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Characterization and Identification of Tropical Lipid-Producing Microalgae Isolated from Abandoned Kaolin and Tin Mine Site in Belitung Island, Indonesia

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Abstract: Recently, microalgae have attracted world attention to produce fatty acids as an alternative source of new and renewable energy. The fatty acids characteristics in these microorganisms are varied depending on the strain and cultivation condition. Therefore, it is necessary to determine the potential strain with high lipid content for bioenergy purposes. This research aimed to characterize two tropical lipid-producing microalgae from the abandoned kaolin and tin mine sites in Belitung island. These cultures were identified as *Chlamydomonas* sp.1 and sp.2 based on microscopic observation and 18s rDNA sequence analysis. The phylogenetic study showed that sp.1 was related to *Chlamydomonas moewusii* while sp.2 formed a clade with *Chlamydomonas applanate*. Furthermore, sp.2 reached a higher biomass concentration (6 mg/mL) with lipid content about 39% ± 1.4 w/w, than sp.1 (2 mg/mL with about 32% ± 2 w/w) lipid content. The GC analysis showed that both strains contained a high amount of C16 to C18 fatty acid, which is suitable for biodiesel production.

Keywords: *Chlamydomonas*, fatty acid, biodiesel, Belitung Island.

從印度尼西亞勿里洞島廢棄高嶺土和錫礦場分離的熱帶產脂微藻的表徵和鑑定

摘要：近來，微藻以生產脂肪酸作為新能源和可再生能源的替代來源引起了全世界的關注。這些微生物中的脂肪酸特性因菌株和培養條件而異。因此，有必要確定具有高脂質含量的潛在菌株用於生物能源目的。本研究旨在表徵來自勿里洞島廢棄高嶺土和錫礦場的兩種熱帶產脂質微藻。根據顯微鏡觀察和 18s 重組脫氧核糖核酸 序列分析，這些培養物被鑑定為衣藻物種 1 和物種 2。系統發育研究表明物種 1 與莫武衣藻相關，而物種 2 與扁平衣藻形成一個進化枝。此外，物種 2 達到更高的生物量濃度 (6 毫克/毫升)，脂質含量約為 39% ± 1.4 重量比，比物種 1（2 毫克/毫升，約 32% ± 2 重量比）脂質含量更高內容。氣相色譜法分析表明，這兩種菌株都含有大量的碳 16 至碳 18 脂肪酸，適用於生物柴油生產。

关键词：衣藻、脂肪酸、生物柴油、勿里洞島。

1. Introduction

Studies in the cell and molecular biology of microalgae have accelerated, primarily due to their potential as a suitable feedstock for biofuel production [1]. Biodiesel is a mixture of fatty acid methyl ester (FAME) produced through transesterification [2]. Conventionally, it was usually produced from plants oils and animal fats, but not from microalgae. On the other hand, microalgae are known for producing lipids in high amounts per dry weight basis. Therefore, several parties are attempting to produce and commercialize biodiesel from microalgal lipid [3].

These lipids have gained attention in recent decades due to their potential applications in many areas, including energy, nutraceutical, and pharmaceutical purposes [1]. Microalgae are known to synthesize a large number of fatty acids, including saturated (SFA), mono-unsaturated (MUFA), and poly-unsaturated fatty acids (PUFA). Microalgae can accumulate lipids more than 50% of their dry weight under stress conditions [4]. Also, microalgal biomass has the potential to be used as feedstock for biodiesel production [2]. However, not all lipids from these microorganisms are satisfactory for making biodiesel [3]. Therefore, the characterization and identification of lipids in

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microalgal cells are necessary to ensure their suitability and feasibility for biodiesel-production purposes.

The overall production cost is still relatively high in the current situation, thereby limiting its mass-scale application. Therefore, a significant effort needs to be made to reduce the production cost [5]. There are many approaches to solving such issues, including selecting an excellent strain, low-cost harvesting of microalgal biomass, improving the biomass and lipid productivities, and maximizing the values of by-products [6].

