Characterization of microalgae from lowlands in South Sumatera (Indonesia) as a potential source for biodiesel production

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Abstract. Biodiesel production technology from microalgae is widely considered as a potential and efficient method. This research was conducted to characterize microalgal species isolated from some lowlands in South Sumatera and investigated their potential for biodiesel production. Five microalgal culture isolate were selected and identified as strains of Chlorella sp PKB, Chlorella sp PPP, Chlorella sp SB, Crucigenia quadrata PTA and Scenedesmus sudetica PTA. These isolates were determined the growth rates, biomass and total lipid content. Under similar environmental condition, dried weight biomass of isolate were 1.30; 0.88; 0.74; 0.5; 0.26 respectively. Total lipid content of isolates were 35.2; 28.5; 25.6; 17.8; 8.4 respectively. Chlorella sp PKB showed the highest lipid content comparison others, whereas Chlorella sp SB showed the highest dried biomass. The results suggest that only Chlorella sp PKB can be a possible candidate species for biodiesel production.

1. Introduction
Currently, energy generated from fossils fuels is about 90% and only 10% of energy is produced from renewable energy sources [1]. Based on the ever-increasing energy demand it is predicted that the conventional oil reserves will vanish after 2050 [2]. Therefore, there is an urgent need to develop alternate energy sources towards the mitigation of the energy crisis. Biodiesel production technology from microalgae is widely considered as a potential and efficient method. Microalgae are photosynthetic microorganisms that are able to convert sunlight, water and carbon dioxide into biomass [3]. Microalgal biomass contained important ingredients that are useful, such as protein, carbohydrates, lipids and nucleic acids [4]. Many microalgal species contain high levels of lipids, even reaching 90% [5]. The lipid content in these microalgae can be further converted into biodiesel [3].

Utilization of microalgae as a source of biodiesel have many advantages compared to other raw material sources. Microalgae have high photosynthetic efficiency, high biomass productivity, higher growth rates than plants, high CO₂ fixation and O₂ production, can be grown in climates, water and various chemical compositions, including unsuitable land used for agriculture. In addition, the growth of microalgae can reduce nutrients and other contaminants from various sources of waste water, thus benefiting the environment and savings for bioremediation of waste water [6,7].
Screening of microalgae species as a key step toward the commercialization of microalgae as a producer of biodiesel still plays an important role because it is estimated that there are more than 50 thousand species of microalgae, but only about 30 thousand species have been studied and analyzed [8]. The ability of microalgae to produce large quantities of lipids and oils is species / specific strains rather than specific genera [9]. Four microbial strains of *Botryococcus* sp isolated from freshwater lakes and ponds in Thailand showed different lipid levels when grown in a nitrogen-rich medium and lack of nitrogen [10].

Some of the expected microalgae characteristics of high-lipid microalgae, high growth rate, are easily harvested and able to adapt to local environmental conditions [11]. Local microalgae species are expected to have a competitive advantage in local geographic, climate and ecological conditions. This characteristic plays an important role in the success of large-scale production. Water samples from aquatic environments experiencing fluctuations and / or erratic conditions provide a greater opportunity to isolate the microalgae that accumulate high lipids. This condition is likely to provide strong and opportunistic algae (rapid growth) with superior survival (eg, accumulation of lipid deposits) [12].

Our previous research was revealed the lowlands in South Sumatera are abundance with microalgae mainly Bacillariophyceae and Chlorophyceae [13]. Observation under fluorescens microscope and nile red staining against microalgae isolates from South Sumatra lowlands showed microalgae with lipid deposits, namely *Cyclotella atomus*, *Chlorella* sp and *Ankistrodesmus gracilis* [14]. This research was conducted to characterize microalgae isolated from some lowland areas in South Sumatra to investigated their potential use as biodiesel feedstock.

