RJC

RASĀYAN J. Chem.
43-52| Special Issue | 2022
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

MICROALGAL LIPID FROM *Chaetoceros calcitrans* AND ITS CONVERSION TO BIODIESEL THROUGH *Ex* AND *In-situ* TRANSESTERIFICATION

P. Purkan[1,✉], H. Nurlaila[1], A. Baktir[1], S. Hadi[1] and W. Soemarjati[2]
[1]Department of Chemistry, Faculty of Science and Technology, Airlangga University, Surabaya, Indonesia
[2]Brackishwater Aquaculture Development Center Situbondo, Indonesia
✉Corresponding Author: purkan@fst.unair.ac.id

ABSTRACT

The manufacture of biodiesel from lipids can be carried out by ex and in-situ methods. The difference lies in the continuity of lipid extraction and its transesterification into biodiesel, either separately or simultaneously. The paper reported the FAME productivity from lipid *Chaetoceros calcitrans* that were created by both methods. The research was started by culturing *Chaetoceros calcitrans* in seawater media with the addition of trace elements, it produced a dry cell weight of 15 g/L on the fifth day of fermentation. The highest yield of lipid was 61.40% (w/w) obtained when the extraction was carried out from the biomass in a mixed solvent between n-hexane and 96% ethanol (1:1) assisted by a combination of hydrothermal acid and ultrasonication treatments. The transesterification of lipid that was carried out with methanol and 6% (v/v) H2SO4 catalyst for one hour at 60ºC in an ex-situ process, produced FAME of 19.15% (w/w). Meanwhile, the in-situ method which joined the lipid repealing and transesterification steps simultaneously produced 21.59% (w/w) of biodiesel. The in-situ process exhibited higher FAME than the ex-situ. The main FAME component on the biodiesel of *Chaetoceros calcitrans* was methyl oleic ($C_{17}H_{33}COOCH_3$, C18:1(Δ^9)). With insight into the cell's pre-treatment and simultaneous process of the in-situ method, it is feasible to apply the techniques for biodiesel production in the future.

Keywords: *Chaetoceros calcitrans*; Microalgae; Hydrothermal Acid; Ultrasonication; Biodiesel.

RASĀYAN *J. Chem.*, Special Issue, 2022
This manuscript is focusing **SDG-7:** Affordable and Clean Energy

INTRODUCTION

Energy is a main necessity in many sectors, such as the transportation sector. Either of the energy sources that be used in it is diesel. Diesel is the fuel that resulted from the fractional distillation of crude oil, it was formed at 250-400ºC and classified in C_{15}-C_{18} (long chain carbon). It could be supplied from fossil fuel as unrenewable energy sources, in other words, if it was used continuously then the supply of fossil fuel will increasingly be depleted. Furthermore, it was sourced from fossil fuel in an unfriendly environment, because its combustion process produces gas emissions such as CO (carbon monoxide), CO_2 (carbon dioxide), NO_x (oxide nitrogen), SO_x (oxide sulphur), and other air pollution. Gas emissions could trigger the greenhouse effects that create environmental problems.[1,2] The effort to obtain environmentally friendly unrenewable energy sources is very required as alternative energy. Biodiesel is worth developing as an alternative fuel because its combustion process does not produce aromatic compounds.[3] Biodiesel is a fatty acid alkyl ester (FAAE) that is produced by a transesterification reaction between triglycerides and alcohol with a catalyst, such as acid, base, or enzyme.[3] Utilization of vegetable oil such as corn oil, bean oil, coconut oil, and palm oil sourced from plant biomass has been widely used as raw materials for biodiesel production, however, the uses can influence the food sector, especially on the level of basic needs of the community.[4,20] In order to the equilibrium of foodstuffs sources as basic needs of the community could still be maintained, then need to explore the other abundant sources of raw materials in nature for biodiesel production, one of which is to take advantage of microalgae oil that was contained in its biomass. At this time, biodiesel can be produced from microalgae biomass. It has substantial potency as the raw material of biodiesel production because it contains a high yield of lipids, it was shown that 70% of wet microalgae biomass produced a 136900 L/ha yield of lipids.[3-5] Microalgae are prokaryotic or eukaryotic photoautotrophic unicellular microorganisms, that requires CO_2, nutrient, and light during photosynthesis.[6,7,19] In its process, CO_2 was

http://doi.org/10.31788/RJC.2022.1558063
This work is licensed under a CC BY 4.0 license.

