Screening for Lipid Depositor of Indonesian Microalgae Isolated from Seashore and Peat-land

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Abstract

The screening for finding the lipid producer of indigenous Indonesian tropical microalgae that obtained from peat-land at Riau, Sumatra and seashore at Lombok has carried out. Preliminary screening was done by selection on faster growing algae from 44 (forty-four) isolates. 10 (ten)isolates were chosen for further analysis. Out of 10 selected isolates shows that five isolates have indication rapid growth and high content of lipid, furthermore those chose isolates were observed comprehensively, namely LIPI11-2-AI005, LIPI11-2-AI010, LIPI11-2-AI015, LIPI11-2-AI018, and LIPI11-2-AI019. The microalgae are belong to the eukaryotic microalgae and its seem to be closed to Chlorophytes that has cell nuclear with an envelope, light green chloroplast, thick cell wall, round and elongated cell features. Cultivation of the microalgae in laboratory condition (1 L) has shown that lipid content was about 20-35% base in cell dry weight. The highest lipid content was found in the isolate LIPI11-2-AI018 of 30,74% per dry weight cell. LIPI11-2-AI018 therefore was test for scale up cultivation reach the culture volume of 5 L in the cylindrical photobioreactor, and expected to be source of triglycerides and lipids for biodiesel ingredient.

Keywords: Algal characteristic, Chlorophytes, Laboratory cultivation, lipid screening process, lipid content

1.Introduction

A trend to find the alternative sustainable energy sources that not competing with food and to

fulfill the clean lifestyle requirements has develop the utilization of marine biomass, including microalgae. Microalgae are believed can be suitable candidate for energy sources in the future in Indonesia as tropical and island country. Properties of microalgal biomass such as a high content of carbohydrate, lipid and protein, has potential use for energy sources which are able to convert be oil, alcohol compounds or hydrogen, depend on the processes. The advantages of microalgae cultivation are higher photosynthesis efficiencies, higher oil yields, requires less water and can be undertaken on non-arable land [1,2], since microalgae are photosynthetic microorganisms which convert inorganic compounds such as CO₂ to biomass (or organic compound) with help of sunlight [3].

The obstacles employing microalgae as sources of energy are selection good strains which has to growth rapidly and content high lipid or other components, cultivation or scale up techniques with without any problem in decreasing the targeted compound and biomass, harvesting techniques and processes post-harvesting [4]. Therefore specific selection microalgae strain for lipid production and cultivation techniques are challenging due to the variety, characterization, and comparable conditions [5]. Lipid produced by microalgae generally include neutral lipid, polar lipid wax esters, sterols and hydrocarbon [6]. In several report, microalgal lipid depositor are ranging from the chlorophytes, diatoms, rhodophytes and crysophytes taxa [7]. It's seems the lipid accumulation mainly has appear inside the vacuole and less inside others cell organelles. This yield of the lipid productivity in microalgae cultivation is also crucial factor for consideration algal energy system. Lipid production is affected by many environmental condition such us light, pH, temperature, and salinity, nutrients, and CO₂ concentration [8].

This study was designed to screen the indigenous tropical microalgae strain as potential sources of biodiesel feedstock. Screening strategy was carried out base on their survival rate and synthesized of lipid compound. The ability of microalgae to survive in diverse and extreme conditions is reflected in the tremendous diversity and sometimes unusual pattern of cellular lipids obtained from these microalgae[9]..Comprehensive studies are needed in attempt to get information of the microalgal energy.

2.Material and methods

2.1 Collection the samples and isolation of microalgae

Field work for taking samples was done by collecting the waters from seashore, salt farm, pond, lake at Lombok Island (West Nusa Tenggara) and Giam Siak Kecil (Riau Province). Collection was also done by picking up the sample using cotton stick thereafter soaking in the enrichment or selected sterile media. The other method was done by collecting and filtering the water samples, therefore, it's concentrated using vacuum pump with 45 µm filter paper pore size.

