlet extraction method. The pond water algae biodiesel also was tested on gas chromatography and palmitic acids have the highest percentage of ester content (Nautiyal et al., 2014). Ultrasound assisted extraction were being tested on *N. oculata* microalgae and further transesferication process analyzed by gas

chromatography (Adam et al., 2012). Only three fatty acids detected in the biodiesel sample such as myristic, palmitic and strearic acids. Our analysis by ultrasonication extraction method also showed the palmitic acids are the highest ester content in the *Spirulina* biodiesel. From all the findings, palmitic acids are the major component in all biodiesel samples observed in this study. Different methods of lipid extraction also affect the biodiesel yield and conversion.

[TABLE 5]

[FIGURE 4]

[FIGURE 5]

[FIGURE 6]

4.0 Conclusion

This study provides preliminary investigation about the efficiency for different extraction method of algae. Different species will react differently towards the method of extraction based on the size, diameter and cell walls of the microalgae. In conclusion, ultrasonication proves the best method to extract lipid from microalgae for both species *Chlorella* and *Spirulina*. The transesterification study was successful for both species of microalgae and obtained from the 1750-1735cm^{-1} and 1320-1000 cm^{-1} frequency. The main components of fatty acids in biodiesel produced were detected by gas chromatography which consists of undecanoic, palmitic, linoleic and linolenic acids.

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