converted into biomass by solar energy or other lighting sources. Microalgae produce high biomass.[8] Microalgae biomass contains high triglycerides (a form of lipid) in the cell. The lipid content that resulted from it is higher than from plant biomass. It contains fatty acids (C_{14}-C_{22}).[9,10] The components of fatty acids in microalgae oil are different, it depends on the microalgae strain.[11,20] Either of the microalgae that can be used as the raw material for biodiesel production is *Chaetoceros calcitrans*, a Bacillariophyceae (Diatomophyceae) microalgae class and size 6-8 µm.[11] This research uses Indonesia's local strain of *Chaetoceros calcitrans* that was obtained from Brackishwater Aquaculture Development Center Situbondo, East Java. Besides, it was abundant in Indonesia, it can be cultivated easily, its growth rate of it is relatively fast and it can produce high biomass.[13,19] *Chaetoceros calcitrans* has been reported that contain 12-40% (w/w) lipid total from dry biomass and be produced by Bligh and Dyer method.[14] These microalgae contain 8.7% (v/v) of lipid total (0.9% (w/w) from dry biomass) of polyunsaturated fatty acid (PUFA) [and 34% (v/v) from a fatty acid total of eicosapentaenoic acid (EPA).[15] Kwangdinata has produced biodiesel from lipid, extraction result of *Chaetoceros calcitrans* dry biomass by ultrasonication method as cell pre-treatment then transesterification reaction use base catalyst (KOH), it obtains a yield of lipid was 16.23% (w/w) from dry biomass and yield of biodiesel was 35.35% (w/v) from lipid total.[17] Ultrasonication is a physical method of cell disruption through ultrasonic waves (>20 kHz). In this method, during the ultrasonication process will be formed microbubbles then they collide randomly, that namely cavitation. It causes lysis on the structure of the cell wall.[16] Ultrasonication method is able to improve the yield of lipids until 50-500% on both *Nannochloropsis oculata* and *Chlorella* Vulgaris.[17] The other cell disruption method has been done by Park, it was hydrothermal acid as cell pre-treatment before lipid extraction, which result in 337 mg/g cell from dry biomass.[17] Hydrothermal acid is a chemical method of cell disruption by using strong acid (such as HCl or H_2SO_4) to hydrolyze the cell wall at a high temperature (120°C) to upgrade the ability of the cell wall lysis so that the components that are contained in microalgae cell (in this focus is lipid) could be extracted perfectly.[16,17] The purpose of this study is to determine the FAME productivity from lipid *Chaetoceros calcitrans* that was created by the ex and in-situ methods accompanied by hydrothermal acid and ultrasonication treatments for the lipid extraction. The cell pre-treatment was adopted to disrupt these microalgal cells so the lipid could be released effectively. The catalyst of H_2SO_4 6% (v/v) and KOH 6% (w/v) was used on the lipid conversion to biodiesel. The components of FAME in the biodiesel were identified by the instrument of Gas Chromatography-Mass Spectrophotometry (GC-MS).

EXPERIMENTAL

Cultivation of Microalgae

The *Chaetoceros calcitrans* microalgae that were used in the research were obtained from Brackishwater Aquaculture Development Center Situbondo, East Java. The culture of *Chaetoceros calcitrans* with Aadensity 3.5×10^{-5} cell/mL was cultivated in the sterile seawater media with an additional fertilizer of 1 mL/L. The one liter of diatom fertilizer was composed of a mixture of 75 g of KNO_3, 5 g of NaH_2PO_4, 5 g of Na_2EDTA, and 3.15 g of $FeCl_3$. The oxygen to support the culturing process was prepared by an aerator. The growth of *Chaetoceros calcitrans* cells during the culturing was counted by a hemocytometer.

Lipid Extraction from *Chaetoceros calcitrans* With and Without Cell Pre-Treatment

The *Chaetoceros calcitrans* microalgae biomass as 10 grams was added by 400 mL of a mixed solvent of n-hexane and ethanol 96% (1:1) in a polypropylene tube then it was vortexed and centrifuged at 5000 rpm for 10 minutes respectively. The residues were removed, then supernatant which has two layers was taken. The organic phase (upper layer) was evaporated then the yield of lipid was counted gravimetrically. In other work, it was also done lipid extraction assisted by hydrothermal acid and ultrasonication treatment. Assisting of hydrothermal acid was performed by adding 250 µL of 2% (v/v) H_2SO_4 to the biomass cells immersion in n-hexane and ethanol 96%, then connected by autoclave treatment in 120°C for 10 min.

While the extraction which was assisted by ultrasonication was carried out by contacting the biomass cells immersion in the sonication device on 100% amplitude power for 10 mins. A lipid that resulted from all treatments was determined and compared to each other. A combination of two-cell pre-treatment was also done on the lipid extraction.

Biodiesel Production with Ex-situ Transesterification
A Lipid that resulted from extraction with various cell pre-treatment was added with methanol in volume comparison 5:1, then added with 2,5 mL of H_2SO_4 6% (v/v) as a catalyst into three necks round flask then was refluxed in 60°C for an hour. The result of transesterification was chilled for 10 minutes, then partitioned with 20 mL of aqua dest and 25 mL of n-hexane, the mixture formed two layers. The biodiesel in the upper phase was separated from n-hexane and glycerides by evaporation. The FAME component was detected by Gas Chromatography-Mass Spectrometry (GC-MS).

Biodiesel Production with In-Situ Transesterification
Dry biomass of *Chaetoceros calcitrans* as much as 10 g was added with 400 mL methanol and 25 mL of a catalyst either H_2SO_4 6% (v/v) or KOH 6% (w/v) into three necks round flask then was refluxed in 60°C for an hour. The result of transesterification was chilled for 10 minutes approximately. Furthermore, it was partitioned with 200 mL of aquadest and 250 mL of n-hexane. Two layers were formed from the portioning work. The lower phase contained excessive methanol, catalyst, and glycerol. Whereas, the upper phase contained biodiesel (fatty acid methyl ester, FAME), n-hexane, and glycerides. The upper phase was evaporated. Next, the biodiesel (FAME) component was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS).