2. Methods

2.1. Sampling and isolation

Water samples for microalgae isolation were collected from 5 different sites of lowland area in South Sumatera by using plankton net. The sampling location details are shown in Table 1. Ten ml of water samples were aseptically transferred to 250 ml erlenmeyer containing 100 ml of sterilized BG-11 medium, then incubated in room temperature with a white fluorescence light intensity of 30 μmol photon / m2 / sec (25 watt TL lamp) and continuous illumination period 24 hours for 4 weeks [15] with modification. The growth of microalgae in medium were characterized by changing of medium color to green or brown, and were confrmed by observations under microscope. The cultures were subsequently subcultured into a new sterile BG-11 medium to be isolated.

Microalgae isolation was conducted by streak plating method [16]. One ml of microalgae culture solution was streaked on petri plate containing BG-11 solidified with 1.5% (w / v) bacteriological agar. Then Petri dishes were incubated in room temperature with continuous illumination period (24 hours) for 3 weeks. The growing colonies were isolated and transferred to a new sterile liquid BG-11 medium then incubated. The purity of the culture was confirmed by repeated plating and by regular observation under a microscope. One isolate was selected from each sampling site consider to their purity and growing in medium. The selected isolates were used to further analyze.
Table 1. Sampling location.

| No | Coordinate point | Administrative area |
|----|------------------|---------------------|
| 1  | S 03°15.454’N E 104°40.080’ | Desa Sakatiga Seberang, Kecamatan Inderalaya (Ogan Ilir) |
| 2  | S 03°05.523’N E 104°43.673’ | Desa Ibul Besar I, Kecamatan Pemulutan (Ogan Ilir) |
| 3  | S 03°05.525’S E 104°43.674’ | Pelabuhan Tanjung Api-Api, Desa Rimau Sungsang, kecamatan Banyuasin II (Banyuasin) |
| 4  | S 03°05.524’S E 104°43.673’ | Desa Sri Tiga, Kecamatan Muara Telang (Banyuasin) |
| 5  | S 03°05.383’S E 104°43.522’ | Desa Karang Anyar, kecamatan Muara Telang (Banyuasin) |

2.2. Determination of growth curve and biomass

Ten ml of microalgal culture suspension were cultivated in 250 ml Erlenmeyer containing of 100 ml of BG-11 medium, then the erlenmeyer were incubated in room temperature with white fluorescence light intensity of 30 μmol photon / m2 / sec and continuous illumination period (24 h) until stationary phase or maximum for 3 weeks. Microalgae growth was calculated in 3-day intervals using a spectrophotometer at 680 nm OD and dry weight.

Microalgae cells were harvested after 3 weeks to calculated the dry weight. Microalgae cells were harvested by using centrifugation speed of 3500 - 5000 rpm then washed twice with distilled water. Microalgae pellets were dried overnight at 105°C [8].

2.3. Extraction of total lipid content

Total lipid content of dried microalgal cells biomass was extracted using Bligh and Dyer method modified by Hidayat [17]. This method extracts lipids from microalgal cells by using a mixture of methanol, chloroform, and water. The culture sample is centrifuged at 3,500 rpm for 10 minutes in a large (200 ml) plastic centrifuge tube. The pelleted cells along with 35ml of supernatant are then transferred to a glass Centrifuge tube (50 ml) to be re-centrifuged at 3,500 rpm for 10 minutes. The supernatant is removed by pipette. The pellet is then resuspended with 4ml of DH2O, then 10ml of methanol and 5ml of chloroform are added, resulting in a 10:5:4 ratio of methanol: chloroform:water. At this ratio, all solvents are miscible and form one layer. After overnight extraction on a shaker table, 5ml of water and 5ml of chloroform are added which results in a 10:10:9 ratio of methanol:chloroform:water. Tubes are centrifuged for 10 minutes at 3,500 rpm. At this solvent ratio, two layers are formed, a water methanol upper layer and a chloroform lower layer. The chloroform lower layer which contains the extracted lipids is then removed by Pasteur pipette and placed into a pre-weighed vial. After the first extraction, 10ml of additional chloroform is added to conduct a second extraction. The additional 10ml of chloroform again results in a 10:10:9 methanol:chloroform:water ratio and two layers are formed. The tube is centrifuged at 3,500 rpm for 10 minutes, and the lower chloroform layer is removed by Pasteur pipette and placed into another pre-weighed vial. The chloroform is evaporated by heating in a 55°C water bath under a constant stream of nitrogen gas. After 1 hour in a 105°C oven, vials are weighed again. The weight difference represents weight of lipids extracted from the culture sample. Percentage of lipid content can be determined by measuring the dry weight of the culture sample at the same time as the lipid analysis. The mass of cells used for the lipid analysis can be determined by multiplying the dry weight by the volume of culture used for the lipid extraction.
sample. The weight of lipids extracted can then be divided by the mass of cells extracted to determine the percent lipid content.

