ISSN: 2229- 6905

Journal of Algal Biomass Utilization
J. Algal Biomass Utln. - Copyright © 2009 PHYCOSPECTRUM - ISSN: 2229 – 6905

Isolation and Identification of *Cercomonaslongicauda* Microalgae from Brackish Water in Padang, Indonesia.

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Abstract

In recent years, microalgae have been developed as a potential source for biodiesel production because of the high amount of oil content. In this research, the brackish microalgae isolated from MuaraPenjalinan, Padang, Indonesia, and analyzed for its suitability as a biodiesel feedstock. PCR results showed that isolated species was *C. longicauda* withsimilarity was 99.8%. *C. longicauda* shows 17% of fat content in stationary phase. Types of fatty acids contained in *C. longicauda* are C16:0, C17:0, C18:0 and C20:0 fatty acids. In varied concentration of $NaNO_3$ in BMM medium, *C. longicauda* has optimum growth at 27.5 mg/L. Based on the content of fat, the types of fatty acids, and growth characteristics of *C. longicauda* microalgae, it was concluded that microalgae can be used as biodiesel feedstock.

Keywords: Microalgae, *C. longicauda*, Nile red, Fat, PCR, Biodiesel.

1. Introduction

Energy as one of the basic needs for humans life are running low, this is caused by a commonly used fuel derived from a non-renewable petroleum (Christi., 2007). Fuel utilization also contribute in increasing of greenhouse gases that affect global warming, it is associated with the CO_2 released into the atmosphere in excessive amounts, the impact of those things are the climate change and the earth's rising temperature (Walter et al., 2012). Therefore, the utilization of eco-friendly alternative energy really is important.

Microalgae can be used as an alternative fuel energy source because it contains a high amount of oils that can be extracted, processed, and converted into transportation fuels by using available technology. Microalgae are able to accumulate oil more than 50% of their dry weight (Walter et al., 2012). Bioenergy production from microalgae is considered as a process to produce a renewable energy to combat the global warming (Elumalal et al., 2011). The advantages of microalgae as a source of bioenergy include the following: (1) It can synthesize and accumulate large amounts of lipids: (2) Rapid growth rate, so that it is possible to win through the needs of bioenergy: (3) It can grow in all types of waters: (4) It can utilize the nutrients for growth such as nitrogen and phosphorous from wastewater: (5) It can reduce the greenhouse gas emissions (Hu Qiang Hu et al., 2008; Li Yanqun et al., 2008).

In this research, sample including microalgae were collected from Muara Penjalinan. Padang, West Sumatera, Indonesia. Isolated microalgae were cultivated in BMM medium and identified the spesies by molecular identification. Lipid containing from isolated microalgae identified by Nile red staining and fat by soxhlet ekstractor. The fatty acids of isolated microalgae were analyzed for biodisel production using GC-MS. The growth of microalgae was observed in several nitrogen cocentrations in order to choose the better concentrations of nitrogen to grow isolated microalgae.

2. Materials and methods

2.1 Sampling

Microalgae samples were collected from MuaraPenjalinan. This assay use plankton net with 30 microns of hole size and three coordinates points which is then collected into one. Collected microalgae were cultivated in BMM media (Bristol's Modified Medium). Media BMM was prepared by dissolving 10 g of $NaNO_3$, 1 g of $CaCl_2.2H_2O$, 7 g of KH_2PO_4, 3 g of K_2HPO_4, 3 g of $MgSO_4.7H_2O$, and 1 g of NaCl in 400 ml of sterile distilled water, and 10 ml of each solution was then dissolved in 1 L of sterile distilled water. Coordinates of sampling points were recorded using a GPS tool.