Belitung is the largest tin producer in Indonesia. Kaolin and tin in Belitung island were mined for a long time ago. However, ex-mining remained amount of holes. The accumulation of rainwater formed kaolin lake. Currently, a well-organized kaolin lake has been utilized for ecotourism, while the abandoned one naturally becomes a habitat for aquatic organisms, including microalgae. Acidic rocks are found abundantly in this area. Therefore, microalgae isolated from this habitat are well-adapted to the acidic environment. Accordingly, this study aims to characterize and identify lipid produced by microalgae isolated from Belitung island. In this study, two potential lipid-producing strains were isolated from Belitung island, Indonesia. They were identified under the genera of Chlamydomonas and could accumulate lipid at a significant percentage per dry weight. The fatty acid methyl ester profile of the microalgal lipid was also analyzed.

2. Materials and Methods

2.1. Microalgae Sampling and Isolation

A water sample was collected from a freshwater pond of the former kaolin and tin mine, respectively, in Belitung, located on the east coast of Sumatera Island. The collected sample's microalgae were then isolated using a direct isolated technique under an inverted light microscope (Olympus CKX41 SF) using Sekiguchi [7] method with modification. Subsequently, two single isolated strains were incubated in a 24-well plate and enriched with AF6 medium. Both were collected with codes BLT-0502 and BLT-0603, respectively. The formed cell colony was transferred into a 10 mL fresh medium and kept as a stock culture. In addition, the algae were cultivated in a single batch in 100 mL working volume for further identification.

2.2. Microalgae Identification

The morphological observation was carried out using an Olympus BX53 microscope with magnification 1000 times. Meanwhile, Scanning Electron Microscopy preparation was conducted using Goldstein's method [8]. That was initiated by fixation using glutaraldehyde 2%, tannin acid 2%, and cacodylate buffer. The process was followed by post-fixation using osmium tetroxide (OsO_4) and sample dehydration with alcohol series. The specimens were observed under a JEOL JSM 5310 LV SEM.

Molecular identification was performed by extracting the total DNA from both strains using the Vivantis GF-1 Plant DNA Extraction Kit. Furthermore, nucleotide sequences of nuclear-encoded small subunit rRNA dene (18 rDNA) were used for molecular phylogenetic analyses with the previously described primer sets and conditions [9]. The sequences of 25 operational taxonomic units (OTUs) were aligned based on Nakada et al. [10] and checked using BioEdit [11]. Also, Bayesian Inference (BI) was performed using MrBayes 3.2.6 with 1,000,000 generations of Marcov Chain Monte Carlo iteration (MCMC) and discarding the first 25% as burn-in. The best substitutional model GTR+I+G fis 18S rDNA was selected by PAUP* 4.0b10 and MrModeltest 2.3. Meanwhile, maximum likelihood (ML) analysis was performed using IQ Tree 1.6.9 with automatic ModelFinder and an ultrafast bootstrap analysis based on 1,000 replications. Finally, the phylogenetic tree data were visualized using FigTree 1.4.3 [11].

2.3. Growth Condition

The isolated microalgae were maintained at 100 mL working volume in the AF6 medium with modification. Furthermore, the composition of AF6 medium per liter is 140 mg $NaNO_3$, 22 mg NH_4NO_3, 30 $MgSO_4$•7H2O, 10 mg KH_2PO_4, 5 mg K_2HPO_4, 10 mg $CaCl_2$•$2H_2O$, 10 mg $CaCO_3$, 2 mg Fe-Citrate, 2 mg Citric Acid, and 5 mL P IV metal solutions. The culture was inoculated to 5 L working volume with continuous aeration and cultured at semi-outdoor conditions. In addition, the microalgae growth was determined by measuring optical density at 680 nm every two days.

2.4. Biomass Production

Biomass production was determined every seven days up to 28 days cultivation and was harvested by centrifugation at 6,000 rpm for 5 min. Subsequently, the washed pellet was dried at 60ºC and provided as dry weight ($g.L^{-1}$).