FAME Detection with GC-MS
The FAME components on ex-situ and in-situ biodiesel were determined by GC-MS at Airlangga University laboratory, using HP-5MS capillary column. In the FAME sample, as much as 200 µL was added by 1 mL of n-hexane in a microtube. As much as 100 µL of the mixture is put into the column, then the running process is turned on until all the recorded data appear.

Statistical Analysis
The data quantity of lipid and biodiesel product were analyzed statistically by ANOVA variance homogeneity and t-independent test with SPSS v.16 Program.

RESULTS AND DISCUSSION

The Growth of *Chaetoceros calcitrans*
The microalgae showed a sigmoid curve in seawater media (Fig.-1). It had an adaptation phase or lag phase until the first day, then followed by the exponential phase until the fifth day, and the last stationer to the death phase on the day after. The highest cell density was achieved as 5.89×10^6 cells/mL on the fifth day (Fig.-1). The microalgae cell showed a cylinder and round shape and has an elliptical valve that appears rectangular and golden green (Fig.-2).

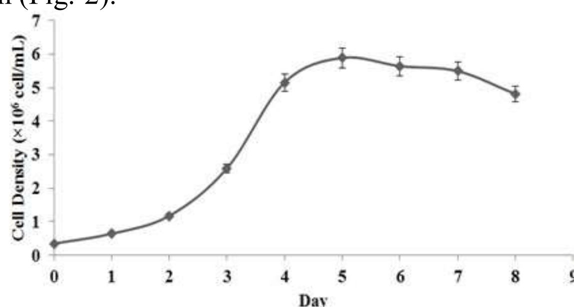

Fig.-1: Growth Curve of *Chaetoceros calcitrans* Microalgae

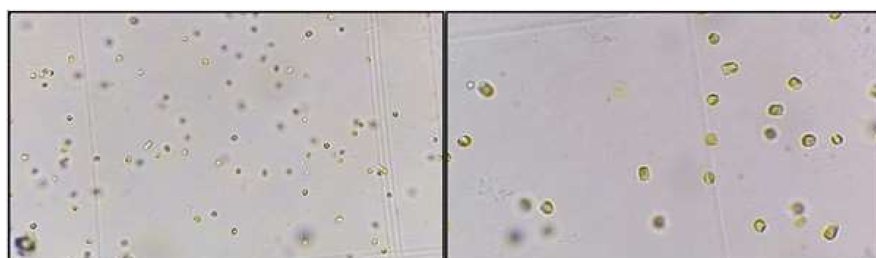
Fig.-2: Microscopic Appearance of *Chaetoceros calcitrans*. (a) 40x and (b) 100x Magnifications.

Lipid Yield

The lipid that yielded from various cell pre-treatment was compared to each other. The use of cell pre-treatment like hydrothermal acid (HA), ultrasonication (US), and a combination of hydrothermal acid with ultrasonication (HA+US) enhanced lipid yield compared to no cell per-treatment (N). Lipid extraction without cell pre-treatment (N) resulted in a lipid yield of 3.08% (w/w) while assisting HA, US, and combination HA with US resulted in lipid yield of 3.08%, 10.65%, 56.94%, and 61.40% (w/w) respectively (Fig.-3). The highest lipid yield was 61.40% (w/w) with an increased percentage was 94.99% that resulted from lipids that were obtained from the combination of both hydrothermal acid and ultrasonication group.

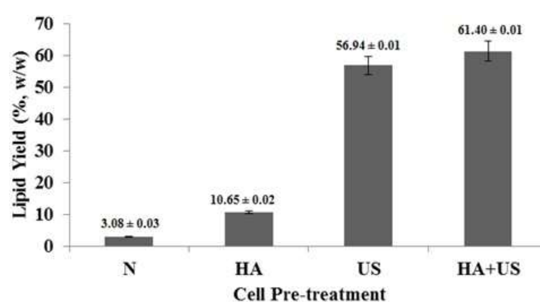

Fig-3: Lipid Yield on Each of Cell Pre-Treatment Variations. N (Without Cell Pre-treatment), HA (Hydrothermal Acid), US (Ultrasonication), and HA+US (The combination of Hydrothermal Acid and Ultrasonication).

Biodiesel (FAME) Yield

The FAME yield obtained by ex-situ transesterification of lipid from various cell pre-treatment with methanol and H_2SO_4 6% (v/v) as catalyst was similar to 17-19% (w/w). The slightly higher fame product was obtained from lipid extracted with the combination treatment of HA and US, which was 19.15% (w/w) (Fig.-4). The lipid extraction and its conversion to FAME in one reaction step had been carried out through an in-situ process by the catalyst of H_2SO_4 6% (v/v) and KOH 6% (w/v) respectively. Transesterification of *Chaetoceros calcitrans* microalgae dries biomass with methanol using a catalyst of H_2SO_4 6% (v/v) resulted in the FAME of 21.59% while using a catalyst of KOH 6% (w/v) resulted in FAME as 20.72% (w/w) (Fig.-5). The use of an H_2SO_4 catalyst is more profitable than KOH in the in-situ process.

