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Isolation, Identification and fatty acid analysis of microalgae from West Sumatera, Indonesia

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Abstract

Increasing of energy demands leads the decline of fossil fuels increase of greenhouse gases levels in the atmosphere. Therefore, many studies have been conducted to search alternative energy sources. Microalgae biodiesel is a renewable resource and can fulfill the demand for transportation fuels. Microalgae contain lipids and fatty acids that can be converted to biodiesel via transesterification reaction. In this research, samples are collected from Sawahan freshwater, Sumatera Barat, Indonesia. Nile red staining was used to select microalgae containing lipid. The species of the isolated microalgae were identified by microscopic and molecular identification. Based on molecular identification, the isolated microalgae has 89% similarity with *Micractinium reisseri.* The major fatty acids of isolated microalgae are C16:0, C18:0, C18:1, C18:2 and C18:3.

Key words: Isolation, Nile red staining, microscope, PCR, Fatty acids

1. Introduction

Increasing of energy demands leads the decline of fossil fuels and increase of greenhouse gases levels in the atmosphere. Therefore, many studies have been conducted to search alternative energy sources. Microalgae biodiesel is a renewable resource and can fulfill the demand for transportation fuels. Microalgae can be used as an alternative fuel energy source because it contains lipids, which can be extracted, processed, and converted into transportation fuels using available technology. Biodiesel production using microalgae offers the following advantages: (1) microalgae have a rapid growth rate and can be grown without plantation on the land: (2) microalgae cultivation consumes less water than land crops: (3) microalgae have the same process with higher plants to reduce the greenhouse effect with capture of carbon dioxide in photosynthesis reaction using energy from light, so that it is environmental friendly: (4) its production does not depend on the weather and can be harvested daily (da-Silva et al., 2009; Gouveia et al., 2009; Guedes et al., 2011; Li et al., 2008).

The ability of microalgae to produce biodiesel depends on lipids and fatty acids which are contained in microalgae. Intracellular lipid of microalgae can be detected by Nile red staining and observed under fluorescence microscope (Chen et al., 2009; Govender et al., 2012). Nile red (*9-diethylamino-5H-benzo[α]phenoxazin-5-one*) is photo stable and intensely fluorescent in organic solvents and hydrophobic environments (Bertozzini et al., 2011). The Nile red fluorescence method has been successfully applied to the determination of lipids in some microalgae (Chen et al., 2009; Govender et al., 2012). The lipid bodies (yellow fluorescence) can be easily located by Nile red in the cell and it is a conventional, qualitative method of lipid identification (Elumalai et al., 2011). In this research, Nile red staining was used to screen microalgae in freshwater samples to select which microalgae contained much intracellular lipid to be isolated.

Lipid and fatty acids are the primary metabolites of microalgae to produce biodiesel. *Chlorella vulgaris* (Gouveia et al., 2009; Sahu et al., 2013), *Spirulina maxima* (Gouveia et al., 2009), *Nannochloropsis* sp. (Gouveia et al., 2009), *Neochloris oleabundans* (da-Silva et al., 2009; Gouveia et al., 2009), *Scenedesmus obliquus* (da-Silva et al., 2009; Gouveia et al., 2009), *Botryococcus braunii* (Yoo et al., 2010), *Scenedesmus sp*. (Sahu et al., 2013), *Nannochloropsis sp*. (Bondioli et al., 2012), *Tetraselmis suecica* (Bondioli et al., 2012) and D*unaliella tertiolecta* (Gouveia et al., 2009) have been studied. *Neochloris oleabundans* (freshwater microalgae) and *Nannochloropsis* sp. (marine microalga) proved to be suitable as raw materials for biofuel production (da-Silva et al., 2009; Gouveia et al., 2009). The conditions of cultivation influence lipid and fatty acids in microalgae. *B. braunii*, cultivated in 10% CO_2 , contains high lipid and oleic acid (Yoo et al., 2010) and suitable for biodiesel production. Fatty acids are the major components in forming biodiesel. Previous research has done to observe fatty acids in some microalgae. *Desmodesmus sp*, *Desmodesmus elegans*, *Scenedesmus sp*, *Scenedesmus sp.*, *Chlorella sp.* and *Chlorococcum macrostigmatum* contain similar fatty acids. The major fatty acids in some microalgae were C16:0, C16:4, C18:1, C18:2 and C18:3 (Abou-Shanab et al., 2011; Kaur et al., 2012; Sahu et al., 2013).