Concentrated water containing algae were transferred to enrichment medium. Meanwhile, the filter paper was cut and also soaking into enrichment or selected sterile media directly, following Sekiguchi's protocol [10]. Further procedures for isolation, characterization and screening were done at laboratory. Microalgae were isolated manually by seduce the single algal cell using micropipetting technique under the light inverted microscope with 20x objective lens (Olympus CKX41). The expected algal single cell was transferred several times into micro-plates well for purification, following Sekiguchi's protocol [11].

2.2 Screening for rapid grow microalgal strain

53 isolates obtained from Lombok Island and Riau are choosed for potential rapid grow strain in several medium. Six media were employed for screening, there are AF6 (pH 6.6), Allens (pH 2.5 adjust by adding H₂SO₄), BG-11 (pH 7.4), MKM (brackish medium), f/2 (marine medium) and Zarouk medium (pH 8~9). Ten (10) isolates fast growing were choose for further analyses, namely LIPI11-2-AI005; LIPI11-2-AI007; LIPI11-2-AI010; LIPI11-2-AI015; LIPI11-2-AI018; LIPI11-2-AI019; LIPI11-2-AI020; LIPI11-2-AI021; LIPI11-2-AI023 and LIPI11-2-AI035. Stock cultures were prepared by cultivation at 24 well microplate with 250 μ L initial culture (working volume 2.5 mL) at 25°C for 7-14 days, with light intensity as 6000 lux on 12 hours light and 12 hours dark conditions. Therefore, 5 selected isolates will scale up for lipid producer evaluation. The specific growth rate analyses of five strain was calculate from the math model of linear regression by time (days) and cell density (cell mL⁻¹) as specified by Li et al [12], i.e.

where N_t is cell density at time (t) and N_0 is cell density at the start of the exponential phase (t_0).

2.3 Characterization

Characterization of isolated algal strains was performed by their morphological features using light microscope including following features; cells, chloroplast, cell architecture and reproduction type. 100x objective lens was used for light microscopy observation (Olympus BX53) [13].

2.4 Scale up algal cultures

Selected microalgal strains were pre-cultured in 100 mL sterilized bottle reactor and incubated in 300C with light intensity of 30 Watt/m² in alteration of 12 hours dark and 12 hours light regime. After 5-7 days, when the cell density reaches 0.5 of absorbance (680nm), sample was transferred to the 1L culture vessel. This culture would be the stock for scale up cultivation to 5 L that was transferred gradually. The procedures were similar to the all isolates with their appropriate selected media. For running the experiment, the isolates were cultivated in cylindrical vessel reactor with 5 L working volume during 30 days in triplicate. Observed parametersare growth rate,

proximate biomass and environmental condition such as temperature, pH and light intensity at per three days. The growth rate of each strain was determined by cell turbidity that measured by spectrophotometry (Shimadzu UV-1700) at 680nm, comparing to medium as blank control [14]. The environment factors were measured by portable kit for pH, temperature and light intensity.

2.5 Lipid Productivity and Proximate value

First stage screening of lipid content in the cell was use the fluorescence staining detection by lipophilic Nile red dye performance. Nile red is almost non-fluorescent in water but becomes intensely fluorescent, with a strong red emission when in a lipid-rich environment although the fluorescence emission is environmentally sensitive. Intracellular fat vacuoles, filled with neutral lipids such as cholesterol, lipoproteins and triglycerides will fluoresce green while polar lipids, such as phospholipids, will fluoresce red. All of candidate isolates were subjected to Nile red dye at rate of 1 mL microalgae culture added with 10 μ L of Nile Red in Acetone (500 μ g/ml), thereafter was mixed by vortex for 1 minute. Settling and incubation was done for 10 minutes. Observation was done under fluorescence microscope (Olympus, BX53). The larger appearance of red bright color was selected for further research, following Greenspan et.al. method [15].