2.2 Isolation of Microalgae

Serial dilution and Capillary Pipette is a technique that can be used for isolation process (Welch ., 1980; Belcher Hilary et al., 1982). In this research, the isolation was performed by combining the serial dilution technique and a capillary pipette technique. In serial dilution, isolation of microalgae was carried out by taking 1 ml of sample and putting into 9 ml of media, the sample is allowed to grow and then identified with a microscope. After the dominant microalgae were grown, the microalgae cells were taken by using capillary pipette and identified with a microscope. This microalgae cell was transferred into microplate that has been filled with BMM medium and incubated at 30° C with ± 3000 lux of lamp intensity. Microalgae were isolated and allowed to grow for few days and it was then observed with a microscope until monoculture is produced.

2.3 Nile Red Staining

0.1 ml of 33 µg/ml Nile red (*9-diethylamino-5H-benzo [α] phenoxazin-5-one*) in DMSO is mixed 0.6 ml of culture and stored in light proof room for ± 3 minutes. The color of lipids observed with a fluoroscence microscope at wavelength of 470 nm (ZEISS).

2.4 Molecular Identification

DNA of microalgae was extracted by using Dneasy® plant mini kit (Qiagen). DNA extract was amplified by using PCR (Takara, japan). with 18s rRNA, FW1 (5'-CCTGGTTGATCCTGCCAG -3 ') and rev1 (5'-TTGATCCTTCTGCAGGTTCA-3') as primers (Xun Yang et al., 2012). PCR was performed for 35 cycles; initialing was performed at 95^{o} C for 5 min, denaturation was performed at 95^{o} C for 30 seconds, annealing was performed at 56.3^{o} C for 30 seconds, extension was performed at 7^{o}C for 2 min, and termination was performed at 72^{o}C for 7 minutes. PCR products were electrophoresed through 1% of agarose gel. DNA bands were extracted by using NucleoSpin® Gel and PCR Clean-up kit. Direct sequencing was directly performed on DNA from electrophoresis and DNA sequences that were obtained from NCBI gene data (Blast). Sequence alignment of isolated species and several species that have similarity was observed by using ClustalX2. Distance analysis between species was performed by using MEGA5 program. Phylogenetic tree was made by using Neighbor-Joining assay.

2.5 Fat Content

Fat content in microalgae can be extracted by using Soxhlet method (Wiley., 2013). 0.5 g of sample was dried in an oven at 80^{o} C, inserted into soxhlet tool and extracted with hexane for 6 hours. Fat extract was dried in an oven at 105^{o}C and it was then cooled and weighed. Fat content was calculated by using gravimetry method.

2.6 Analysis of Fatty Acids

1 ml of isolated microalgae was centrifuged at 3000 rpm for 10 minutes (TOMY LC-200) and it was then separated with its supernatant. Pellets were dried with nitrogen gas and added into 5 ml of internal standard (C19: 0) 0.5 mg/ml. Esterification of methyl was performed by the addition of 0.1 ml of 0.5% HCl-methanol, and 0.4 ml of dehydrated methanol. Fatty acid methyl esters (fames) were extracted by using n-hexane and separated by centrifugation. n-hexane phase was dried with nitrogen gas. For GC-MS analysis, 1 ml of sample was added into 20 ml of hexana and it was then inserted into the GC column. GC-MS column that was used is DB WAXTR (30 m, φ = 0.250 m) at a temperature of 260^{o}C (Matsunaga et al., 2009).

2.7 Cultivation and Growth of Microalgae

Isolated microalgae cultivated in BMM media. To observe the effect of differences of nitrogen composition in growth medium of microalgae, cultivation was performed in BMM medium with varied amounts of $NaNO_3$. Variation 1 was ($NaNO_3$ = 22.5 mg/L), variation 2 was ($NaNO_3$ = 25 mg/L), and variation 3 was ($NaNO_3$ = 27.5 mg/L) (Afny Varitha et al., 2013). Microalgae growth was observed every day for 13 days by using a spectrophotometer and measured at a wavelength of 570 nm.

3. Results

3.1 Sampling

Sampling sites took place in MuaraPenjalinan located in Pasie Nan Tigo, Koto Tangah, Tabing, Padang, West Sumatra. The estuary was a direct meeting between Batang Air Dinginriver and Indian Ocean. The sampling location have coordinates in three points (S 00^{o}51'51,9 "^{o}20'00.6 E100"), (S 00^{o}51'52,3 "E100^{o}20'05.5") and (S 00^{o}51'41,6 "E100^{o}20 '20.2 ").

