ISOLATE OF HETEROTROPHIC MICROALGAE THRAUSTOCHYTRIUM AUREUM AS A POTENTIAL SOURCE FOR DOCOSAHEXAENOIC ACID (DHA)

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ABSTRACT

Docosahexaenoic acid (DHA) is one of essential fatty acids that are beneficial to health. Nowadays, the source of docosahexaenoic acid (DHA) is mainly obtained from fish which are extracted into fish oil products. However, the fish oil products still have some drawbacks in term of purity, acceptable flavor for costumers, and also their production process are not environmental friendly. As an alternative solution, heterotrophic microalgae can be used as a potential source for DHA due to their excellence compared to fish oil products. The aim of this study is to isolate the heterotrophic microalgae that can produce DHA. The heterotrophic microalgae were isolated from mangrove fallen leaves (*Rhizophora apiculata*) by using direct planting method. The morphology of pure microalgae colony were observed through light microscope. Fatty acids were extracted and methylated through direct transesterification method. Identification and quantification of DHA were conducted by using gas chromatography. The results were four isolates of heterotrophic microalgae, which were named by the code MTKC1, MTKC2, MTKC3, and MTKC4. The extract of MTKC2 that only showed the content of DHA with value of 9.2% of total fatty acids. Therefore MTKC2 is a potential source for DHA The MTKC2 was further identified by using molecular biology method and confirmed as *Thraustochytrium aureum*.

Keywords: docosahexaenoic acid (DHA), heterotrophic microalgae, *Thraustochytrium aureum.*

INTRODUCTION

Marine environment with all its uniqueness and biodiversity is a source for the search of potential new drugs. A number of marine microorganisms have been investigated for bioactive metabolites. Among these are, marine bacteria, actinomycetes (Bull and Stach, 2007; Fenical and Jensen, 2006), fungi (Bhadury *et al.*, 2006;. Newman and Hill, 2006; Julianti *et al.*, 2011, Jeon *et al.*, 2013), cyanobacteria (Tan 2007) and microalgae (Yang *et al.*, 2010, Dahmasa *et al.*, 2011).

One of pharmaceutical products derived from marine environments is docosahexaenoic acid (DHA). DHA is a polyunsaturated fatty acid omega-3 that very beneficial as essential nutrients. DHA is usually used in the form of supplements or food fortification especially in baby food, therefore it has high economic value. DHA can be isolated from fish oil, oil seals (seal oil), and a group of microorganisms microalgae, especially heterotrophic microalgae. However, DHA from microalgae have advantages over from the first two sources, such as the ability to grow very fast, has a higher purity, lower content of chemical contaminants such as heavy metals and dioxins, as well as more environmentally friendly in their production processes. In terms of smell and taste, DHA from microalgae products more accepted by consumers because it does not smell fishy (Batten *et al.*, 2011).

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Currently, Indonesia is the third country in APEC membership who has significant potential in the production of microalgae (Batten *et al.*, 2011). However the utilization of the heterotrophic microalgae still limited. Heterotrophic organisms use chemical energy from organic forms of carbon (glucose) for metabolic activity. Carbon is very important in the growth of microalgae (Chisti, 2007; Milne *et. al*, 2012). Heterotrophic microalgae are also spread by the process of decaying mangrove leaves, this is the result of the carbon processes. So that more mangrove leave blight expected to be more heterotrophic produced (Bongiorni *et. al*, 2004).

Many recent studies show that heterotrophic conditions can produce a variety of microalgae metabolites at all scales because of the high levels of omega 3 fatty acids DHA and EPA, as well as reduced production costs (Chen *et al.*, 2007). Species of heterotrophic microalgae capable of producing foreign DHA are *Schizochytrium* sp.,T*hraustochytrium striatum*, *Crypthecodinium cohnii*, and *Ulkenia* sp (Dahmasa *et al.*, 2011).

Driven by the above backgrounds, this present study conducted a heterotrophic microalgae exploration that began with the isolation of the heterotrophic microalgae from Indonesia marine environment that can produce docosahexaenoic acid (DHA).

MATERIALS AND METHODS

Samples of mangrove fallen leaves (*Rhizophora apiculata*) were collected from mangrove area Tritih Kulon, Cilacap. The heterotrophic microalgae were isolated from the leaves by using direct planting method. Firstly, the leaf sample was washed by using sterile artificial seawater. A small portion size (1.5 x 1.5 cm^2) of the leaf was placed onto the surface of the agar in Petri dishes. The agar media containing yeast extract, peptone, dextrose, sterile artificial sea water as well as the antibiotic streptomycin sulfate and penicillin G. The inoculated Petri dishes were incubated at room temperature (25°C) for 1-3 days. The growing of microalgae colony was observed carefully and then transferred into a new media by strike method and the transferred was repeated until getting the pure culture. The Microscopic observation was conducted during isolation process to ensure that isolates were microalgae. Microalgae are generally microscopic micelles (diameter between 1-10 μm) belonging to the algae class and live as colonies and single cells. The morphology of the unicellular form of microalgae has no clear organ function division in its component cells (Metzger and Largeau 2005).