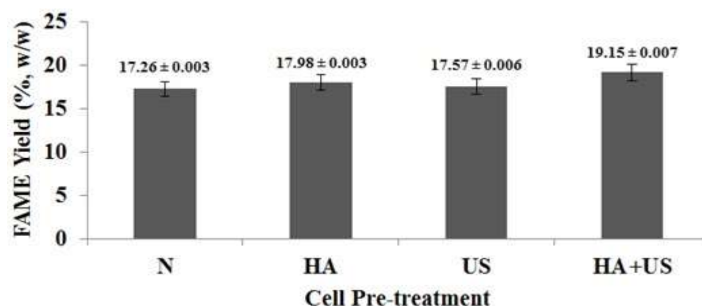

Fig.-4: FAME Yield on Each of Cell Pre-Treatment Variations Based on Result of Ex-Situ Transesterification. N (Without Cell Pre-treatment), HA (Hydrothermal Acid), US (Ultrasonication), and HA+US (The combination of Hydrothermal Acid and Ultrasonication).

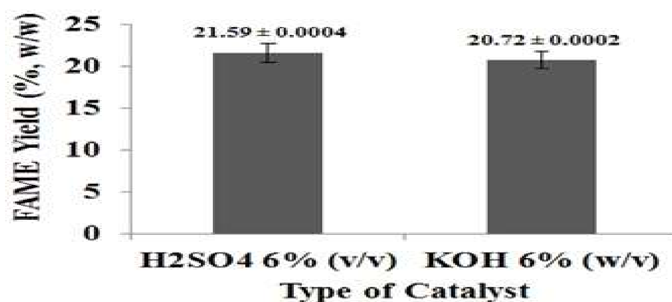

Fig.-5: FAME Yield on Using of Different Catalysts Based on Result of In-Situ Transesterification.

Component on Biodiesel Products

The result of GC-MS characterization of both ex-situ and in-situ biodiesel products showed in Table-4. The highest biodiesel is based on the total percentage of chromatogram peak area obtained by ex-situ transesterification using a combination of hydrothermal acid and ultrasonication treatments. The biodiesel products consist of methyl palmitic ($C_{15}H_{31}COOCH_3$, C16:0), methyl linoleic ($C_{17}H_{31}COOCH_3$, C18:2($\Delta^{9,12}$)), methyl oleic ($C_{17}H_{33}COOCH_3$, C18:1(Δ^9)) and methyl stearic ($C_{17}H_{35}COOCH_3$, C18:0) that had a percentage of chromatogram peak area as 13.501%, 15.568%, 66.498%, and 4.434% respectively. The highest biodiesel (FAME) component in this group was methyl oleic with a retention time of 26.175 minutes (Fig.-6).

Table-4: The ex-situ and in-situ Biodiesel Products on Various Treatments

Method	Treatment	Retention time (min)	FAME composition	Percentage of FAME component (%)	Total area percentage of FAME (%)
ex-situ (using H_2SO_4 6% as Catalyst)	Without Cell Pre-treatment (N)	24.253	Methyl Palmitic (C16:0)	5.172	10.882
		26.17	Methyl Oleic (C18:1(Δ^9))	5.71	
	Hydrothermal Acid (HA)	24.247	Methyl Palmitic (C16:0)	22.536	52.588
		26.17	Methyl Oleic (C18:1(Δ^9))	26.462	
		26.421	Methyl Stearic (C18:0)	4.382	
	Ultrasonication (US)	21.397	Methyl Myristic (C14:0)	1.649	65.273
		24.247	Methyl Palmitic (C16:0)	20.391	
		26.112	Methyl Linoleic (C18:2($\Delta^{9,12}$))	5.013	
		26.169	Methyl Oleic (C18:1(Δ^9))	32.823	
		26.427	Methyl Stearic (C18:0)	5.397	
	Hydrothermal Acid and Ultrasonication (HA+US)	24.247	Methyl Palmitic (C16:0)	13.501	100
		26.112	Methyl Linoleic (C18:2($\Delta^{9,12}$))	15.568	
		26.175	Methyl Oleic (C18:1(Δ^9))	66.498	
		26.421	Methyl Stearic (C18:0)	4.434	
in-situ	H_2SO_4 6%	24.247	Methyl Palmitic (C16:0)	14.66	33.686
		26.17	Methyl Oleic (C18:1(Δ^9))	29.476	
	KOH 6%	24.247	Methyl Palmitic (C16:0)	14.549	24.242

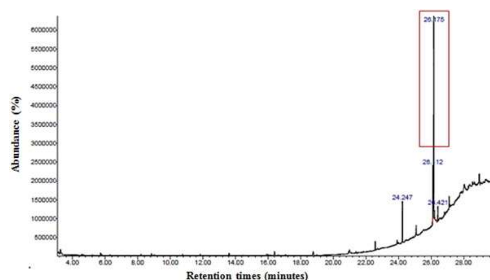

Fig.-6: GC Chromatogram of Methyl Oleic on Biodiesel Product. The Retention Time of 26.175 mins Were Marked by Reddish Rectangular