Thereafter, while the culture volume reach 1L and more, the lipid content were determined by modified Bligh and Dyer method [16]. The extraction of microalgae biomass was use mixed solvent of methanol:chloroform:water (1:2:1) that found as best solvent. After vigorously vortex the biomass with the solvent resulting three layers of organic, aqueous layers and settling biomass containing the water. The aqueous (contain chloroform) layer were collected and evaporated to dryness, resulting the crude oil (lipid). Gravimetrically the dry crude lipid was storage. Total carbohydrate from microalgae biomasses were determined by phenol-sulphuric acid assay that measured at λ 490 nm [17]. Protein determination was using Bradford assay [18] that measured by spectrophotometric at λ 595 nm.

3. Result and Discussion

Indonesia has promoting for build the microbes culture collection (InaCC) including the microalgae which is expecting launch at 2015/2016 year. Indonesian Institute of Sciences (LIPI) is an entity saved for the tasks of depositary authority and, in front of function evaluation of the microorganisms, extending international implications, continuous accumulation of files and materials. Microorganism are depositing under the Budapest Treaty (role of protection of reference material in production, contracts of collaboration, etc.). Outlook of InaCC is hoping for good depositary of microbes and their properties, serve for industry base on microbes sources and public education. Screening and analysis the isolates function is necessary step for depositary facility in

order to give the initial information of their property. In this studies are focusing on screening the microalgae of InaCC collection isolates for lipid depositor, an energy precursor.

3.1 Collection, isolation and purification of Microalgae

Lombok and Sumatera Islands have choose for taking the raw material samples due to the consideration of geographic condition and position the islands between two ocean and plates that have possibility for collecting floristic algae in those area. Typical tropical Islands Lombok and Giam Siak Kecil have annual climate that divided into two general wet and dry seasons. The wet season usually during October - March of the following year. The total annual rainfall is at 1088 mm and annual humidity averages at 84.6 percent. Dry season starts as early as March - September. Indonesia has approximately 12 hours of daylight every day throughout the year [19](BMKG, 2013). The sampling was taking in early wet season which have intention available for collecting algal resistant to drought and high sunshine exposure. The algae supposed to be synthesize lipid. 53 isolates were isolated from the areas that belong to 24 green algae, 12 cyanobacteria and 17 red algae. Isolation was performed by direct cell picking up under microscope and therefore purified using serial rinse in the wells containing selected medium. The process for purification need a time more than 1 month duration. From 53 isolates were selected 10 isolates that have high lipid synthesize activity, and therefore for scaling up were selected 5 isolates (Table 1). The best five of strains were used for lipid production microalgae were LIPI11-2-AI005, LIPI11-2-AI010, LIPI11-2-AI015, LIPI11-2-AI018, and LIPI11-2-AI019. Microalgae morphologies were observed microscopically using light microscope. All 5 isolates belong to the green algae or chlorophytes that have character as follows; LIPI11-2-AI005 shows that isolate has ovoid cell form with $\pm 6 \mu\text{m}$ in diameter, green and clear chloroplast. LIPI11-2-AI005 belong to genus *Chlorella*. Isolate LIPI11-2-AI010 has long bar cell form with sharp end, $\pm 50 \mu\text{m}$ in length and halter chloroplast, belong to class chlorophyceae. Isolate LIPI11-2-AI015 has green color, round cell form with $\pm 4 \mu\text{m}$, and clear pyrenoid and chloroplast. LIPI11-2-AI015 belong to genus *Chlorella*. Isolate LIPI11-2-AI018 has ovoid cell form, green color, with $\pm 10 \mu\text{m}$ in diameter and round chloroplast form, belong to genus *Coelastrella*. Isolate LIPI11-2-AI019 has ovoid cell form with $\pm 8 \mu\text{m}$ in diameter and U shape of chloroplast, belong to genus *Coelastrella* (Figure 1).