The pure isolate of microalgae was fermented using YPG broth media (5 g yeast extract, 5 g peptone, dextrose 10 g in 1 L artificial seawater). Fermentation was carried on an orbital shaker with a speed of 150 rpm for 14 days at room temperature. The biomass was separated from the medium by centrifugation at a rate of 3000 rpm for 30 minutes. The drying process of the biomass was carried out by using an oven at 80^{O}C for 3 hours.

In the process of DHA extraction, the dried biomass of microalgae were extracted and methylated through direct transesterification method. In situ transesterification method is a method that combines the extraction and transesterification process into a single process so that the use of solvent and time spent can be reduced (Yokochi *et. al*, 1998). The biomass is added with a mixture of alcohol and acid or bases that will result in the reactive extraction of lipids as FAME (fatty acid methyl esters).

The in situ-transesterification in this study was conducted in the reaction tube. In the reaction tube, 1 gr of the dried biomass was added 0,5 mL of dichlorometane (CH2Cl2) soluiton and 1 mL HCl 2M in methanol and then homogenized by using vortex. The mixture solution was reacted in a water bath at temperature of 60^{o}C for 3 hours. After 3 hours, 2 mL of saturated solution of NaCl and 1 mL of n-hexane were added and homogenized. The mixture of reaction was allowed to separate to form methyl ester of DHA or in general namely is FAME (fatty acid methyl ester) in n-hexane. Then, DHA in n-hexane was separated by using vortex and keep in temperature of 4^{o}C

Identification and quantification of DHA were conducted by using gas chromatography (Shimadzu 2010) by using system of column CP-SIL8 -CB with length 30 m and width 0.25 mm, helium motion phase, FID detector, Split injection mode, detector and injector was set at 260^{o}C. The isolate of microalgae that produced DHA was further identified by using molecular biology method. Identification of molecular

biology, DNA extraction was performed using ZR fungal or bacteria DNA Miniprep kit. DNA amplification was performed using Polymerase Chain Reaction (PCR) using primers 18s rDNA/ITS. The resulting PCR products were characterized first using DNA electrophoresis and visualized with UV transilluminator. The sequence analysis of DNA was performed by Lab 1st Base, Singapura.

RESULTS AND DISCUSSION

Heterotrof microalgae isolation technique is done by using direct planting method by cutting small part with size (1,5 x 1,5 cm^2) from leaf litter of mangrove, which is then placed on the surface of medium in petri dish containing solid media. The technique has been described by Fan *et al.* (2002), because the possibility for microalgae grows faster and does not spread, making it easier to inoculate. The artificial sea water was adjusted with a pH of 6 to get the balance of salt concentration and acidity that was suitable environment for the growth of heterotroph microalgae. The antibiotics streptomicyn and Penicillin G Na were added in in order to inhibit the growth of bacteria.

The colony of microalgae was observed after 2 days of incubation (Figure 1). The observed microalgae colony was carefully transferred to the new media and repeated until a pure culture (Figure 2).

The isolate of microalgae are mostly obtained from the brown leaves. This may be due to the process of decomposition of mangrove leaves can be associated with the presence of heterotrophic microalgae. Mangrove leaves play a role in the process of heterotrophic microalgae carbon cycle

Figure 1. The colony of microalgae observed growing, sign by the red arrow.

(detrivores). Therefore, according to (Bongiorni *et al.*, 2004), the more rotten a mangrove leave it is expected more heterotrophic microalgae produced.

Based on characterization by using the microscopic method, among the pure isolates of microalgae was concluded that there were 4 isolates with different microscopic characteristics. The isolates were given name by the code MTKC1-4 (Figure 3). There are several characteristics used to distinguish the microalgae division such as colony form, whether there is or no flagella, and pigment type. Morphological characteristics can be reviewed from the microalgae live habitat. According to Li and Watanabe (2001), taxonomic characteristics may be subject to change due to environmental conditions in culture.