In this research, samples including microalgae were collected from Kuranji and Sawahan freshwater, Sumatera Barat, Indonesia. Samples were screened by Nile red staining and microalgae containing much lipid were isolated. Isolated microalgae were cultivated in BBM medium and identified the species by microscopic and molecular identification. The fatty acids of isolated microalgae were analyzed for biodiesel production using GC-MS and the total fatty acids are determined qualitatively using FTIR. The growth of microalgae was observed in several nitrogen concentrations in order to choose the better concentration of nitrogen to grow isolated microalgae.

2. Methods

2.1. Sampling and isolation

Samples of microalgae were collected from Sawahan freshwater, Sumatera Barat and stored in BBM medium. Isolation was carried out using capillary pipette technique. Microalgae were seeded in microplate 48 wells and kept in incubation for 2 weeks. The intensity of light was ±3000 lux and the temperature was maintained at 30^{o}C.

2.2. Nile red staining

Nile red (*9-diethylamino-5H-benzo[α]phenoxazin-5-one*) was dissolve in acetone (1 mg in 1 ml acetone) was used to stain microalgae. Microalgae (0.5 ml) were centrifuged and washed with physiological saline solution several times. Nile red was added in resuspended microalgae in physiological saline (1 : 100 v/v) and incubated for 20 minutes (Elumalai S., 2011). Lipid staining was observed under fluorescence microscope (ZEISS).

2.3. DNA extraction, PCR amplification, electrophoresis and sequencing

DNA of microalgae was extracted using Dneasy® mini plant kit (Qiagen). The extract of DNA was amplified in PCR (Takara, Japan): initialing in 96^{o}C for 5 minutes, denaturation in 96^{o}C for 30 s, annealing in 57^{o}C for 30 s, extension in 72^{o}C for 30 s, and termination in 72^{o}C for 4 minutes. All reactions were carried out for 35 cycles using universal primer for the amplification of the D1-D2 rRNA region, fw1 (5'-AGCGGAGGAAAAGAAACTA-3') as forward primer and rev1 (5'-TACTAGAAGGTTCG-ATTAGTC-3') as reverse primer were used. Electrophoresis was carried out in agarose gel 1 %. The bands were extracted using NucleoSpin® Gel and PCR Clean-up kit and cloned using pGEM®-T easy vector kit. DNA plasmid was extracted using Wizard® Plus Minipreps DNA Purification kit. Sequencing was carried out using specific primer T7 (5`-TAA-TAC-GAC-TCA-CTA-TAG-GG-3').

2.4. Cultivation and growth of microalgae

Microalgae were cultivated in flask culture in BBM medium. The growth of microalgae was measured everyday using microplate reader OD_{570} (PerkinElmer).

2.5. Fatty Acids Analysis of microalgae

Isolated microalgae (10 ml) were centrifuged with speed 3000 rpm for 10 min (TOMY LC-200) and separated from the supernatant. Pellets were dried with nitrogen gas and 5 µL of internal standard (C19:0) 0.5 mg/ml were added. Methyl esterification was performed by adding 0.1 ml of 0.5% HCl-methanol, 0.4 ml of dehydrated methanol. Fatty acid methyl esters (FAMEs) were extracted using n-hexane and separated by centrifugation. n-Hexane layer was dried with nitrogen gas. For GC-MC analysis, hexane (40 µL) was added and input 1µL sample to GC column. GC-MS analysis used DB WAXTR column (30 m, φ = 0.250 µm) and the temperature was set about 130^{o}C.

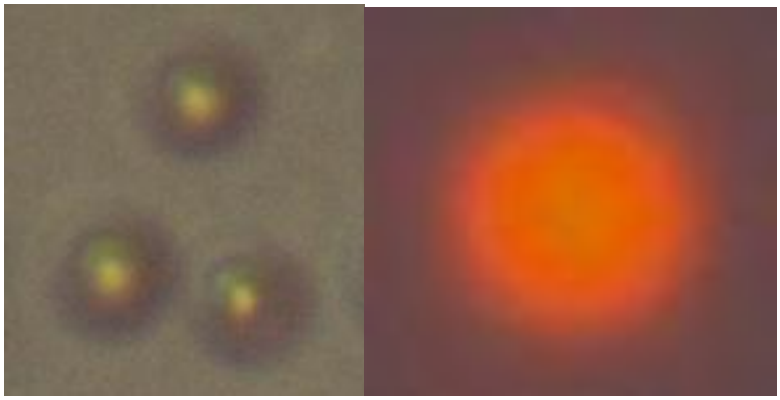
3. Result

3.1. Isolation

One species of microalgae had been isolated using capillary pipette and observed under light microscope (Fig. 1a) based on its growth and lipid content. The species was determined by microscopy and molecular identification.

