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Evaluation on the growth of *Nannochloropsis sp.* from Lampung area in the biogas effluent of tapioca industry

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Abstract. Evaluation on the growth of *Nannochloropsis sp.* in the biogas effluent of tapioca industry (MEBIT) has been conducted. The *Nannochloropsis sp.* that isolated from marine biota in Lampung area was acclimated in the 1, 3, and 6% MEBIT. The evaluation of *Nannochloropsis sp.* was using cell density at 750 nm, chlorophyll a concentration and biomass yields. The *Nannochloropsis sp.* was growth well in the 6% (v/v) MEBIT based on the color of the culture. The OD of *Nannochloropsis sp.* on MEBIT obtained at 750 nm is about 0.14 to 1.48. The chlorophyll a concentration is about 1.27 to 13.51 mg / mL, whereas the concentration of the biomass is 0.311 g/L with a productivity of 0.019 gL⁻¹ d⁻¹, it is higher than when it cultivates in the standard media (BG 11). The protein content in MEBIT is 30.65%, it is lower than in the BG 11 growth (36.41%) of the dry biomass.

1. Introduction

Lampung is the province with a large production of cassava. The amount of cassava produce during 2016 reached 7,387,084 tons [1]. This high production of cassava encouraged the establishment of tapioca industry. There are around 70 tapioca industries have developed in Lampung. The industries produces about 70% waste in the form of solid waste and liquid waste [2].

Liquid waste is recognized as the most impact on the environment because it still contains organic matter such as carbon (C), nitrogen (N), phosphorus (P), etc [2]. The organic contain in liquid waste could be determined as Chemical Oxygen Demand (COD) content. The COD content of liquid waste from tapioca industry was about 7,000 - 30,000 mg / L [2], which is above the quality standard from Ministry of Environment Republic Indonesia No. 5 year 2014 that only 300 mg / L. One best way to reduce it, is by using the waste as a feed in biogas production [2, 3]. In the previous study, the anaerobic biogas system could reduce COD level of liquid tapioca industry waste until 48,16% [2]. However, it still above the tapioca wastewater quality standard of the government. Hence, the biogas effluent which is contained nitrogen, phosphorus and organic matters could be used as a media for microalga cultivation.

Microalgae are photosynthetic microorganisms that produce oxygen and convert CO₂ and water into organic component (such as glucose) for biomass production [3]. The utilization of effluents from anaerobic systems could significantly reduce the nitrogen, phosphorus, organic and inorganic materials in it. Those materials are used by microalgae for biomass productions [3]. Some studies report that



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microalgae such as *Chlorella pyrenoidosa*, *Scenedesmus obliquus* and *Nannochloropsis salina* from freshwater or marine environments could cultivate on effluents from anaerobic processes [4].

Nannochloropsis sp. is one of the prospect marine microalgae that can be cultivate in the biogas effluents with a high productivity of biomass [5]. Its biomass has protein content that can be used as a source of nutrition alternative. On the other hand, cultivation of microalgae using biogas effluents could significantly reduce the nitrogen (N), phosphorus (P), organic and inorganic materials in it [3] which can increase the growth rate and the biomass productivity in a shorter time. In the present study, we utilize the effluents of biogas from anaerobic process of the tapioca industry system for the growing media of *Nannochloropsis sp.* and evaluate the protein content.

2. Methodology

2.1 Chemicals and reagents

The inoculum of *Nannochloropsis sp.*, sterile sea water, biogas effluent of tapioca industrial media (MEBIT) and BG 11 standard media (Blue-Green Medium) [6]. Methanol is used for chlorophyll extraction, reagent for protein analysis [7], BSA (Bovine Serum Albumin) standard solution, and distilled water.

2.2 Microalgae and culture maintenance

The *Nannochloropsis sp.* were taken from mangrove roots on the Dewi Mandapa beach, Pesawaran, Lampung, based on the method of Andersen [8] with modification. The monoculture was maintained in seawater medium containing BG 11 media. The simple photobioreactors were made using erlenmeyer 250 mL in a volume of 100 mL. The cells were grown in air-lift photobioreactor under 2500 lux of light intensity (photoperiod 24:0 h) with free air bubbling at 22 ppt and room temperature [9].

2.3 MEBIT analysis

The effluent used in this study was obtained from one of the traditional tapioca industry in Pesawaran Regency, South Lampung. The effluent is taken from the storage waste before entering into the environment, by grab sampling method [10]. Before being used as a microalgae growth medium, the effluent is filtered using a filter with the pore of 0.45 μm , then was analyzed the content of pH, total dissolved solid, COD, total phosphate, total nitrogen (Kjeldhal), and total oxygen carbon.