3.2 Isolation and Nile Red Staining

One species of microalgae was well isolated and cultivated in BMM media. In Nile red staining process, isolated microalgae were observed to detect lipid content by using Nile red. Figure 1 shows the results of staining. Nile red staining is an easy method that only requires small amounts of microalgae sample. Interactions between lipids and Nile red dye caused a yellow color on microalgae, neutral lipids was a hydrocarbons and triglycerides that was marked by formation of a yellow color whereas the polar lipids was marked by formation of red color (Govender et al., 2012).

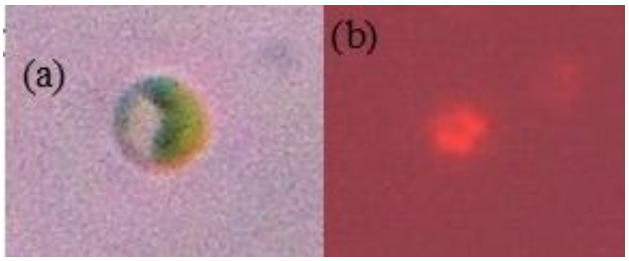

Figure 1. Isolated microalgae (a) with Nile red staining and (b) without Nile red staining.

3.3 Molecular Identification

Determination of species was performed by comparing the sequence (1712 bp) with the data from NCBI (Blast). Alignment was observed by using ClustalX2 program. The differences between species were analyzed by using MEGA5. Phylogenetic tree was made by using Neighbor-Joining assay (Figure 2).

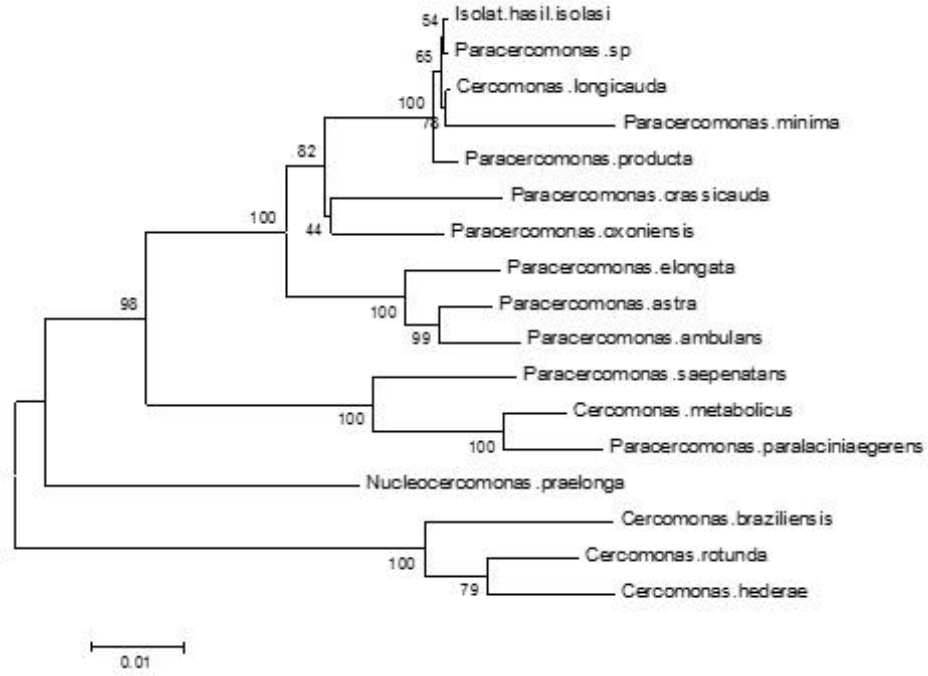

Figure 2. Phylogenetic tree of isolated microalgae

Based on the analysis of P-Distance by using MEGA5 program (Table 1), it is known that isolated species have distances percentage with the species that have close genetic relationship, the percentage is 0.1% with *Paracercomonassp*, 0.2% with *Cercomonaslongicauda*, and 0.5% with *Paracercomonasproducta*. These results show a distance below 1%, so that it has ≥ 99% of similarity. The species that has ≥ 99% of similarity showed a similar species (Bosshard et al., 2003). Under these conditions, it is known that isolated microalgae are *Cercomonaslongicauda* species. Differences of nucleotide in isolated microalgae sequence with the three closest species sequence from BLAST results can be seen in Figure 3.