3.2. Nile Red Staining

Several samples from Sumatera Barat freshwater were screened to obtain which microalgae contained lipid. Fig. 1b shows the result of Nile red staining for isolated microalgae. Isolated microalgae shows yellow color in its cells. It means isolated microalgae contain neutral lipid. Neutral lipids including hydrocarbons and triglycerides were stained in yellow, while polar lipids were stained in red (Matsunaga et al., 2009). Nile red staining is the simple method to see lipid in microalgae. It is easy to be used, and only needs a small amount of sample (Gao et al., 2008). Interaction of Nile red with lipid gives yellow color under fluorescence microscope. It can be used to screen microalgae to decide potential microalgae for biodiesel production (Pick et al., 2012). Rigid cell walls prevent penetration of the fluorescence dye into the cell (Chen et al., 2011; Govender et al., 2012).



a b

Fig. 1. Isolated microalgae (a) without Nile red staining and (b) with Nile red staining Photos were taken using fluorescence microscope λ=515-565 nm (ZEISS).

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3.3. DNA extraction, PCR amplification, Electrophoresis and Sequencing

DNA of isolated microalgae has been extracted using Dneasy mini plant KIT. D1-D2 rRNA region is amplified using universal primer, fw1 (5'-AGCGGAGGAAAAGAAACTA-3') as forward primer and rev1 (5'-TACTAGAAGGTTCG-ATTAGTC-3') as reverse primer. rRNA genes are chosen for identification because they have high copy numbers, can be amplified with truly universal primers and shows a divergence rate (Abou-Shanab et al., 2011; Sonnenberg et al., 2007). Electrophoresis of PCR product showed one band. It means the culture of isolated microalgae is pure and does not contain another species. The species is determined by NCBI BLAST based on the sequence result of the band and compared with microscopy observation. The species of isolated microalgae has 884 bp of rRNA and was confirmed as *Micractinium reisseri* with 89% similarity and the E value is 0.

3.4. Fatty Acid Analysis

Fatty acid is a major component in biodiesel production. Previous study has investigated fatty acids contained in microalgae. Accumulations of fatty acids in microalgae depend on several factors such as medium, light intensity, and temperature. In this study, isolated microalgae was cultivated in BBM medium for 7 days and incubated with continuous light and temperature was maintain at 30^{o}C. Fatty acids contained in isolated microalgae were analyzed using GC-MS. Isolated microalgae contains C18:2 as the major fatty acid (Fig.2). Composition of fatty acids in isolated microalgae has been measured quantitatively by comparing the peak of internal standard, C19:0, with each peak of fatty acids (Table 1).