The Growth of *Chaetoceros calcitrans*

Microalgae cultivation has been planned to increase the production of microalgae cell quantity (cell density) so that it was expected to be able to develop high biomass appropriately. *Chaetoceros calcitrans* is a Bacillariophyceae (Diatomophyceae) microalgae class that finds abundant in Indonesia. It can be cultivated

easily in seawater as a growth medium. The growth rate of the microalgae is relatively fast and it produces high biomass.[13] It has been seen that there occur an increase in cell density from the first day until the fifth day of cultivation (Fig.-1). Both the rate of microalgae cell division and the value of its metabolic activity were constant at the exponential phase that was achieved on the fifth day of cultivation with the highest of its cell density being 5.89×10^6 cell/mL based on the growth curve of *Chaetoceros calcitrans* so that its biomass was harvested on the fifth day of cultivation and it was obtained 15 g/L of dry cell weight of *Chaetoceros calcitrans*. The stationer phase has achieved from the fifth day until the seventh day of cultivation, that both the period of this microalgae cell growth and the value of its cell density was constant. Whereas, the rate of microalgae cell division and value of its metabolic activity decreased at this phase, so the microalgae cell density decreased on the eighth was 4.81×10^6 cell/mL which showed the mortality phase was achieved. A nutrient source in its growth media was decreased even until used up, and toxic accumulation was increased which cause lysis cells to die at this phase, so the value of microalgae cell which has died was more than still live. The microscopic appearance of *Chaetoceros calcitrans* was shown in Fig.-2. Its shape was cylindrical and rough. It has an elliptical valve, looked like rectangular and golden green.

The Effect of Cell Pre-Treatment on Lipid Extraction

Lipid extraction is a method to extract and isolate lipids from microalgae cells. To improve the quantity of lipid which was extracted and isolated from a microalgae cell, then cell pre-treatment was applied before lipid extraction.[16,17] It was aimed to further improve the performance of lipid extraction. Cell pre-treatment of microalgae can be applied to disrupt microalgae cells, such as by lysis cell wall (cell disruption) and lipid dissolution by organic solvent, or a combination of both. Choosing the cell disruption method depends on cell wall conditions, cell size, and the scale of the application used.[16,17] Cell pre-treatment which was applied in this research study was a combination of physical and chemical methods. In the physical method, the lysis cell wall of microalgae occurs due to treatment with physical conditions, such as temperature, vibration, microwave, or ultrasonic wave. The physical method that was used in this research study was the ultrasonication method. Ultrasonication is a physical cell disruption method through ultrasonic waves (>20 kHz) that spreads inside liquid media it results in high pressure. As a result, it was formed micro bubbles during the process so as occur collisions randomly, namely cavitation. It causes lysis on the structure of the cell wall.[18,19] The cavitation effectiveness depends on characteristics of ultrasonic wave, solvent properties, and physical conditions such as pressure and temperature.[11,18,19] Whereas, in the chemical method, using of organic solvent will dissolve lipids in microalgae cells.[7,12] The chemical method that was used in this research study was the hydrothermal acid method. Hydrothermal acid is a chemical cell disruption method that uses strong acids such as HCl or H_2SO_4 to hydrolyze the cell wall, and it was applied at a high temperature (120ºC) by autoclave to upgrade the cell wall lysis ability so that the components contained in microalgae cell can be extracted perfectly.[10,11] The content of lipids in microalgae is defined as lipid concentration that is contained in microalgae cells without considering biomass production.[30] Microalgae can be able to result in high biomass and high lipid.[12,13] If each of the lipid yields of *Chaetoceros calcitrans* microalgae biomass in this study were compared to the lipid yield from other report, the lipids yield by ultrasonication in this study (56.94% (w/w)) was higher than lipid yield by ultrasonication on Kwangdinata's study (16.23% (w/w)) because both the condition of cell pre-treatment and ultrasonication parameter on both of them was different.[19,20] The lipid extraction of *Chaetoceros calcitrans* microalgae obtained by ultrasonication in Kwangdinata's study had the ratio of its biomass to solvent is 1:6 (w/v), it only uses a single solvent as media to extract lipid was ethanol 96%, and the frequency during ultrasonication process was 40 kHz. Whereas, the lipid extraction of *Chaetoceros calcitrans* microalgae obtained by ultrasonication in this research study has the ratio of its biomass to solvent is 1:8 (w/v), it use binary solvent as media to extract lipid was n-hexane: ethanol 96% (1:1, v/v), and the amplitude power during ultrasonication process was 100%. Based on the difference in cell pretreatment and ultrasonication conditions in both studies, if solvent quantity is more and more, the kinds of solvent used for cell pretreatment have more variation, and physical condition during ultrasonication is higher than the lipid yield is higher too. Then, the lipid yield of *Chaetoceros calcitrans* microalgae by hydrothermal acid has never been reported in another study. Therefore, this research study applies the hydrothermal acid method