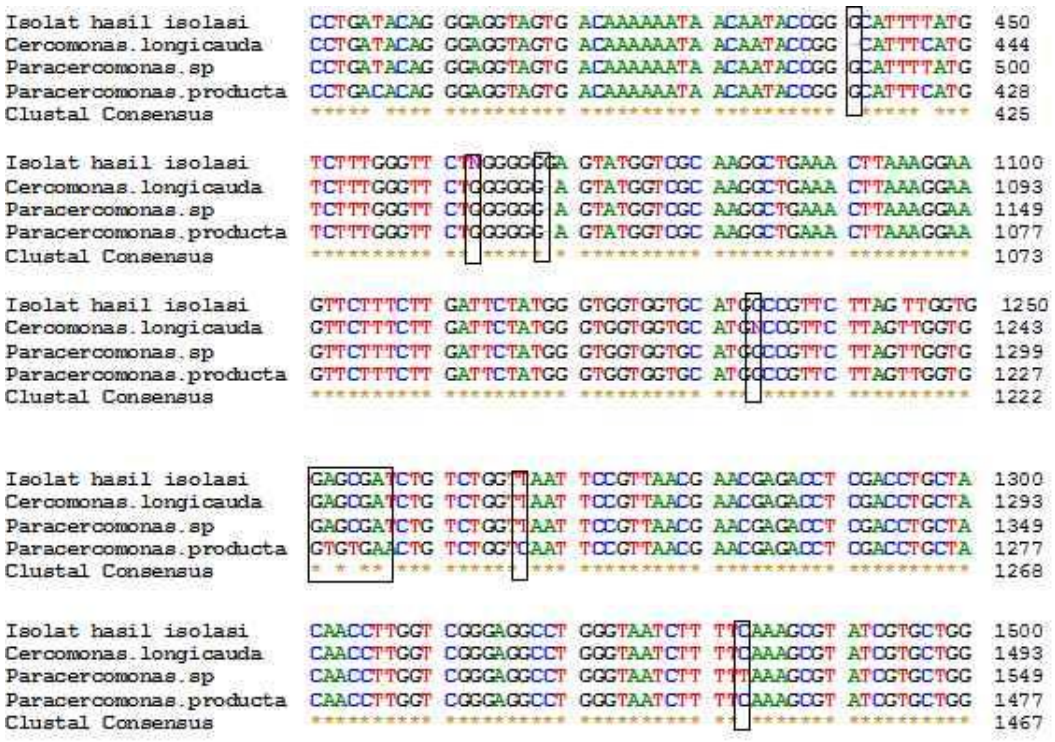

Figure 3. Differences of nucleotide in isolated microalgae sequence with the three closest species sequence

3.4 Fat Content

Lipid microalgae were formed by glycerol esters and fatty acids with a chain length of C_{14}-C_{22}(Kookkhunthod et al., 2011). Fatty acid in microalgae was intracellular molecule because it present in chloroplast. *C. longicauda* shows 17% of fat content in stationary phase.

3.5 Analysis of Fatty Acids

In this research, isolated *C. longicauda* was cultivated in BMM medium for 5 days and incubated at 30^{o}C. Fatty acids were analyzed by using GC-MS. Saturated fatty acids C16:0 and C18:0 present in high quantities (Figure 4). The composition of fatty acid on microalgae is determined by comparing the peak of the internal standard C19:0 and the peak of each fatty acid (Table 2).