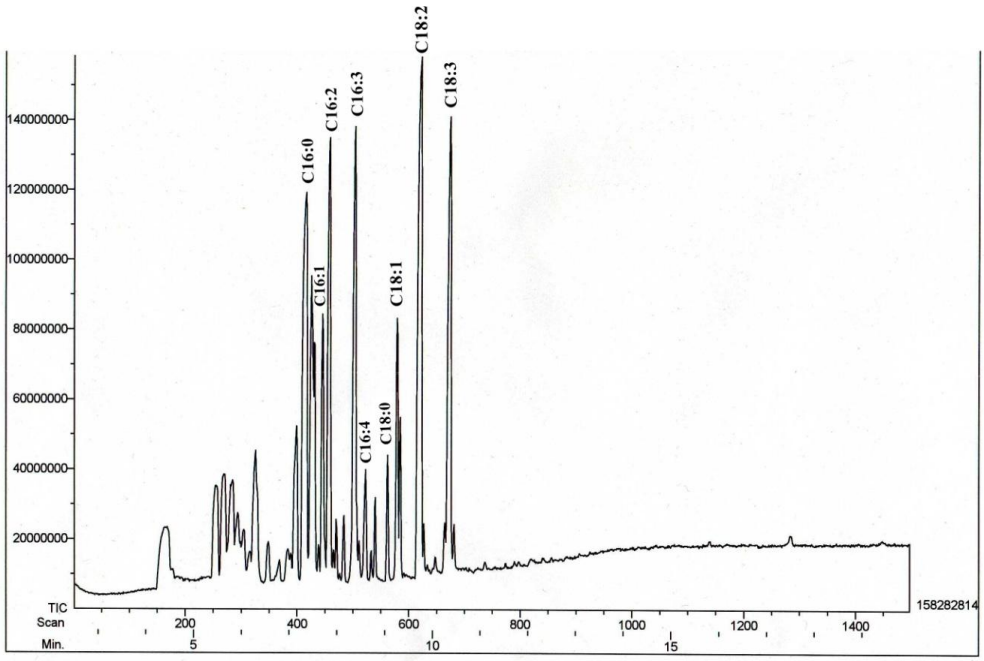

Fig. 2. GC-MS chromatogram of isolated microalgae

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Table 1. Fatty acids composition of isolated microalgae

Fatty acid	Amount of fatty acids (nmol/µL)
C16:0	0.25
C16 : 1	0.17
C16 : 2	0.29
C16 : 3	0.30
C16 : 4	0.08
C18 : 0	0.09
C18 : 1	0.15
C18 : 2	0.34
C18 : 3	0.29

Cultivation and Growth of Microalgae

The growth of microalgae is important for biodiesel production. Microalgae that have rapid growth and higher fatty acids are the best candidate to be the biomass in producing biodiesel (Sahu et al., 2013). Isolated microalgae was cultivated in BBM medium for 21 days and measure its growth rate by microplate reader (OD_{570}). It has rapid growth in 25 mg $NaNO_3$/100 ml BBM for 6 days. Cultivation for more than sixth days need more nutrient and the higher concentration of nitrogren is require because nitrogren is a major nutrient for microalgae growth (Ratha Sachitra K., 2013). Isolated microalgae is cultivated in several nitrogen concentrations. It grows well and survives for 19 days in 75 mg $NaNO_3$/100 ml. This concentration is suitable to growth isolated microalgae for long time (Fig. 3).

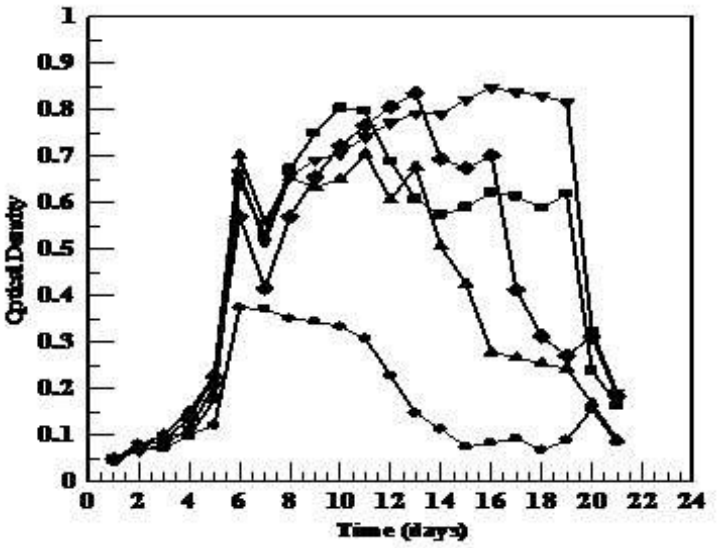

Fig. 3. Growth rate of isolated microalgae in several $NaNO_3$ concentrations (mg) in 100 mL BBM. The mass of $NaNO_3$ are ●; 10, ▲; 25, ■; 50, ▼; 75, ♦; 100

4. Conclusion

Nile red staining can be utilized to screen microalgae containing lipids for a candidate of biodiesel production. Samples from Sawahan freshwater West Sumatera, Indonesia, have microalgae containing lipids. One species of microalgae has been isolated and was identified by microscopy and molecular identification. It has 89% similarity with *Micractinium reisseri* by Gene Bank. GC-MS analysis for fatty acids composition showed it has C16:0, C16:1, C18:1 and C18:2 which are essential for biodiesel production. Isolated microalgae have rapid growth in BBM medium, and can survive for long time in 75 mg $NaNO_3$/100 ml BBM medium.

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