2.5. Total Lipid and Fatty Acid Methyl Ester Profiles

Lipid extraction was conducted using a modified method of Rychebosch et al. [12]. A mixed solution of chloroform and methanol (1:1, volume) was used as a solvent for microalgae lipid extraction. Furthermore, 6 mL of solvent was added to 100 mg microalgae biomass and homogenized using vortex for 30 sec. Water was added to the mix solution for 1/3 part of the total volume and homogenized for 30 sec. the mixture was then centrifuged at 2,000 rpm for 10 min. Subsequently, the aqueous layer was removed, and the solvent layer was transferred into the clear tube. Lipid re-extraction was repeated until the remaining microalgae biomass turned colorless. Also, the solvent

was removed by evaporation in the open air, and the lipid content was gravimetrically determined.

Fatty acids obtained from the extraction were subjected to Gas Chromatography-Mass Spectrometry (GC-MS). Meanwhile, sample preparation was carried out by weighing 0.05 g of the sample and dissolving it with 2.0 mL NaOH in 0.5 M methanol, then heating at 80ºC for 20 min. After adding 2 ml of BF_3 solution in methanol, the sample was reheated at 80ºC for 20 mins. Also, 2 mL of saturated NaCl and 2 mL of hexane were added to the mixture. The sample (2 µl) was then injected into the GC silica gel column. Meanwhile, the GC was conducted using H_2 and N_2 gases as the solvents with an initial temperature of 150ºC and an injector temperature of 200ºC. In addition, a mass spectrum detector was used to measure samples at 250ºC.

3. Result

3.1. Microalgae Identification

Morphological observation showed that both isolates had two flagella of the same length, stigma, papilla at the base, and a real pyrenoid ring (Fig. 1).

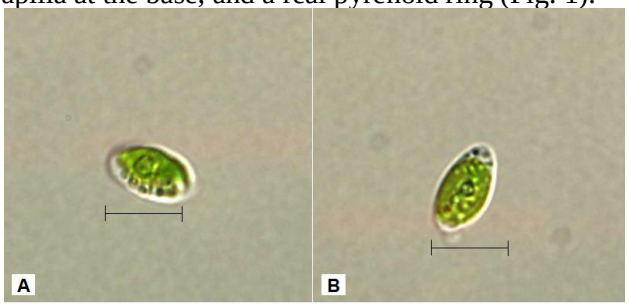

Fig. 1 Microscopic overview of isolated microalgae: A. *Chlamydomonas* sp. 1; B. *Chlamydomonas* sp. 2; scale bar 10 µm

The morphological features found in these two isolates refer to the genus *Chlamydomonas*. The morphological shape of sp.1 (BLT-0502) appeared more rounded than sp.2 (BLT-0603). Based on the habitat and sample location, sp.1 originated from a lake that formed the former kaolin mining. In contrast, sp.2 originated from a tin mining lake. The acidity level of the ex-kaolin lake was 5.78, while ex-tin was 3.49. Furthermore, the SEM figure (Fig. 2) of the isolated microalgae showed that *Chlamydomonas* sp.1 has more round papilla and is shorter than sp.2.

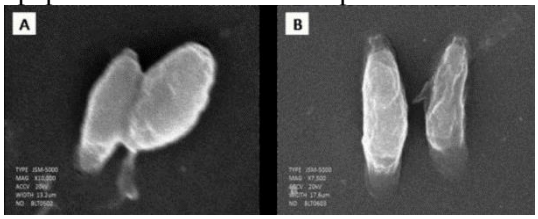

Fig. 2 SEM image of *Chlamydomonas* sp. 1 (A) and *Chlamydomonas* sp. 2 (B) showed different cell shapes and papilla

In the molecular phylogenetic tree based on 18s rDNA (Fig. 1), it was shown that sp.1 and sp.2 strain belong to Volcocales (Chlophyceae, Chlorophyta). In fact, sp.1 formed a robust clade posterior probability (PP) = 1.0, bootstrap proportion (BP) = 94%] in the clade *Moewusinia*, while sp.2 formed a moderate or low supported value (PP = 0.76, BPs = 75%) in the clade *Polytominia* (Fig. 1). All these clades were determined based on PhyloCode [10]. *Chlamydomonas* sp.1 was related to *C. moewusii*, and they formed a robust clade (PP = 1.0, BPs = 90-100%). Besides, this strain formed a group with acidophilic algae (*C. acidophil*us) that may have the same adaptability to grow well in acidic conditions. Therefore, based on the phylogenetic tree (Fig. 3), *Chlamydomonas* sp.2 formed a clade with acid-tolerant algae, *C. applanate* [13].