Table 2. Fatty acid composition of *C. longicauda*

Fatty acid	Peak	Amounts of fatty acids (nmol/µL).
hexadecanoic acid ($C_{16:0}$)	364	0.81
heptadecanoic acid ($C_{17:0}$)	427	0.06
octadecanoic acid ($C_{18:0}$)	497	0.83
eicosanoic acid ($C_{20:0}$)	655	0.07

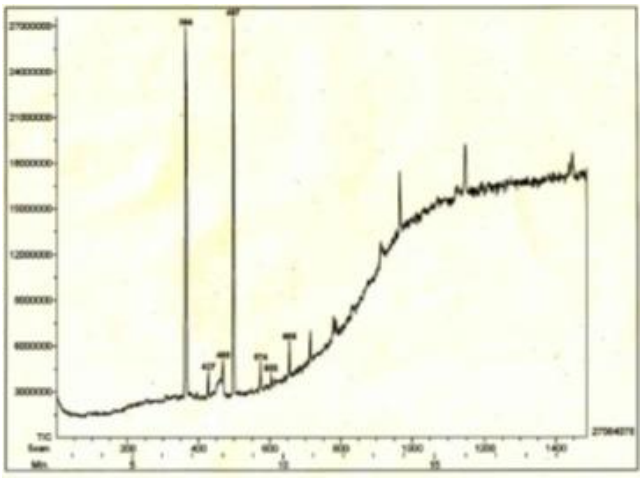

Figure 4. GC-MS chromatograms of *C. longicauda*

3.6 Cultivation and Growth of Microalgae

Microalgae growth is influenced by many factors such as nutrients, light intensity, temperature, salinity and pH. The main nutrient that was required for the production of microalgae was nitrogen and phosphorus (Wijffels et al., 2010). The conditions for biodiesel microalgae are it must has a high fat content and growth characteristics that determine the harvesting time. Microalgae growth characteristic that was observed in this research is the rate of growth by the addition of varied number of $NaNO_3$. The isolated species growth in the varied number of $NaNO_3$ in BMM media has a population peak on day 12 and the optimum growth at 27.5 mg/L of $NaNO_3$(Figure 5).

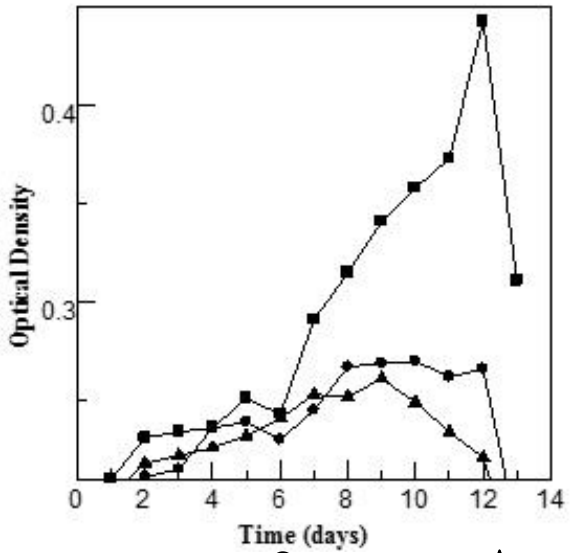

Figure 5. Growth graph of *C. longicauda*in BBM medi , 22.5 mg/LNaNO , 25 mg/LNaNO$_3$, .5 mg/LNaNO$_3$

4. Conclusion

The aim of this research is to isolate microalgae from brackish waters in Padang, West Sumatra. The identification results obtained isolated *C. longicauda* was potential to be used as a strain in biodiesel production because of the fat content and composition of fatty acids. Fatty acid analysis of *C. longicauda* showed that saturated fatty acids such as C16:0 and C18:0 present in high quantities.

Acknowledgement
The authorsexpress sincere thanks to Pertamina Foundation, Indonesia, for the financial support.

Referensi

Afny Varitha et al., (2013)Isolation oil Producing Microalgae *Chlamidomonas snowii* from Tropical Fresh water, Indonesia, *Researh Journal of Pharmaceitical, Biological and Chemical Sciences*, xx, pp.xxx-xxx.