The four isolates of microalgae were further continued to the next step for fermentation. The isolates were growing in liquid media consist of yeast extract, peptone and dextrose, by shaking condition of 150 rpm, at room temperature for 14 days. Microalgae requires some metabolic

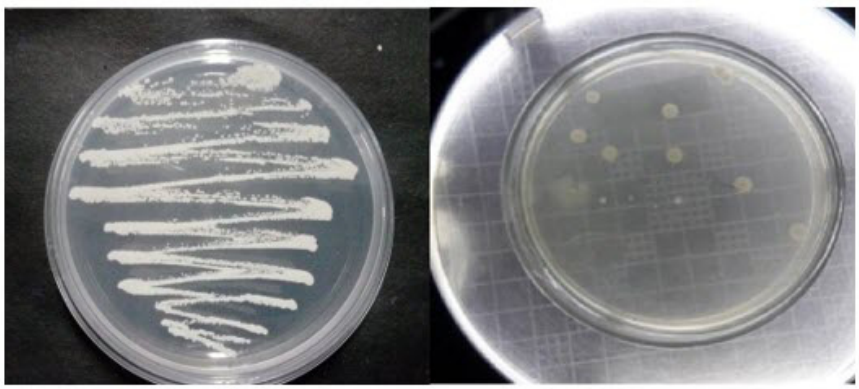

Figure 2. Purification of Microalgae

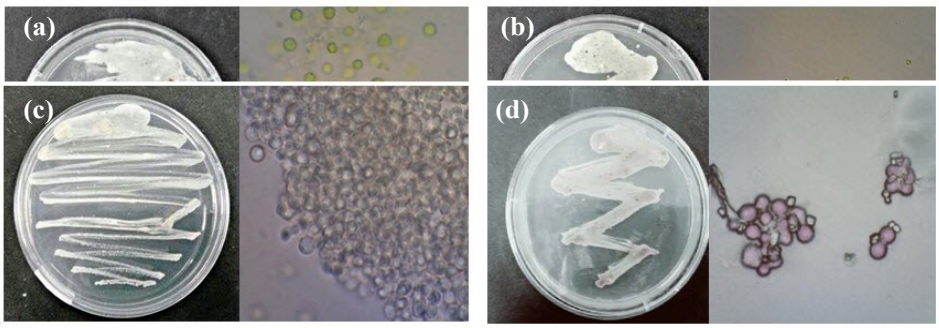

Figure 3. Microscopic characterization: (a) MTKC1, (b) MTKC2, (c) MTKC3 (d)MTKC4

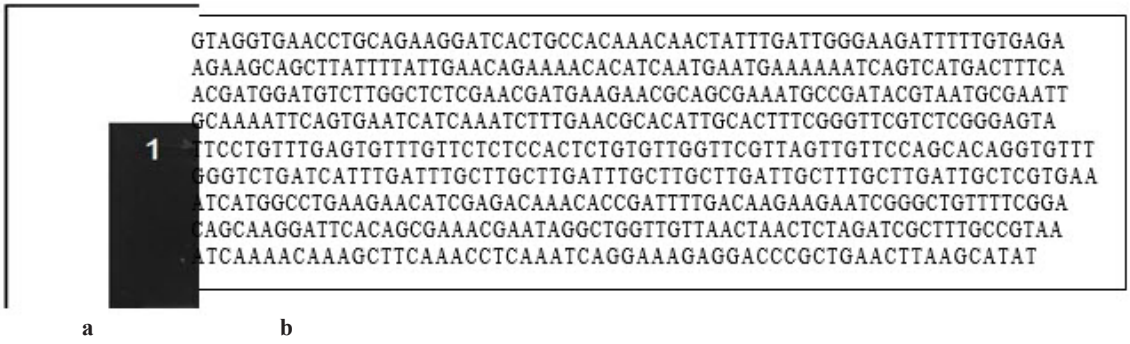

Figure 5. Molecular biology (18s rDNA/ITS) identification of MTKC2: (a) electrophoregram of PCR results, (b) Results of nucleotide sequence

nutrients to produce lipids and fatty acids (Abdel-Raouf *et al.*, 2012, Munoz and Guieysse, 2006). Lipids and fatty acids in microalgae cells depend on various factors including pH, temperature, carbon, nitrogen and growth of heterotrophic microalgae.

Biomass and broth culture were separated after 14 days incubation. The biomass was drying prior to extraction process. The biomass was dried using an oven with a temperature of 80-90^{o}C. According to Ryckebosch *et. al* (2011), the higher temperature on during drying process could be damaged secondary metabolites contained in the biomass. The yield of dried biomass was about 0.5 g/100 mL media.