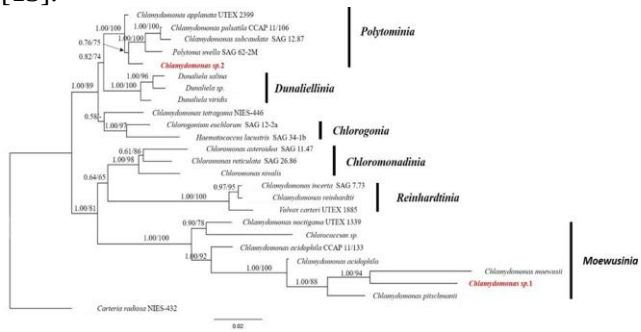

Fig. 3 Bayesian phylogenetic tree of Chlamydomonas sp. 1 and sp. 2 based on 18S rDNA. Numbers at nodes indicate posterior probabilities (≥ 0.50, top left) and bootstrap proportions

3.2. Microalgae Growth

The growth of sp.1 and sp.2 was monitored using a spectrophotometer at 689 nm. The cultivation was carried out for 21 days (Fig. 4), and sp.2 has a higher growth rate than sp.1. Also, the biomass production of both strains was evaluated (Fig. 4). Figure 4 showed that productions were linear to the microalgae growth rate. In addition, the production of sp.2 is three times higher than sp.1. Based on the yield at the end of cultivation (day 21st), sp.1 produced 10 g dry biomass in 5 L culture of 2 mg.mL^{-1}. In comparison, sp. 2 30 g dry biomass in 5 L culture or 6 mg.mL^{-1}.

3.3. Total Lipid and FAME Profiles

The total lipid extracted from sp.1 and sp.2 was 32 ± 2% and 32 ± 1.4%, respectively. Meanwhile, the GC-MS analysis result (Table 1) showed that both *Chlamydomonas* strains contained several fatty acids in the form of methyl ester. The sp.1 contained palmitic, oleic, and alpha-linoleic acids, while sp.2 contained palmitic, stearic, oleic, linoleic, and alpha-linoleic acids.

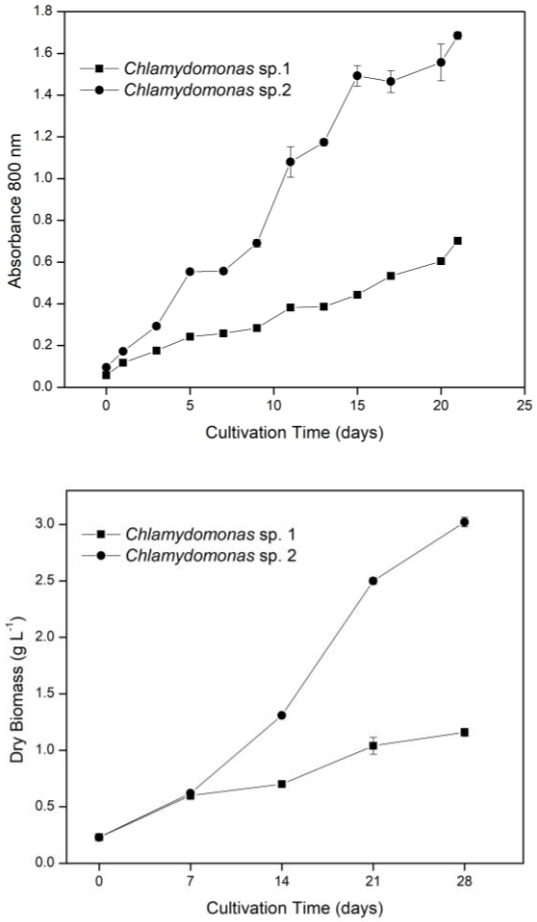

Fig. 4 Growth curve and dry biomass production of *Chlamydomonas* sp. 1 and *Chlamydomonas* sp. 2

