TRIGLYCERIDE COMPOSITION OF SIXTEEN STRAINS OF MARINE DIATOM

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ABSTRACTS

Trigliceride or triacylglicerol (TAG) composition in crude oil of sixteen strain of marine diatom has been detected by spectra analyses on an Electrospray - Ion Trap – Mass Spectrometry (ESI-IT-MS) HCT Bruker-Daltonic GmbH instrument with AgNO3 used as coordination ionization agent. Biomass samples of each microalga strain were taken from early and late stationary cultures in f/2 enriched seawater and algal oils were extracted according to Bligh and Dyer. Results from spectra analysis showed that P-Pt-P ($C_{16:0}$-$C_{16:1}$-$C_{16:0}$) were distinguished in TAG from diatom strains Chaetoceros sp.1, Chaetoceros sp.2, Thalasiossira sp.1, Thalasiossira sp.2, Thalasiossira sp.3, Navicula sp. 1, Navicula sp. 2, Navicula sp. 3, Navicula sp. 4, Nitzschia sp. 2 and Amphora sp. In contrast, TAGs in Melosira sp. included P-P-P ($C_{16:0}$-$C_{16:0}$-$C_{16:0}$) and P-P-O ($C_{16:0}$-$C_{16:0}$-$C_{18:1}$) were identified. TAGs from Chaetoceros sp. were the most varies among samples, i.e. P-Pt-P ($C_{16:0}$-$C_{16:1}$-$C_{16:0}$), A-P-M ($C_{20:4}$-$C_{16:0}$-$C_{14:0}$), P-Pt-Lt ($C_{16:0}$-$C_{16:1}$-$C_{18:3}$), P-Pt-A ($C_{16:0}$-$C_{16:1}$-$C_{20:4}$), D-P-P ($C_{22:6}$-$C_{16:0}$-$C_{16:0}$), A-Ln-P ($C_{20:4}$-$C_{18:2}$-$C_{16:0}$). Various TAGs were also detected in Nitzschia sp.2, i.e. P-Pt-M ($C_{16:0}$-$C_{16:1}$-$C_{14:0}$), P-Pt-P ($C_{16:0}$-$C_{16:1}$-$C_{16:0}$), P-Pt-S ($C_{16:0}$-$C_{16:1}$-$C_{18:0}$), P-Pt-A ($C_{16:0}$-$C_{16:1}$-$C_{20:4}$). TAGs composition in Skeletonema strains that similar to those in Nitzschia sp.1 has longer carbon, *i.e.* P-P-O ($C_{16:0}$-$C_{16:0}$-$C_{18:1}$), P-O-O ($C_{16:0}$-$C_{18:1}$-$C_{18:1}$) and O-O-O ($C_{18:1}$-$C_{18:1}$-$C_{18:1}$). TAGs with longer carbon chain and more double bond including highly unsaturated fatty acid $C_{20:4}$ were increased with culture age in diatoms Chaetoceros sp.1, Chaetoceros sp.2, Thalasiossira sp.2, Navicula sp.1 and Nitzschia sp. 2.

Keywords: diatom, TAG, ESI-IT-MS, f/2, early and late stationary

I. INTRODUCTION

Nowadays development of microalgae as a source of fuel to substitute future fossil fuel crisis have attracted attention worldwide (Benneman, 2008; Patil *et al.,* 2008; Chisti, 2007; Guschina and Harwood, 2006; NREL, 2005). The interest in microalgae, for alternative second generation biofuel sources, is less competitive with food and feed production. Aquatic Species Program supported by the U.S. Department of Energy (DOE) has collected over 3000 algal strains and screened for their oils as renewable source of biodiesel. Biodiesel from algae contains no sulfur, nontoxic and highly biodegradable. There were 300 species of green and diatoms algae reported by DOE as promising strains for development of biofuel. Panggabean *et al.* (2009) and Bayu *et al.* (2009) also reported some native Indonesian diatoms to be potential renewable feedstocks. Those findings were based on their capabilities for oil production.

Diatoms (Bacillariophyta) are greatly diverse and abundant as the green algae, including 285 genera (Round *et al.*, 1990). Diatoms belong to Ochrophytes based on the presence of golden brown (ocher) fucoxanthin besides chlorophyll a and c, chrysolaminarin and the silicious cell coverage –the frustule- of various ornamentation which reflecting taxonomic diversity. Their existance is very important to contribute global primary productivity in oceans, lake and various

environments. They live as planktonic, benthic, periphytic (on plant leave or seaweed surfaces), epizoic (on crustaceans, whales etc.), endozoic (within foraminifera), moist teresstrial habitats, polar ice etc. Several diatom strains of Indonesian waters are maintained in the culture collection of Research Centre for Oceanography, Indonesian Institute of Science.