2.4 Cultivation of *Nannochloropsis sp.* on BG 11 and MEBIT

The media used for the cultivation of *Nannochloropsis sp.* in this study consist of BG 11 [6] and MEBIT. In this study nutrient were added in the form of agriculture fertilizer includes TSP (1mL/L; 10 ppm) urea (1 mL/L; 20 ppm), and Za (1 mL/L; 30 ppm) to the MEBIT [11]. The acclimatization of *Nannochloropsis sp.* in MEBIT were prepare in the variations of MEBIT concentration start from 1, 3 and 6% (v/v). The 10% v/v *Nannochloropsis sp.* inoculum was growing in it [11]. The MEBIT with the best growth of *Nannochloropsis sp.* then use as cultivation media and the BG 11 (standard media) is used as a control in the working volume of 2000 mL with the same conditions as previously [9].

2.5 Observation of growth of *Nannochloropsis sp.*

2.5.1 Optical Density. Optical Density (OD) is determined using variant carry spectrophotometer 50 probes. The absorbance of 1 mL inoculum *Nannochloropsis sp.* taken using a pipette volumetric was measured at 750 nm [12]. Observation of the growth based on OD is carried out for 16 days, beginning day 0 of cultivation.

2.5.2 Measurement of chlorophyll a. Before measuring the chlorophyll a, the maximum wavelength of chlorophyll a should be determined. The method for determining the maximum wavelength of chlorophyll a refers to Amaral [13]. Inoculum stock of *Nannochloropsis sp.* was taken from 0 mL to 8 mL and added media to a volume of 10 mL. The sample was extracted using methanol and the extract was measured using a Variant Carry spectrophotometer 50 probes in the wavelength range of 200 nm

to 800 nm. The maximum wavelength use to observe the growth of *Nannochloropsis* sp using the chlorophyll a. Chlorophyll a was measured for growth observation of *Nannochloropsis* sp. refer to Becker's method [14]. Inoculum from each cultivation medium (BG 11 and MEBIT) were extracted using methanol. Samples (pellet of *Nannochloropsis* sp) were added with 1 mL of methanol and then ultrasonic for 15 minutes before centrifuged to obtained the extract. The absorbance was measured at maximum wavelength. The growth based on chlorophyll a observations was carried out for 16 days, beginning with day 0 (t0) cultivation. Chlorophyll a could be calculated using equation in Becker's method [14].

2.6 Harvesting *Nannochloropsis* sp.

Culture of *Nannochloropsis* sp. harvested using centrifugation techniques [15]. Wet biomass were dried using a freeze-dryer for 21 hours then weighting. Furthermore, the biomass productivity of *Nannochloropsis* sp. was calculated [15].

2.7 Determination of Protein Content by Lowry Method

The preparation of reagent (A, B, C, D, standard solution) and analysis of protein was performed using the Lowry method [7]. The freeze dried biomass of *Nannochloropsis* sp. was weight about 50 mg then dissolved in 50 ml of phosphate buffer pH 7, sonified for 60 minutes, then centrifuged at temperature of 4°C at 4500 rpm for 10 minutes. The filtrate was separated from the deposit, taken as much as 1 mL and added 3 mL of distilled water then mixed with 5 mL of reagent C. The mixture was stirred in 10 s using vortex then left 15 min at room temperature, then added quickly 0.5 mL of reagent D and perfectly stirred, allowed to stand for 30 min on dark room temperature. Absorption is measured using spectrophotometer at 650 nm. The Bovine Serum Albumin (BSA) standard curves are used to determine the concentration of protein in *Nannochloropsis* sp. BSA standard solutions are made with the concentration of 0, 20, 40, 60, 80 and 100 ppm. The protein content of *Nannochloropsis* sp. could be determined as mentioned previously [16].

3. Results dan Discussin

3.1. Characteristics of MEBIT

Before using as a growing media for *Nannochloropsis* sp., the MEBIT was analysis to determine the chemical compositin, including total organic carbon (TOC), total nitrogen (TN), total phosphate (TP), TDS and pH. The chemical composition of the MEBIT are presented in table 1. The chemical compositions of the MEBIT reveal the organic matters that existed for the growth of *Nannochloropsis* sp.