3. Results and discussion

3.1. Isolation and identification of microalgae

This research had isolated five the fastest grow in medium and easy to isolated of green microalgae. The green microalgae (Chlorophyceae) was choosen as this class contains high neutral lipids content, which reaches 70% of the total lipids in the cell [18], abundant in nature, easily isolated and grows rapidly in laboratory than other groups [9]. These isolates were identified as Chlorella sp PKB, Chlorella sp PPP, Chlorella sp SB, Crucigenia quadrata PTA and Scenedesmus sp RLI (Table 2).

| No | Microalgae         | Family       | Habitat characteristic of sampling location |
|----|--------------------|--------------|---------------------------------------------|
| 1  | Chlorella sp PKB   | Oocystaceae  | Brackish                                    |
| 2  | Chlorella sp PPP   | Oocystaceae  | Freshwater                                  |
| 3  | Chlorella sp SB    | Oocystaceae  | Brackish                                    |
| 4  | Crucigenia quadrata| Scenedesmaceae| Saline                                      |
| 5  | PTA                | Scenedesmaceae| Freshwater                                  |
|    | Scenedesmus sp RLI|              |                                             |

Table 2. Description of microalgal isolates.

Microscopic observation of microalgae isolates morphology showed Chlorella sp cells are round, light green or dark green, cell diameters are 2 - 10 μm with 1 chloroplast (Fig. 1A - 1C). Crucigenia quadrata cells are oval or triangular shape, light green colour, possess 1 chloroplast, cell length are 3-15 μm, 2 - 12 μm width, senobia diameter are 5 - 31 μm, four cells are bound and arranged to form a colony with an empty space in the center of the colony (Fig. 1D - 1E). Scenedesmus sp cells are oval, green colour, 5 - 30 μm long, 2-10 μm width and four cells are arranged in parallel to form a colony. In general, Scenedesmus cells form 1 colony but sometimes 4-16 cells combine to form a long linear line (Fig. 1F).

![Microscopic morphological observation of isolates](image)

Figure 1. Microscopic morphological observation of isolates (A) Chlorella sp PKB, (B) Chlorella sp PPP, (C) Chlorella SB, (D-E) Crucigenia quadrata PTA, (F) Scenedesmus sp RLI.
Chlorella sp species was always found in all water samples. It revealed that Chlorella sp is exist in variety habitats, both in freshwater or brackish water, and able to grow well in BG-11 medium. Culture medium is a factor that plays an important role in microalgae culture. In addition to the selection of the right species, other fundamental factors that determine growth rates, product yields and biochemical composition of microalgae are medium selection [19].

3.2. Growth curve of microalgae isolate
Five isolates showed different growth curve when cultured in BG-11 media for 14 days (Fig. 2). The first phase is induction or lag phase which little increase in growth rate as cell absorption (nm) occurs. In the second phase the growth rate increases exponentially this is depends on many factors such as algal species or type, light intensity and medium temperature. The third phase is the constant phase in which the cell density become relatively constant. Finally declining growth rate when the cell divisions decreases because some factors become influential to growth rate such factors as NPK nutrients concentration, pH, dissolved CO2, light and contamination risk.