Belcher Hilary et al., Culture Collection of Algae and Protozoa, *Culturing Algae*, 25.

Bosshard et al., (2003)Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation),*J. Clin. Microbiol*, 41,pp. 4134-4140.

Christi., (2007)Biodisel From Microalgae, *Biotechnology advance*, 25, pp.294-306.

Elumalal et al., (2011)Isolation, Identification, Morphological Studies and Lipid Granules Staining (Nile Red) of Different Micro-Algae for Biodisel Production from Fresh Water and Saline Water, *Journal of Experimental Science*,7. pp. 26-29.

Govender et al., (2012)BODIPY staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae, *Bioresource Technology*,114, pp.507-511.

Hu Qiang Hu et al., (2008)Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances, *The Plant Journal*, 54,pp.621-639.

Kookkhunthod, et al., (2011)Heterothrophic Growth of Chlorella sp KKU-S2 For Lipid Production using Molasses as a carbon Substrat,*IPCBEE*, 9.pp.xxx-xxx.

Li Yanqun et al., (2008). Biofuels from Microalgae, *Biotechnol*, 24, pp. 815-820.

Matsunaga et al., (2009)Characterization of marine microalga, *Scenedesmus sp*. strain JPCC GA0024 toward biofuel production, *BiotechnolLett*, 31,pp.1367–1372.

Walter et al., Microalgal biotechnology : Potential and Production. Berlin/Boston, Library of congress cataloging-in-publication data.

Welch., (1980). Ecological effect of waste water, *Cambridge Univ Press*,352.

Wijffels et al., (2010) An Outlook on Microalgal Biofuels, *Science*, 329.pp.796-798.

Wiley., Handbook of Microalgal Culture: Applied Phycology and Biotechnology, *Blackwell publishing*, 588.

Xun Yang et al., (2012), Characterization and Identification Of freshwater Microalgal Strains Toward Biofuel Production, *Bioresources*, 7 (1),pp. 686-695.

Table 1 . Distance matrix of isolated microalgae

Paracercomonas.astra																
Paracercomonas.ambulans	0,015															
Paracercomonas.elongata	0,020	0,023														
Isolat.hasil.isolasi	0,038	0,042	0,040													
Cercomonas.longicauda	0,039	0,043	0,040	0,002												
Paracercomonas.sp	0,038	0,042	0,040	0,001	0,002											
Paracercomonas.producta	0,040	0,042	0,041	0,005	0,004	0,005										
Paracercomonas.minima	0,058	0,062	0,059	0,020	0,019	0,020	0,022									
Paracercomonas.crassicauda	0,047	0,051	0,047	0,033	0,033	0,033	0,034	0,052								
Paracercomonas.oxoniensis	0,044	0,045	0,045	0,026	0,027	0,026	0,027	0,045	0,031							
Cercomonas.metabolicus	0,082	0,080	0,082	0,082	0,081	0,080	0,083	0,101	0,080	0,078						
Paracercomonas.paralaciniaegerens	0,086	0,086	0,086	0,085	0,086	0,084	0,088	0,105	0,083	0,080	0,018					
Paracercomonas.saepenatans	0,077	0,077	0,077	0,073	0,074	0,073	0,076	0,093	0,082	0,070	0,040	0,038				
Nucleocercomonas.praelonga	0,083	0,086	0,086	0,077	0,078	0,077	0,080	0,097	0,080	0,077	0,093	0,098	0,088			
Cercomonas.rotunda	0,119	0,119	0,117	0,107	0,107	0,107	0,107	0,126	0,109	0,105	0,113	0,123	0,115	0,097		
Cercomonas.hederae	0,124	0,126	0,121	0,113	0,112	0,112	0,113	0,131	0,113	0,107	0,117	0,126	0,122	0,104	0,024	
Cercomonas.braziliensis	0,124	0,125	0,118	0,113	0,113	0,112	0,113	0,132	0,111	0,106	0,117	0,125	0,118	0,103	0,045	0,033