Table 1. The chemical composition of the MEBIT

No.	Parameter	Unit (mg/L)
1	pH	6.7
2	TDS	149
3	COD	256
4	TP-PO ₄	45
5	TN	198
6	TOC	53.2

3.2 Acclimatitation of *Nannochloropsis* sp.

In order to understand the best composition of MEBIT for the growth of *Nannochloropsis* sp., we need to acclimate it in several concentrations of MEBIT. The variation of MEBIT concentrations of 1, 3 and 6% v/v was used in acclimatitation of *Nannochloropsis* sp. the during 0 to 6 days. The *Nannochloropsis* sp growth could be seen in figure 1. The *Nannochloropsis* sp. already growing on the day 1, it can be seen from the change in culture color. Figure 1 also shows that growth of *Nannochloropsis* sp. is the best in 6% v/v MEBIT, which has a greener color in the culture. This is due to the levels of organic

compounds such as N and P in the 6% v/v MEBIT is greater than in 1 and 3% v/v, therefore, the growth of *Nannochloropsis sp.* more optimum in 6% v/v MEBIT.

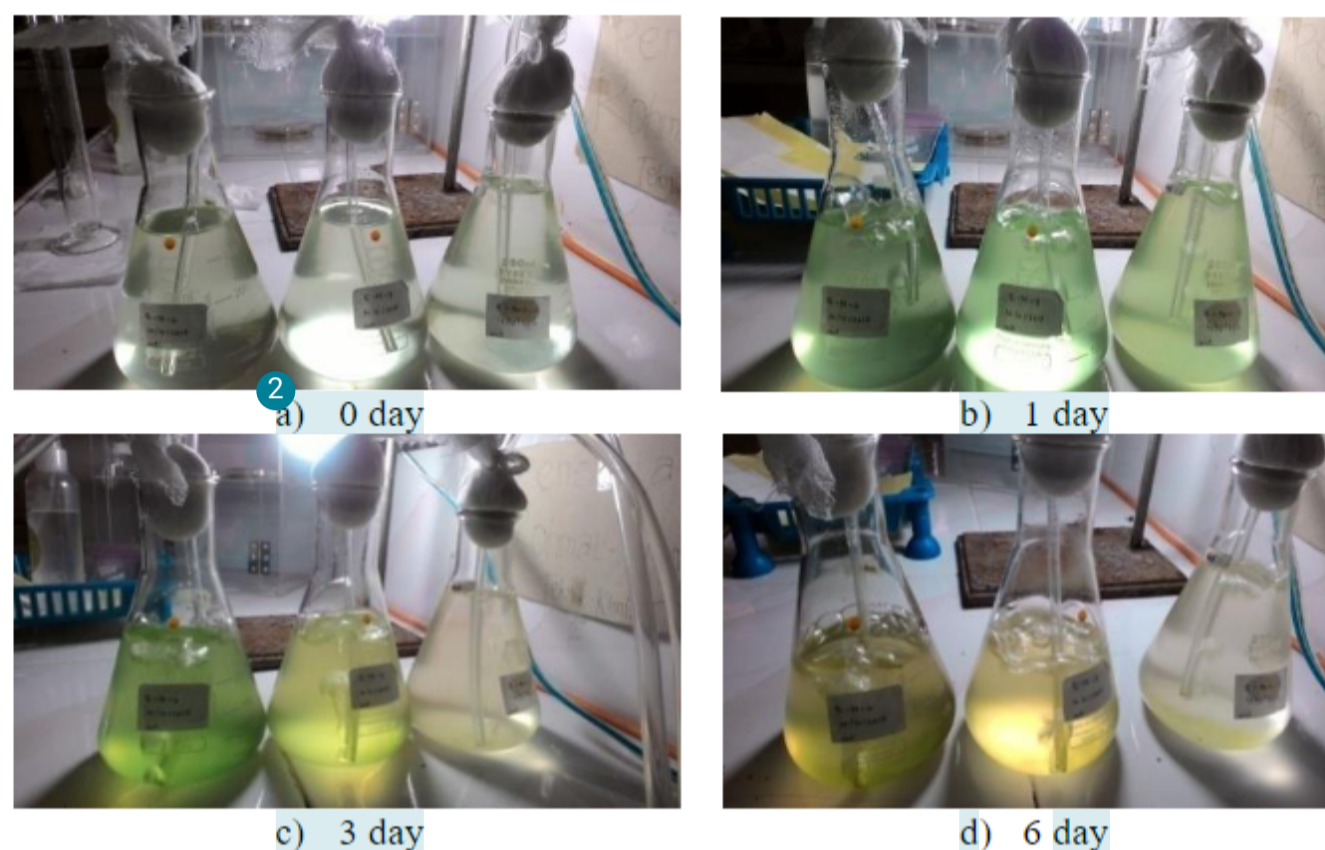


Figure 1. Acclimatization of *Nannochloropsis sp.* in different MEBIT concentration. The 0, 1, 3, 6 day refers to the day of acclimatization.