to extract lipids on *Chaetoceros calcitrans* microalgae biomass too, and was obtained lipid yield by hydrothermal acid in this study was 10.65% (w/w). It can be determined that lipid yield by hydrothermal acid in this study was lower than by ultrasonication in both this study and Kwangdinata's study. Because of that, this research study applies the combination of hydrothermal acid and ultrasonication as cell pre-treatment before lipid extraction to upgrade lipid quantity. Based on Table-1, the combination method resulted the highest lipid were 61.40% (w/w) with an increasing percentage was 94.99% than by just a single method of cell pre-treatment such as without cell pre-treatment, just hydrothermal acid, or just ultrasonication. A single method of cell pre-treatment which was applied in microalgae cells to disrupt (to lysis) the microalgae cell wall before lipid extraction, can result in lipid certainly, however, it's lipid quantity which was obtained is less than the maximum. With apply the combination of both hydrothermal acid and ultrasonication methods as cell pre-treatment before lipid extraction, then ultrasonic wave which was applied to lysis this microalgae cell wall during the ultrasonication process will upgrade cell wall lysis ability in its cell wall that has been hydrolyzed previously by using of strong acid for the hydrothermal acid process. During the hydrothermal acid process, the existence of strong acid was able to hydrolyze its cell wall perfectly with the existence of high temperature. It causes the microalgae cell wall to begin to appear broken slightly and lipids can exit from the cell then it was mixed and dissolved in an organic solvent. In other that, the existence of strong acid during the hydrothermal acid process causes color alteration on the lipid-solvent organic complex from green into yellow-pale golden. It was caused by two atoms of hydrogen in strong acid will shift magnesium nuclei on the chlorophyll structure, so the chlorophyll structure was decomposed to become pheophytin, a derivative compound of chlorophyll that has a yellow pale golden color. However, this process is less than the maximum to obtain high lipids. When entering into the ultrasonication process, the microalgae cell wall that has been appeared broken slightly was disrupted again by the ultrasonic waves. It causes formed microbubbles that collide randomly with each other during the ultrasonication process, namely cavitation which causes lysis on the structure of the microalgae cell wall.[15,16] So that, the lipid quantity which resulted from this combination method of cell pre-treatments was higher than by just a single method of cell pre-treatment.

Lipid Components in *Chaetoceros calcitrans*

Based on Table-4 related to FAME components, lipid components that were contained in each of the cell pre-treatment groups were identified as fatty acids. The fatty acids that were contained in each cell pretreatment group were more and more than without cell pretreatment groups (only use n-hexane: ethanol (1:1, v/v) binary solvent). When dry biomass of *Chaetoceros calcitrans* microalgae was extracted its lipid without preceded by cell pre-treatment, the process produced the main component of palmitic and oleic acids in its lipid.

The lipid product increased when the extraction was preceded by cell pre-treatments. Extraction assisted by hydrothermal acid yielded an additional product of oleic acid besides palmitic and stearic acids. Meanwhile, ultrasonically assisted extraction produces additional myristic, oleic, and linoleic acids. Joining hydrothermal acid and ultrasonication as pre-treatment methods yielded lipid components of palmitic, stearic, oleic, and linoleic acids. Each lipid obtained without cell pretreatment groups contained saturated and unsaturated fatty acids. Myristic acid ($C_{13}H_{27}COOH$, C14:0), palmitic acid ($C_{15}H_{31}COOH$, C16:0), and stearic acid ($C_{17}H_{35}COOH$, C18:0) were classified as saturated fatty acids which have not double bond in a long hydrocarbon chain so that the molecule structure on its carbon chain is straight and unbranched. Whereas, oleic acid ($C_{17}H_{33}COOH$, C18:1(Δ^9)) and linoleic acid ($C_{17}H_{31}COOH$, C18:2($\Delta^{9,12}$)) were classified as unsaturated fatty acids which have one or more double bonds on a certain number of carbon (in this case is a number of the double bond) in along hydrocarbon chain, where each of double bonds was linked by methylene group (-CH=CH-CH_2-CH=CH-). So, the existence of a double bond along the hydrocarbon chain causes the molecule structure on its carbon chain to be bent and branched. Oleic acid is classified as monounsaturated fatty acid (MUFA) because it has just a double bond on the ninth number of carbons.

Whereas, linoleic acid is classified as polyunsaturated fatty acid (PUFA) because it has two double bonds on both the ninth and the twelfth number of carbons. All of them is appropriate that fatty acids in nature contain an even amount of carbon generally.[16-19]

The Difference of FAME from *ex* and *in-situ* Process

To produce biodiesel from microalgae biomass, then the stage of that was biodiesel production (by transesterification) and biodiesel purification (separation of biodiesel product which is still mixed with glycerol).[16,17,20,21] Biodiesel is a fatty acid alkyl ester (FAAE) that is obtained from the reaction of triglycerides with alcohol using a catalyst, such as acid, base, or enzyme.[5] Triglycerides which is contained in microalgae cell is very potential to be developed as bioenergy, due to they could be transformed into FAME and glycerol in the transesterification process by using a catalyst.[5,11] The function of the catalyst is used to increase the reaction rate efficiently in a short time. If it is done without the role of a catalyst, it requires a high temperature to force the transesterification reaction to take place normally, so the biodiesel can be produced.[5.11] Some main factors that influence both transesterification reaction and yield of biodiesel (alkyl esters) such as the molar ratio of both triglycerides and alcohol, both volume and type of alcohol that be used, both types and concentration of catalyst that be used, reaction temperature, pressure, and time. Microalgae biomass contains high triglycerides in cells where it contains fatty acids C_{14}-C_{22} which can be converted into fatty acids alkyl esters by transesterification.[12,13] In this research study, biodiesel was produced from Indonesia's local strain of *Chaetoceros calcitrans* microalgae that was obtained from Brackishwater Aquaculture Development Center Situbondo, East Java by ex-situ and in-situ transesterification. Based on the result of ex-situ and in-situ biodiesel yields, it was proven that the quantity of in-situ biodiesel yield was higher than the quantity of ex-situ biodiesel yield. This is appropriate with Pragya's study that in-situ biodiesel production is capable to upgrade the number of biodiesel products than ex-situ biodiesel production.[15,16] In addition, in-situ biodiesel production is easier and faster and its process is more effective than ex-situ biodiesel production.[14,20] It was caused that both lipid extraction and transesterification occur simultaneously in one stage in the same system[19,20], so it is called direct biodiesel production, which is different from ex-situ biodiesel production where lipid extraction at first and then proceeds to transesterification so that its process was occur separately in two stages on different systems so it is called indirect biodiesel production. Moreover, in-situ biodiesel production can be applied to minimize the cost and time that be required for lipid extraction.