Diatoms store carbon in the form of natural oils or as polymer of carbohydrates known as chrysolaminarin. The natural oils from diatoms and algae are in the form of triacylglycerols (TAGs). TAGs consist of three long chains of fatty acids attached to a glycerol backbone. TAGs with methanol through transesterification reaction a chemical compound known as an alkyl ester or generically known as biodiesel. Biodiesel is an alternative to petroleum diesel fuel. The fatty acids components of different microalgal strains determine the fuel produced, because individual fatty acid chains is species or strain specific (Pernet *et al.*, 2003; Hu *et al.*, 2008). TAG content and variation could be change as they grow or at different physiological condition. Therefore variations in fatty acids component in algal lipid would be necessary to take into consideration. The objective of this present study is to determine fatty acids components of TAG of typical diatom strains from two stages of culture.

II. METHOD

Sixteen strains of marine diatoms (Table 1) originally taken from micro-algal culture collection of Research Centre for Oceanography, LIPI, were used in the experiment. Several group of algal strains were cultured during February through March 2012 and others were cultured during May though June 2012 in our laboratory. Each strain were grown in two

1L seawater with salinity of 27ppt enriched with f/2 media (Guillard, 1975). The cultures were aerated and incubated in air condition room (23-25 ^{o}C) under 3000 lux illumination for 12 hrs/day. At early and late stationary phases, 50mL cultures were filtered through GF/F membrane for biomass measurement and 300 mL of culture stock for algal oils extraction according to method of Bligh and Dyer (1959) using chloroform : methanol Proportion. The TAG components of the algae oil were analyzed by electron spray ionization-ion trap-mass spectrometry (ESI-IT-MS) on HCT Brucker-Daltonic GmBH instruments with AgNO$_3$ used as coordination ionization agent (Sandra *et al.*, 2002)

III. RESULT AND DISCUSSION

3.1. Biomass and Lipid Production

Biomass obtained from early stationary phase were varied from 142 mg/L for *Amphora* sp. to 698 mg/L for *Skeletonema* sp. 1. Biomass were mostly increased from early to late stationary phase up to 880 mg/L in *Skeletonema* sp. 3 (Figure 1) and the increment was greatest for *Amphora* sp. (> 200%). However lipid content which expresses as dry weight/ L (Figure 1) or percentage of biomass dry weight did not follow biomass production rate (Figure 2). The strain *Skeletonema* sp.1, *Skeletonema* sp. 2, *Nitzschia* sp. 1, *Thalassiosira* sp. 1 and *Thalassiosira* sp. 3 have high biomass production rates, but low lipid content. On the other hand those algae with lower biomass dry weight (*Chaetoceros*, *Melosira*, *Navicula* and *Amphora*) produced higher lipid content. Lipid content varied from 1.39% in *Thalassiosira* sp. 3 to 26% in *Amphora* sp. after reaching early stationary phase and from 0.76% in *Skeletonema* sp. 1 to 20% in *Chaetoceros* sp. 1 after late stationary phase. Lipid accumulation

during stationary phase was observed in most of centric diatoms (*Chaetoceros, Melosira* sp., *Skeletonema* sp. 3 and *Thalassiosira*) (Figure 2) which also reported in several literatures (Bayu *et al.*, 2010; Nurachman *et al*., 2012 b, Panggabean *et al*., 2009; Pratiwi *et al*., 2009). Highest lipid accumulation was observed in *Melosira* sp., that was up to 4.5 fold from the early stationary harvest. It was in contrary with the naviculoids or pennate diatoms (*Navicula*, *Nitzschia* and *Amphora*), further culturing to late stationary phase did not increase but decreased their lipid content. Top lipid producer are *Chaetoceros* sp. 1 (20%) and *Amphora* sp. (26%) that represented the centrics and naviculoids repectively among our samples examined. Those percentage were derived from 38.2 and 30.8 mg/L lipid product from *Chaetoceros* sp.1 and *Amphora* sp respectively. Higher lipid product was resulted from *Thalassiosira* sp. 2 (65.78 mg/L) which