3.3 Cultivation of *Nannochloropsis sp.*

Nannochloropsis sp. growth was observed using OD 750 nm and the chlorophyll a content. The OD is known as absorbance or turbidity [15]. The quantity of light absorbed by the cell suspension were associated with the biomass concentration [17]. Yet, the presence of pigments could influence the measurement. Errors could be minimized by measuring the cell density outside the range of chlorophyll wavelength, which is at 750 nm. The observation of *Nannochloropsis sp.* growth in BG11 and MEBIT media was presented in figure 2. The red line shows the growth of *Nannochloropsis sp.* on MEBIT media and purple lines is the growth on BG 11 media as well as green and blue lines are the control of each media. The absorbance OD obtained from *Nannochloropsis sp.* in BG 11 media is 0.12-1.17, while at the MEBIT media is 0.14-1.48. The growth period of microalgae could be measured based on biomass and the number of cells in the media. Based on the growth curve, the growing phases of *Nannochloropsis sp.* on MEBIT was going on until the day 12, but there was a decrease on the day 14, whereas on BG 11, the growing phases occur until the day of 16. The difference in the growing phase is due to the availability of nutrient and interference with suspended solids. Optical Density does not merely represent the number of microalgae cells but also representing suspended solids that are in the media [16].

3.4 Extraction of Chlorophyll a

Microalgae are a group of microorganisms that dominant contain the chlorophyll a [15]. Therefore, the concentration of chlorophyll a, could represent the concentration of the microalgae. Determination of concentration of microalgae using chlorophyll extraction is to reduce the interference of suspended solids. The determination of maximum wavelength of chlorophyll a for *Nannochloropsis sp.* can be seen in figure 3. It can be seen that the absorbance peaks appears at wavelengths of 430 nm and 665 nm. The absorbance at a wavelength of 665 nm commonly is chlorophyll a, while 430 nm is absorbance of chlorophyll a and karetenoid [16]. Thus, the wavelength of 665 nm is more specifically of chlorophyll

a. The absorbance at 650 nm indicates the presence of chlorophyll b but in a small concentration [16]. The peak intensity increases in proportion to the quantity of microalgae. For example, the absorbance of 2 ml of microalgae at 665 nm is about 0.1 while the absorbance of 8 ml of microalgae at 665 nm is about 0.6. Therefore, increasing the concentration of chlorophyll a is directly proportional to the increasing in the volume of microalgae.