Isolated Chlorella strains from different habitats also show different growth curves. Chlorella sp PKB, Chlorella sp PPP and Chlorella sp SB were able to adapt quickly in the medium, therefore they experience a fairly short lag phase. Chlorella sp PPP and Chlorella sp PKB started entering the exponential phase on the 10th day, while Chlorella sp SB continued to grow until the end of the culture period (day 14). Scenedesmus sp RLI and Crucigenia sp PTA undergo longer phase of adaptation in media. This is due to Crucigenia sp PTA was isolated from saline water habitat, therefore it is suspected that Crucigenia sp PTA was not suitable to be grown in BG-11 media.

![Figure 2. Growth curve of microalgae isolate after cultured in BG-11 media for 14 days.](image)

Growth curve (Fig. 2) revealed that chlorella sp was grow faster compared to Scenedesmus sp and Crucigenia sp. This can be possible due to size of Chlorella sp that smaller than Scenedesmus sp and Crucigenia sp, therefore easier to divide exponentially number of cells. The larger cell size requires greater energy to increase the number of cells. Allometric relationship between microalgae cell growth rate with cell size is an acceptable reason to explain the difference in maximum growth rate among various species. Bouterfas et al. studied the effects of radiation and irradiation of the cell growth rate showed that the smaller microalgae species (Selenastrum minutum) grows faster than greater (Cosmarium subprotumidum) [20].

3.3. Biomass and total lipid content
Cultivation was conducted to collected as much biomass. Media composition will affect the biomass and lipid content produced. Microalgae biomass production and total lipid content are an essential
indicator for biodiesel production at a specific lipid content. A Microalgae species needs about 30% lipid of dry weight to be a possible candidate for biodiesel production [21]. Microalgae dry biomass and total lipid content of isolated microalgae were showed in Table 3.

Table 3. Biomass and total lipid content.

| Parameters               | Microalgae       |
|--------------------------|------------------|
|                         | Chlorella sp PKB | Chlorella sp PPP | Chlorella sp SB | Scenedesmus sp RLI | Crucigenia sp PTA |
| Dry biomass weight (g L⁻¹) | 0,74             | 0,88             | 1,30             | 0,5                | 0,26              |
| Total lipid content (%)  | 35,2             | 28,5             | 25,6             | 17,8               | 8,4               |

The highest total lipid contain was found in Chlorella sp PKB, while the lowest one was in Crucigenia quadrata PTA. The highest dry weight biomass was found in Chlorella sp SB, while Crucigenia sp PTA was the lowest yield for both biomass and lipid content.

In general, microalgal biomass is inversely proportional to lipid levels. If cell biomass is high, lipid levels are low. The growth curve (Fig. 2) shows that at the end of the incubation period (day 14), Chlorella sp SB was still in an exponential growth phase, that is a phase of cells dividing exponentially rapidly, resulting in high biomass. Whereas Chlorella sp PPP and Chlorella sp PKB were in the stationary phase, that is a condition when nutrients (mainly nitrogen) have started to decrease, caused growth becomes slow. Under this nitrogen deficiency conditions, algal cells often accumulate excess carbon metabolites in the form of lipids. As stated by Amaro et al. [5] and Widjaja et al. [22] that lack of nitrogen (or phosphate) will limit cell growth but increase lipid levels [5]. Bellou et al. reported that while nutrients are still available in culture media, Chlorella vulgaris synthesizes proteins used for cell division and growth [23]. In optimum conditions, microalgae do more protein synthesis for DNA synthesis which is then used as material in cell division process [24]. When the nutrients in the culture media are running out then the microalgae will accumulate more photosynthetic results in form lipid. In media with low nitrogen concentrations, the lipid content will be higher. For example, lipid content in Chlorella vulgaris grown under suitable conditions are 14-30% (w / wDW) while under nutrient deficiency conditions are 70%, 63% in Chlorella emersonii and 56% in Chlorella minutissima [5].

4. Conclusion

This research revealed that Chlorella sp PKB has the highest total lipid content and above 30% (35,2%), as a standard biodiesel feedstock. It can be concluded that Chlorella sp PKB can be a possible candidate species for biodiesel production.

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