only 15% of its high biomass yield. Moderate lipid contents includes *Melosira* sp. (18%) from the centrics and *Navicula* sp. (17-22%) from the naviculoids were derived from 49 mg/L and 35-43 mg/L lipid respectively. Earlier lipid accumulation also observed in the *Navicula* sp. of Nurachman *et al.* (2012b). Thus the use of the naviculoids may be more economical in terms shorter culture biomass production. Achievable yield of about 142-880 mg/L biomass with 1-26% oil content (as triglycerides) in this study was lower than our former results (Bayu *et al.*, 2010; Nurachman *et al*., 2012 a and b, Panggabean *et al*., 2012). The low TAG production was due to different culture conditions, *i.e.* lower temperature and light intensity. Low light intensity favours the formation of the membrane polar lipid associated with chloroplast (Brown *et al*., 1996, Orcutt and Patterson, 1974, Sukenik *et al*., 1989).
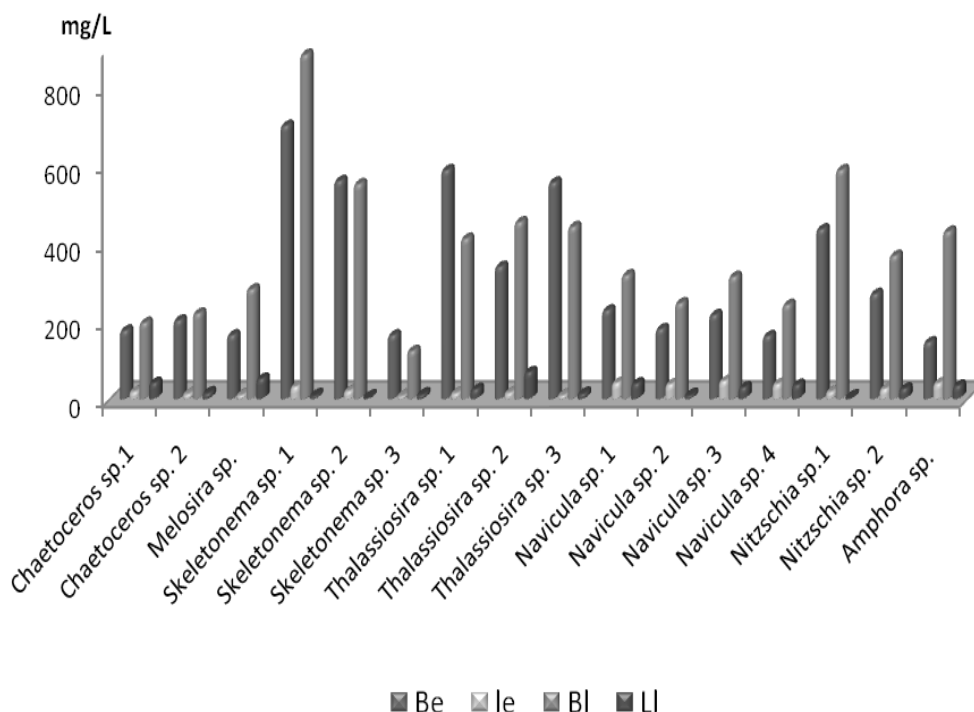

Figure 1. Biomass and lipid (mg/L dry weight) yield of sixteen diatom strains from early (Be and Le) and late (Bl and Ll) stationary phase.
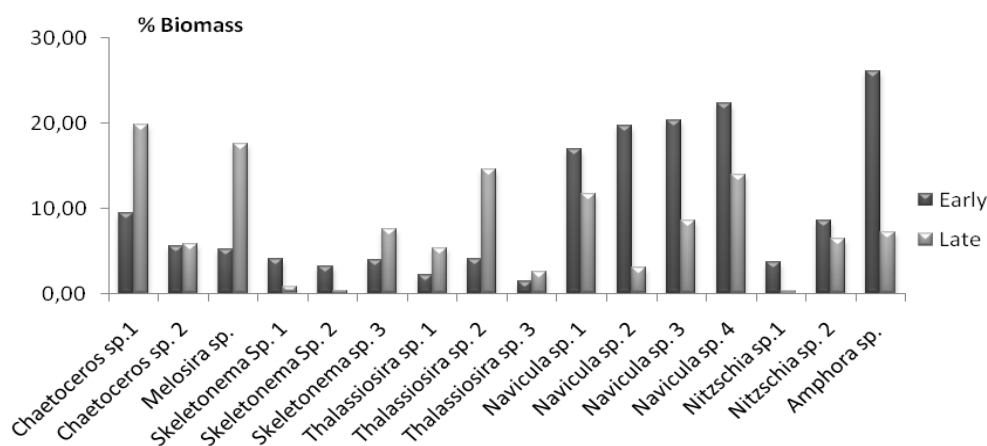
Figure 2. Lipid content (% biomass) of sixteen diatom strains from early and late stationary phase.