The transesterification reaction was conducted to convert DHA to an ester product, fatty acid methyl ester (FAME) that can be detected by using gas chromatography (Figure 4). The mechanism of chemical reaction occurred when DHA or other fatty acids that was extracted by solvent dichloromethane bind with methanol to form fatty acid methyl ester (FAME) with HCl as acid catalyst. The catalyst accelerated the esterification reaction. The results showed that only extract of MTKC2 containing DHA with a value of 9.2% of total fatty acids (Table 1). The DHA content is good enough when compared with the results of previous studies. Some papers reported the content of DHA from other microalgae such as Criptomonas sp contained 6.6% of DHA, Rhodomonas sp. of 4.6% (Renaud et al 1999) and from Isochrysis galbana of 12.7% (Custodio *et. al*, 2014). Dahmasa et al (2011) reported that *Schizochytrium* sp has the highest DHA content of 40-45% after optimization the condition of cultivation.

Based on the results of the GC analysis, it was also known that there was another omega 3 fatty acid, eicosapentanoic acid (EPA), in the extracts of MTKC1 and MTKC3 (Table 1). The effect of nutrients such as nitrogen sources can affect EPA production. Heterotrophic conditions and the presence of peptone, yeast extracts and enzymes were known to increase EPA production (Wen and Chen, 2001). Reanud et al (1999) reported that EPA produced from the type of microlaga Fragilaria sp. of 6.8% and Lang et al (2011) reported the type of Heterosigma akashiwo was 14.8%. DHA or EPA was not detected in extract of MTKC4. The MTKC2 was further identified by using molecular biology method and confirmed as Thraustochytrium aureum, GenBank accession number FJ533161 (Figure 5).

Table 1. The Result of Omega-3 Content Analysis

No	Microalgae	Omega-3	Detection Limit	Result (%)
1	MTKC 1	DHA	< 0.1	-
		EPA	-	5.2
2	MTKC 2	DHA	-	9.2
		EPA	< 0.1	-
3	MTKC 3	DHA	< 0.1	-
		EPA	-	8.9
4	MTKC 4	DHA	< 0.1	-
		EPA	< 0.1	-

CONCLUSION

As a result of the isolation of microalgae from mangrove fallen leaves, 4 types of heterotrophic microalgae, namely MTKC1-4 were obtained. Among those isolates only MTKC2 that showed the content of DHA with a value of 9.2%. Therefore, the MTKC2 is a potential source for DHA production and identified as *Thraustochytrium aureum*.

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Tanggal Pengujian : 15 Juli 2016

Hasil Pengujian

1. Hasil Fermentasi Mikroalga (1)

No	Parameter Uji	Hasil	Batas deteksi (LoD)	Satuan	Metode
1.	Asam Linolenat	12,07	-	%	Kromatografi Gas
2.	Asam Eicosatrienoat	<0,1	0,1	%	Kromatografi Gas
3.	Asam Arachidonat	<0,1	0,1	%	Kromatografi Gas
4.	EPA	5,18	-	%	Kromatografi Gas
5.	DHA	<0,1	0,1	%	Kromatografi Gas

2. Hasil Fermentasi Mikroalga (2)

No	Parameter Uji	Hasil	Batas deteksi (LoD)	Satuan	Metode
1.	Asam Linolenat	15,89	-	%	Kromatografi Gas
2.	Asam Eicosatrienoat	3,23	-	%	Kromatografi Gas
3.	Asam Arachidonat	<0,1	0,1	%	Kromatografi Gas
4.	EPA	<0,1	0,1	%	Kromatografi Gas
5.	DHA	9,20	-	%	Kromatografi Gas

3. Hasil Fermentasi Mikroalga (3)

No	Parameter Uji	Hasil	Batas deteksi (LoD)	Satuan	Metode
1.	Asam Linolenat	10,33	-	%	Kromatografi Gas
2.	Asam Eicosatrienoat	<0,1	0,1	%	Kromatografi Gas
3.	Asam Arachidonat	<0,1	0,1	%	Kromatografi Gas
4.	EPA	8,85	-	%	Kromatografi Gas
5.	DHA	<0,1	0,1	%	Kromatografi Gas

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4. Hasil Fermentasi Mikroalga (4)

No	Parameter Uji	Hasil	Batas deteksi (LoD)	Satuan	Metode
1.	Asam Linolenat	<0,1	0,1	%	Kromatografi Gas
2.	Asam Eicosatrienoat	<0,1	0,1	%	Kromatografi Gas
3.	Asam Arachidonat	<0,1	0,1	%	Kromatografi Gas
4.	EPA	<0,1	0,1	%	Kromatografi Gas
5.	DHA	<0,1	0,1	%	Kromatografi Gas

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