Fatty Acid Methyl Esters (FAME) Components on both ex-situ and in-situ Biodiesel Products

The amount of FAME components from the ex-situ biodiesel product from the cell pre-treatment group appeared to be more than that produced by the no-cell pre-treatment group. The FAME components that emerged from the group without pre-treated cells were methyl palmitate and methyl oleate. Meanwhile, from the cell pre-treatment group, there was an increase in the number of FAME components apart from methyl palmitate and methyl oleate. The ex-situ biodiesel product with hydrothermal acid treatment showed FAME components such as methyl palmitate, stearate, and oleate. While in the ultrasonic treatment, there are additional components of methyl myristate and linoleate. Furthermore, the ex-situ biodiesel product from the combination treatment of hydrothermal acid and ultrasonication displayed FAME components of methyl palmitate, stearate, oleic and linoleic. The in-situ biodiesel product using an acid (6% H_2SO_4) or alkaline (6% KOH) catalyst shows the presence of methyl palmitate and methyl oleic components in the FAME, this result is the same as that shown by the ex-situ process without pre-treating the cells (Table-4). Based on the results of GC-MS characterization, the highest biodiesel product in both ex-situ and in-situ processes is indicated by the largest percentage of chromatogram peak area. The greatest value was obtained from a treatment combination of hydrothermal acid and ultrasonication, where its biodiesel has a component of methyl palmitic (C16:0), linoleic (C18:2($\Delta^{9,12}$), oleic (C18:1(Δ^{9})) and stearic (C18:0), with the total percentage of chromatogram peak area were 13.501%, 15.568%, 66.498%, and 4.434% respectively. The highest biodiesel (FAME) component in this group was methyl oleic. Methyl oleic as the highest biodiesel (FAME) component was not only contained in this treatment group but also it was contained in the other treatment groups too such as in the transesterification of lipid on without cell pre-treatment group, hydrothermal acid group, ultrasonication group, acid catalyst group, and base catalyst group, where they were obtained 5.710%, 26.462%, 32.823%, 66.498%, 29.476%, and 21.313% respectively. This explains that of them, the highest FAME component on each biodiesel product was methyl oleic. If methyl oleic on each of the in-situ biodiesel products were compared to each other than the highest of it was obtained on in-situ transesterification which use acid catalyst (H_2SO_4 6%), that was 29.476% methyl oleic. If methyl

oleic on each of them were compared to each other than the highest of it was obtained on transesterification of lipid from the combination of both hydrothermal acid and ultrasonication group, which was 66.498% methyl oleic. In another word, methyl oleic was a main FAME component on each biodiesel product that resulted from biomass of *Chaetoceros calcitrans* microalgae.

CONCLUSION

The microalgae of *Chaetoceros calcitrans* were able to grow well in seawater media, yielding a dry cell weight of 15 g/L on the fifth day of fermentation. Lipid extraction from the biomass with a mixture of organic solvents, i.e., n-hexane and 96% ethanol (1:1) resulted in the highest lipid as 61.40% (w/w) when the extraction assisted by a combination of hydrothermal acid and ultrasonication cell pre-treatments. Transesterification of the lipid with methanol using a catalyst of H_2SO_4 6% (v/v) for one hour at 60ºC could result in the FAME as 19.15% (w/w) in the ex-situ process. Meanwhile, the in-situ method that combined lipid repealing and transesterification steps simultaneously, produced the FAME of 21.59% (w/w). The in-situ process exhibited higher FAME than the ex-situ. The FAME component in the biodiesel of *Chaetoceros calcitrans* was methyl oleic ($C_{17}H_{33}COOCH_3$, C18:1(Δ^9)). Insight into the cell pre-treatment and direct conversion of FAME from the cell biomass, it is feasible in the future to develop the techniques used in biodiesel production.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the late Dr. Abdulloh MSi who was involved in this research. This project was supported by Faculty's Flagship Research (Penelitian Unggulan Fakultas, PUF), Universitas Airlangga by Rector's Decree No.1408/UN3/2019.

CONFLICT OF INTERESTS

The authors state no conflict of interest in the research.