3.2. Fatty Acids Composition

Fatty acids are building blocks for the formation of various types of lipids, which may include neutral lipids, polar lipids, wax esters, sterols, hydrocarbons and derivates prenyl *e.g.* carotenoids and phytylated pyrrole chlorophyls (Hu *et al.*, 2008). Lipid from growing algae are mostly membrane polar glycerol-based lipids, which constitute about 5-20% of dry weight. Under stress condition, they shift their membrane lipids into storage neutral lipids in the form of triacyl glycerol (TAG) (Brown *et al.*, 1993; Grimma *et al.*, 1992; Parish and Wangersky, 1987, 1990; Lombardi and Wangersky, 1991,1995; Leonardos and Geider, 2004; McGinnis *et al.*, 1997).

Component of fatty acids are either saturated or unsaturated with double bonds on the carbon chain backbone. They are classified into medium chain C10-C14; long chain C16-C18 and very long $\geq$C20 species fatty acids and derivatives (Hu *et al.*, 2008). Fatty acid composition of lipid alga is species or strain specific and varies with different culture conditions (Guschina and Harwood, 2006; Chisti, 2009).

The analysis of fatty acid composition in this study was conducting using the total lipid extracts rather than examining individual lipid classes. The results showed that TAGs in most diatom strains (Bacillariophyta) consisted of long-chain C_{16} fatty acids (Table 1). The C_{16} component in *Chaetoceros* sp.1, *Chaetoceros* sp.2, *Thalasiossira* sp.1, *Thalasiossira* sp.2, *Thalasiossira* sp.3, *Navicula* sp. 1, *Navicula* sp. 2, *Navicula* sp. 3, *Navicula* sp. 4, *Nitzschia* sp. 2 and *Amphora* sp. were predominated by saturated and mono-unsaturated P-Pt-P ($C_{16:0}$-$C_{16:1}$-$C_{16:0}$), but in *Melosira* sp. was predominated by saturated P-P-P ($C_{16:0}$-$C_{16:0}$-$C_{16:0}$). Other TAG species from *Melosira* sp., also contained longer C_{18} mono-saturated fatty acid P-P-O ($C_{16:0}$-$C_{16:0}$-$C_{18:1}$) also occured. TAGs species from *Skeletonema* and *Nitzschia* sp.1 are mostly contained C_{18} carbon chains, i.e. P-P-O ($C_{16:0}$-$C_{16:0}$-$C_{18:1}$), P-O-O ($C_{16:0}$-$C_{18:1}$-$C_{18:1}$) and O-O-O ($C_{18:1}$-$C_{18:1}$-$C_{18:1}$). On the other hand, the chain length in *Nitzschia* sp.2 varied from C_{14}-C_{20}, i.e. P-Pt-M ($C_{16:0}$-$C_{16:1}$-$C_{14:0}$), P-Pt-P ($C_{16:0}$-$C_{16:1}$-$C_{16:0}$), P-Pt-S ($C_{16:0}$-$C_{16:1}$-$C_{18:0}$), P-Pt-A ($C_{16:0}$-$C_{16:1}$-$C_{20:4}$).

Variation in chain length and degree of saturation in *Chaetoceros* sp.1 oils are much greater than those in the other diatoms, which were particularly detected from the early stationary phase specimens, namely from medium-chain

C_{14} saturated to very-long-chain C_{20-22} poly-unsaturated fatty acids (PUFA) or highly-unsaturated fatty acids (HUFA). TAGs detected in *Chaetoceros* sp.1. are P-Pt-P ($C_{16:0}$-$C_{16:1}$-$C_{16:0}$), A-P-M ($C_{20:4}$-$C_{16:0}$-$C_{14:0}$), P-Pt-Lt ($C_{16:0}$-$C_{16:1}$-$C_{18:3}$), P-Pt-A ($C_{16:0}$-$C_{16:1}$-$C_{20:4}$), D-P-P ($C_{22:6}$-$C_{16:0}$-$C_{16:0}$), A-Ln-P ($C_{20:4}$-$C_{18:2}$-$C_{16:0}$) The very long PUFA above C_{18} is typical marine algae which can not be synthesized by higher plants (Hu *et al*., 2008).