3.2 Microalgae cultivation and Intracellular lipid detection.

Each microalgae can grow rapidly under specific cultivation condition and nutrients. Microalgae growth is directly affected by the availability of nutrients and suitability of the cultivation condition to microalgae isolate. In addition, initial inoculum density also leads better microalgae growth during cultivation time (Li et al, 2011). Five strains of microalgae were cultivated on AF6

medium and incubate at 12:12 (light:dark) condition. Measurements of the microalgae growth were measured as turbidity cells at OD (optical density) of λ 680 nm wavelength. The specific growth rate (μ_{max}) of LIPI11-2-AI005; LIPI11-2-AI010; LIPI11-2-AI015; LIPI11-2-AI018; LIPI11-2-AI019 were 0,065; 0,097; 0,157; 0,088; and 0,062 respectively. The result exhibited LIPI11-2-AI015 have growth rate faster than other selected four (4) microalgae. As is faster growing algae LIPI11-2-AI015 reached the stationer phase earlier than other microalgae isolate.

Detection intracellular lipid were performed by staining of dye Nile red which have been use to evaluate the lipid content of animal cells and microorganism, such as mammalian cell, bacteria, yeasts, zooplankton and microalgae. Nile red is a lipid-soluble florescent dye. Nile red emits a yellow gold fluorescence when dissolved in neutral lipid which is the best substrate to produce biodiesel [20,21].

Fluorescence microscope observation of five microalgae strains shows all microalgae strain emit yellow glow fluorescence, but different intensity. Based on the fluorescence result, LIPI11-2-AI018 gives high and bright intensity of yellow gold. The lowest intensity of Nile red stain is isolate LIPI11-2-AI010 (Figure 2). This result used as the preliminary lipid detection which all the strains have intracellular lipid but the yield of each strain were different one another. Nile red observation can used as early detection of lipid content in microalgae cell [22].

3.3 Proximate value and lipid productivity

Selecting of fast growing, productive strains, optimized for the local climate condition is a fundamental for biodiesel feedstock production from microalgae. In this experiment, proximate value of each strain are observed. The highest biomass production ($\text{g dry weight L}^{-1}$) is $1,221 \pm 0,05$ found on LIPI11-2-AI015 (Table 2). Proximate value observed are protein content, carbohydrate content and lipid content. The highest average protein and carbohydrate production is LIPI11-2-AI005 with value 290.27 ± 10.54 ppm and 250.62 ± 3.74 ppm (Figure 3). Carbohydrate is a main product from photosynthesis and carbon metabolism. Accumulation carbohydrate occurred in plastid or forming cell wall (as cellulose and pectin). Carbohydrate composition especially starch and cellulose are different significantly to each microalgae strains [23]. In addition, protein and lipid also major product of the algal metabolisms.

Lipid content of microalgae cultures in this study ranged from 13,1 % to 30,74% base of their dry weight (Figure 4). Total lipid content of LIPI11-2-AI005; LIPI11-2-AI010; LIPI11-2-AI015; LIPI11-2-AI018 and LIPI11-2-AI019 respectively are 16,57%; 13,1%; 20%; 30,74% and 19,57% per dry weight. The highest total lipid content is $30,74\% \pm 0,006$ which is found in LIPI11-2-AI018. Comparing the current existing data, found some microalgae species can produce high lipid content about 50-80% of dry weight [24,25]. It seems the lipid synthesise has correlation with carbohydrate

synthesize process. Briefly, Acetyl co A that produce from carbohydrate metabolism, could convert to lipid by lipogenesis process by certain situation, therefore tailoring the process to go carbohydrate or lipid direction is possible. On the other hand, abiotic factor such as pH, temperature, and light intensity are suggested influenced the microalgae ability to produce lipid. And mostly, selection of the algal strain with high specific growth rate, and easy cultivation are important factor for lipid synthesis of microalgae [26,27].