Chlamydomonas sp.1 had higher C18:3 fatty acid content than others found in the sample, whereas sp.2, the C18:2 content was higher than other fatty acids. Unsaturated fatty acid produced by sp.1 was 36.3% of the area, and sp.2 was 40.35%. Both strains have higher unsaturated fatty acids content than saturated.

4. Discussion

Morphological observation using light microscope overview (Fig. 2), SEM (Fig. 3), the result of molecular identification, supported by different biogeographic conditions, showed that sp.1 and sp.2 are different types. *Chlamydomonas* is an abundant polyphyletic genera [10, 14-18]. The strains examined in this study were distantly related to the authentic strain of *Chlamydomonas* and located in the separated clade of *C. reinhardtii*. Therefore, a further taxonomical study using polyphasic approaches can lead these strains to new species or combinations.

Table 1 Fatty acid profile of *Chlamydomonas* sp. 1 (BLT0502) and *Chlamidomonas* sp. 2 (BLT0603)

Fatty acids	***Chlamydomonas* sp. 1**	***Chlamydomonas* sp. 2**
C16:0	18.16	11.42
C16:3	-	11.81
C18:1	16.13	-
C18:2	-	16.8
C18:3	20.17	11.74

Chlamydomonas is a genus chlorophytes phylum in order of Volvocales and the family Chlamydomonadaceae. These microorganisms are unicellular microalgae with a pair of flagella of the same length, a cup-like chloroplast located at the base, a pyrenoid in the middle, and generally stigma [19]. Amoah et al. [20] and Meng et al. [21] showed that *Chlamydomonas* had been widely used in various applications, including lipid providers in biofuel production.

The ability of both isolates to be cultivated outdoors indicates that both *Chlamydomonas* have the potential to cultivate on a larger scale. Also, less energy is needed for outdoor cultivation because the laboratory's optimized cultivation system is not always following the condition in the field.

The lipid content of microalgae vary, generally between 20-50% [3]. *Chlamydomonas* sp.1 has a 32 ± 2% lipid content, while sp.2 has 39 ± 1.4%. In that range, both isolates were classified as microalgae with moderate lipid [22]. The content of the two *Chlamydomonas* was different, and the diversity of fatty acid compositions in the two microalgae. Meanwhile, several factors influence lipid accumulation and fatty acid composition, such as nutrition, temperature, light intensity, growth phase, and physiological mechanisms [23]. This study ignored nutritional factors, temperature, and intensity as both species have the same treatment for these three factors. Therefore, differences in the lipid content are more influenced by the growth phase and physiological characteristics of each microalga.

Meng et al. [21] showed that *Chlamydomonas reinhardtii* has a total lipid accumulation of 66.7% of its dry weight, making it a candidate for biodiesel production. Also, growth rates and high lipid content are the two main parameters in determining the rate of lipid and biodiesel productivity [24]. A high rate will generate a biodiesel production process that is more techno-economically feasible. The results on sp.1 and sp.2 showed that these two microalgae are potential candidates for biodiesel production with a total lipid level of over 25% and a relatively fast growth rate.

5. Conclusion

Two tropical microalgae from abandoned kaolin and tin mine sites were identified belonging to Chlamydomonas genera. The phylogenetic study revealed that these microalgae are closely related to *C. moewusii* and *C. applanata*, respectively. Besides, the fatty acid profiles indicated that these microalgae were potentially used as biodiesel sources with total lipid content of 32% and 39%, respectively. A more detailed taxonomical study using a polyphasic approach should be conducted for species-level determination by involving another gene such as chloroplast and internal transcribed spacer (ITS) gene and comparing the ITS secondary structure. Additionally, further research on

biomass and lipid productivity enhancement needs to be performed from a large-scale perspective.

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