TAGs with very-long-chain HUFA $C_{20:4}$ in *Chaetoceros* sp.1, *Chaetoceros* sp.2, *Thalasiossira* sp.2, *Navicula* sp.1, and *Nitzschia* sp. 2 increased during late stationary phase, which also observed in other diatoms (Hatate *et al*., 1998; Chrismada *et al*., 1993; Bayu *et al.*, 2009; Tonon, 2002). The oils of high PUFA contents are not stable and more susceptible to oxidation during storage which not suitable for diesel fuel, according to EU standard requirement EN 14214 and EN 14213 (Chisti, 2007). With the current technology, *i.e.* partial catalytic hydrogenations of the oil, the unsaturation of more than four double bonds can be reduced (Jay *et al*. 2005; Dijkstra 2006 cited by Chisti, 2007).

Table 1. Triacyl glycerol from sixteen strains of diatoms.

No.	Strain Name	Specimens from early stationary phase	Specimens from late stationary phase
1	Chaetoceros sp.1	P-Pt-P (C16:0-C16:1-C16:0) A-P-M (C20:4-C16:0-C14:0) P-Pt-Lt (C16:0-C16:1-C18:3) P-Pt-A (C16:0-C16:1-C20:4) D-P-P (C22:6-C16:0-C16:0) A-Ln-P (C20:4-C18:2-C16:0)	P-Pt-P (C16:0-C16:1-C16:0) P-Pt-A (C16:0-C16:1-C20:4) P-Ln-P (C16:0-C18:2-C16:0)
2	Chaetoceros Sp. 2.	P-Pt-P (C16:0-C16:1-C16:0) S-Pt-P (C18:0-C16:1-C16:0)	P-A-P (C16:0-C20:4-C16:0)
3	Melosira sp.	P-P-P (C16:0-C16:0-C16:0) P-P-O (C16:0-C16:0-C18:1)	nd
4	Skeletonema sp. 1.	nd	P-P-O (C16:0-C16:0-C18:1) P-O-O (C16:0-C18:1-C18:1) S-Ln-S (C18:0-C18:2-C18:0)
5	Skeletonema sp. 2.	P-P-O (C16:0-C16:0-C18:1) P-O-O (C16:0-C18:1-C18:1)	nd
6	Skeletonema sp. 3.	P-O-O (C16:0-C18:1-C18:1) O-O-O (C18:1-C18:1-C18:1)	nd
7	Thalasiossira sp.1.	P-Pt-P (C16:0-C16:1-C16:0)	nd
8	Thalasiossira sp.2.	P-Pt-P (C16:0-C16:1-C16:0) P-Pt-A (C16:0-C16:1-C20:4)	P-Pt-P (C16:0-C16:1-C16:0) A-P-M (C20:4-C16:0-C14:0) P-Pt-Lt (C16:0-C16:1-C18:3) P-Pt-A (C16:0-C16:1-C20:4)
9	Thalasiossira sp.3.	P-Pt-P (C16:0-C16:1-C16:0)	P-Pt-P (C16:0-C16:1-C16:0) P-Pt-O (C16:0-C16:1-C18:1)
10	Navicula sp. 1.	P-Pt-P (C16:0-C16:1-C16:0)	P-Pt-P (C16:0-C16:1-C16:0) P-Pt-A (C16:0-C16:1-C20:4)
11	Navicula sp. 2.	nd	P-Pt-P (C16:0-C16:1-C16:0)
12	Navicula sp. 3.	P-Pt-P (C16:0-C16:1-C16:0) S-Pt-P (C18:0-C16:1-C16:0)	P-Pt-P (C16:0-C16:1-C16:0) P-Pt-S (C16:0-C16:1-C18:0)
13	Navicula sp .4.	nd	P-Pt-P (C16:0-C16:1-C16:0)
14	Nitzschia sp.1.	P-P-O (C16:0-C16:0-C18:1) O-S-O (C18:1-C18:0-C18:1)	P-O-O (C16:0-C18:1-C18:1) O-O-O (C18:1-C18:1-C18:1)
15	Nitzschia sp. 2.	P-Pt-M (C16:0-C16:1-C14:0) P-Pt-P (C16:0-C16:1-C16:0) P-Pt-S (C16:0-C16:1-C18:0) P-Pt-A (C16:0-C16:1-C20:4)	P-Pt-A (C16:0-C16:1-C20:4)
16	Amphora sp.	P-Pt-P (C16:0-C16:1-C16:0) Ln-P-P (C18:2-C16:0-C16:0)	nd

IV. CONCLUSION

Tags in the diatom studied were mostly contained long chain C_{16} saturated and mono-saturated fatty acid. Longer C_{18} fatty acids also occurred in chain forming diatom *Melosira*, *Skeletonema costatum* and *Nitzschia* sp.1. Very long PUFA above C_{18} was observed from *Chaetoceros*, *Thalassiosira*, *Navicula* and *Nitzschia* sp.2. The unsaturation was increased during stationary phase.

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