4. Conclusion

Five strains of microalgae (LIPI11-2-AI005; LIPI11-2-AI010; LIPI11-2-AI015; LIPI11-2-AI018; LIPI11-2-AI019) were studied for screening the suitable microalgae strain for lipid production as potential biodiesel feedstock. The specific growth of LIPI11-2-AI005; LIPI11-2-AI010; LIPI11-2-AI015; LIPI11-2-AI018; LIPI11-2-AI019 were 0,065; 0,097; 0,157; 0,088; and 0,062 respectively. Isolate LIPI11-2-AI015 has highest specific growth about 0,157 and the highest biomass production. Total lipid content of LIPI11-2-AI005; LIPI11-2-AI010; LIPI11-2-AI015; LIPI11-2-AI018 and LIPI11-2-AI019 respectively are 16,57%; 13,1%; 20%; 30,74% and 19,57% base on the dry weight cells. This result show that LIPI11-2-AI018, potentially used as lipid production for biodiesel feedstock.

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Table sheet

Table 1. Qualitative data of 10 strains microalgae growth in 6 medium for screening the rapid growth strains.

Isolates Code	Media					
	Allens	AF6	BG-11	ZM	MKM	F/2
LIP111-2-AI005	-	+++	++	-	+	-
LIP111-2-AI007	-	+	+	-	-	-
LIP111-2-AI010	-	+++	+	-	+	-
LIP111-2-AI015	-	+++	++	-	++	+
LIP111-2-AI018	-	+++	++	-	++	+
LIP111-2-AI019	-	+++	++	-	+	-
LIP111-2-AI020	-	+	++	-	+	-
LIP111-2-AI021	-	+	+	-	+	-
LIP111-2-AI023	-	+	+	-	-	-
LIP111-2-AI035	-	+	+	-	+	+

Note : +++ Rapid growth

+ Less growth

++ Growth

- Not Growth

Table 2. Dry weight (g/L) of five microalgae strains

Time	Dry biomass (g/L)				
Day	LIPI11-2-AI005	LIPI11-2-AI010	LIPI11-2-AI015	LIPI11-2-AI018	LIPI11-2-AI019
0	0.232 $\pm$ 0.06	0.203 $\pm$ 0.07	0.126 $\pm$ 0.11	0.054 $\pm$ 0.13	0.050 $\pm$ 0.14
3	0.329 $\pm$ 0.02	0.245 $\pm$ 0.03	0.154 $\pm$ 0.09	0.064 $\pm$ 0.11	0.046 $\pm$ 0.01
9	0.379 $\pm$ 0.01	0.304 $\pm$ 0.14	0.198 $\pm$ 0.07	0.109 $\pm$ 0.06	0.212 $\pm$ 0.10
15	0.416 $\pm$ 0.03	0.334 $\pm$ 0.02	0.209 $\pm$ 0.03	0.142 $\pm$ 0.15	0.401 $\pm$ 0.07
21	0.471 $\pm$ 0.07	0.304 $\pm$ 0.05	0.627 $\pm$ 0.01	0.308 $\pm$ 0.14	0.532 $\pm$ 0.09
27	0.510 $\pm$ 0.07	0.483 $\pm$ 0.01	1.221 $\pm$ 0.02	0.473 $\pm$ 0.01	0.787 $\pm$ 0.05
30	0.513 $\pm$ 0.08	0.489 $\pm$ 0.01	1.105 $\pm$ 0.03	0.535 $\pm$ 0.09	1.119 $\pm$ 0.04

FIGURE SHEET

A

B

C

D

E

Figure 1. Microscopic features of (A) LIPI11-2-AI005; (B) LIPI11-2-AI010; (C) LIPI11-2-AI015; (D) LIPI11-2-AI018; and (E) LIPI11-2-AI019

Figure 2. Nile red observation on fluorescence microscope respectively (A) LIPI11-2-AI005; LIPI11-2-AI010; LIPI11-2-AI015; LIPI11-2-AI018 and LIPI11-2-AI019

Figure 3. Growth curves of five microalgae strains cultivated in a the experiment and 12:12 light:dark condition for 30 days

Figure 4. Lipid content during cultivation time