AUTHOR CONTRIBUTIONS

Purkan Purkan is involved in coordinating the writing of publication articles and data mining, Hamidah Nurlaila acted as research assistant and data collector, Afaf Baktir supports as data interpreter and editor, then Sofijan Hadi assisted in collecting data and writing the draft manuscript. The last Wiwie Soemardjati was involved in sample preparation and data collection. The research profile of the authors can be verified from their ORCID ids, given below:

Purkan Purkan https://orcid.org/0000-0002-3784-7008

Afaf Baktir https://orcid.org/0000-0002-8756-0523

Sofijan Hadi https://orchid.org/0000-0002-0653-8667

Hamidah Nurlaila https://orcid.org/0000-0001-5349-7856

Wiwie Soemardjati https://orcid.org/0000-0002-1620-8696

Open Access: This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

REFERENCES

1. Demirbas, and M.F. Demirbas, *Energy Conversion and Management*, **52**, 163(2011), https://doi.org/10.1016/j.enconman.2010.06.055
2. L.D.S.Teresa, L. Gouveia, and A. Reis, *Applied of Microbiology and Biotechnology*, **98**, 1043(2014), https://doi.org/10.1007/s00253-013-5389-5
3. R. Katiyar, B.R. Gurjar, R.K. Bharti, A. Kumar, S. Biswas, and V. Pruthi, *Renewable Energy*, **113**, 1359(2017), https://doi.org/10.1016/j.renene.2017.06.100
4. P. Purkan, E. Huruniawati, S. Sumarsih, *Journal of Chemical Technology and Metallurgy,* **52(6)**,1079(2017).

5. T.R. Soeprobowati, and R. Hariyati, *International Journal of Advances in Chemical Engineering and Biological Sciences*, **1(1)**, 37(2014), https://doi.org/10.15242/IJACEBS.C1113051
6. R. Kwangdinata, I. Raya, and M. Zakir, *The Scientific World Journal*, **2014**,1(2014), https://doi.org/10.1155/2014/231361
7. G.V. Subhash, M. Rajvanshi, B.N. Kumar, S. Govindachary, V. Prasad, and S. Dasgupta, *Bioresource Technology*, **244**, 1304(2007), https://doi.org/10.1016/j.biortech.2017.07.024
8. M.V. Derakhshan, B. Nasernejad, M. Dadvar, and M. Hamidi, *Asian Pacific Journal of Chemical Engineers*, **9**, 629(2014), https://doi.org/10.1002/apj.1790
9. J.Y. Park, Y.K. Oh, J.S Lee, K. Lee, M.J. Jeong, and S.A. Choi, *Bioresource Technology*, **153**, 408(2014), https://doi.org/10.1016/j.biortech.2013.12.065
10. S.Y. Lee, J.M. Cho, Y.K. Chang, and Y. Oh, *Bioresource Technology*, **244**, 1317(2017), https://doi.org/10.1016/j.biortech.2017.06.038
11. Y.K Zhu, S.R. Nugroho, Z. Shakeel, B. Li, Martinkauppi, and E. Hiltunen, *Renewable and Sustainable Energy Reviews*, **78**, 391(2017), https://doi.org/10.1016/j.rser.2017.04.089
12. P. Grosso, F. Valentão, Ferreres, and P.B. Andrade, *Marine Drugs*, **13**, 3182(2015), https://doi.org/10.3390/md13053182
13. R. Kiran, Kumar, and D. Deshmukh, *Energy Convers Management*, **88**, 1228(2014), https://doi.org/10.1016/j.enconman.2014.06.022
14. W. Klinthong, Y. Yang, C. Huang, and C. Tan, *Aerosol and Air Quality Research*, **15**, 712(2015), https://doi.org/10.4209/aaqr.2014.11.0299
15. R. Huang, J. Cheng, Y. Qiu, T. Li, J. Zhou, and K. Cen, *Energy Conversion and Management*, **105**, 791(2015), https://doi.org/10.1016/j.enconman.2015.08.036
16. L. Zhu, F. Hiltunen, E. Antila, J. Zhong, Z. Yuan, and Z. Wang, *Renewable and Sustainable Energy Reviews*, **30**, 1035(2014), https://doi.org/10.1016/j.rser.2013.11.003
17. N. Pragya, K.K. Pandey, and P.K. Sahoo, *Renewable and Sustainable Energy Reviews*, **24**, 159(2013), https://doi.org/10.1016/j.rser.2013.03.034
18. P. Purkan, I.T. Lestari, R. Arissirajudin, R.R.P. Ningsih, W. Apriyani, H. Nurlaila, S. Sumarsih, S. Hadi, W. Retnowati and S.W. Kim, *Rasayan Journal of Chemistry*, **13(4)**, 2074(2020), https://doi.org/10.31788/RJC.2020.1345697
19. P. Purkan, E. Nidianti, A. Abdulloh, A. Safa, W. Retnowati, W. Soemarjati, H. Nurlaila and S.W. Kim, *Open Chemistry*, **17(1)**, 919(2019), https://doi.org/10.1515/chem-2019-0102
20. P. Purkan, A. Safa, A. Abdulloh, A. Baktir, R. Arissirajudin, H. Hermansyah and S.W. Kim, *Rasayan Journal of Chemistry*, **14(3)**, 2097(2021), https://doi.org/10.31788/RJC.2021.1436317
21. Purkan, Ihsanawati, Natalia D, Syah YM, Retnoningrum DS, Kusuma HS, *Ukrainian Biochemical Journal*, **88(5)**, 71(2016), https://doi.org/10.15407/ubj88.05.071

[RJC-8063/2022]