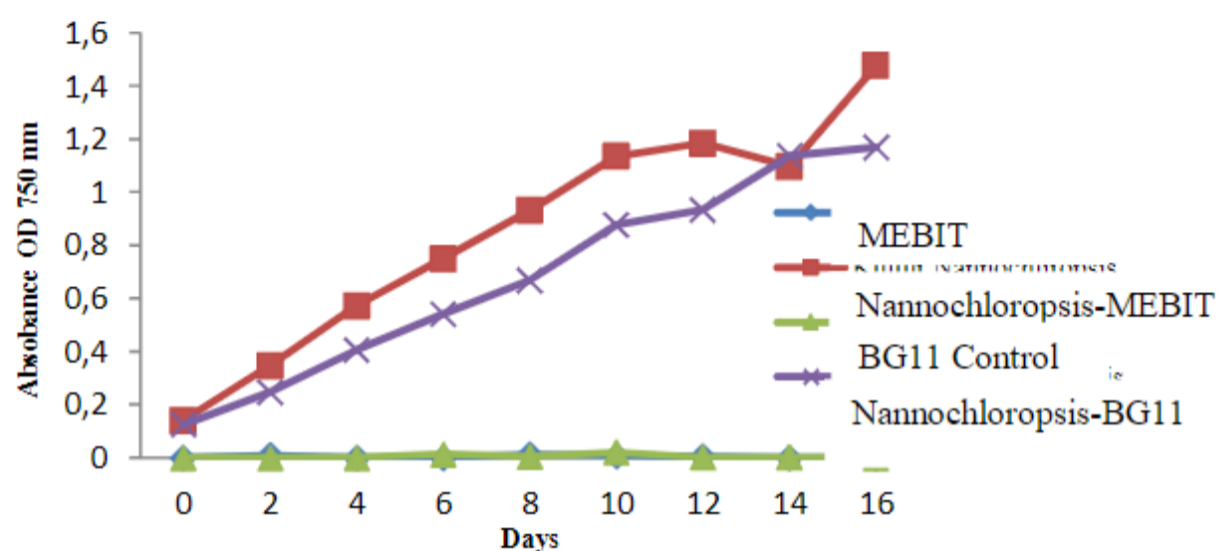


Figure 2. Curve of *Nannochloropsis sp.* growth in MEBIT and BG11 media based on OD absorbance.

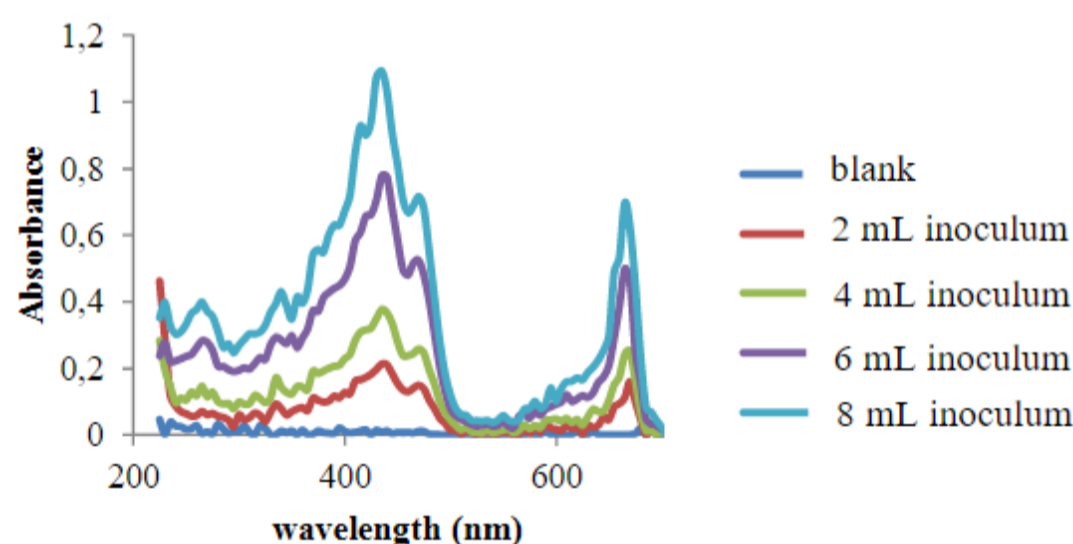


Figure 3. Maximum wavelength of chlorophyll a for *Nannochloropsis sp.*

3.5 Concentration of Chlorophyll a

Samples were extracted using methanol and was centrifuged to separate the pellet and supernatant. The supernatant absorbance represent the concentration of chlorophyll a, as presented in Figure 4. The concentration of chlorophyll a in *Nannochloropsis sp.* growth in BG 11 media is about 0.65-7.65 $\mu\text{g} / \text{mL}$, lower than those grown in MEBIT which is about 1.27-13.51 $\mu\text{g}/\text{mL}$. The difference may caused by differences in macronutrients for chlorophyll builder such as nitrogen and phosphate [17].

3.6 Biomass Production of *Nannochloropsis sp.*

Biomass production of *Nannochloropsis sp.* that was cultivated on MEBIT was higher than cultivated on BG11 in a 16 day of harvesting. *Nannochloropsis sp.* which is cultivated in MEBIT produced biomass concentrations of 0.311 g / L and the productivity of 0.019 g L⁻¹ d⁻¹ whereas in BG 11 it has a biomass of 0.256 g/L and 0.016 gL⁻¹ d⁻¹ in productivity. The difference is likely due to the difference of the constituent nutrients of each media. BG 11 media with the availability of nutrients (N, P, C) around 33.91 mg/L is obtained from nitrogen in NaNO₃ and Co(NO₃)₂·6H₂O, phosphate in Na₂HPO₄·2H₂O and

carbon in Na_2CO_3 , while in MEBIT media the availability of nutrients (N, P, C) around 39.1 mg/L derived from nitrogen in urea ($(\text{NH}_2)_2\text{CO}$, phosphate in TSP ($\text{Ca}(\text{H}_2\text{PO}_4)$) and organic matter (COD).

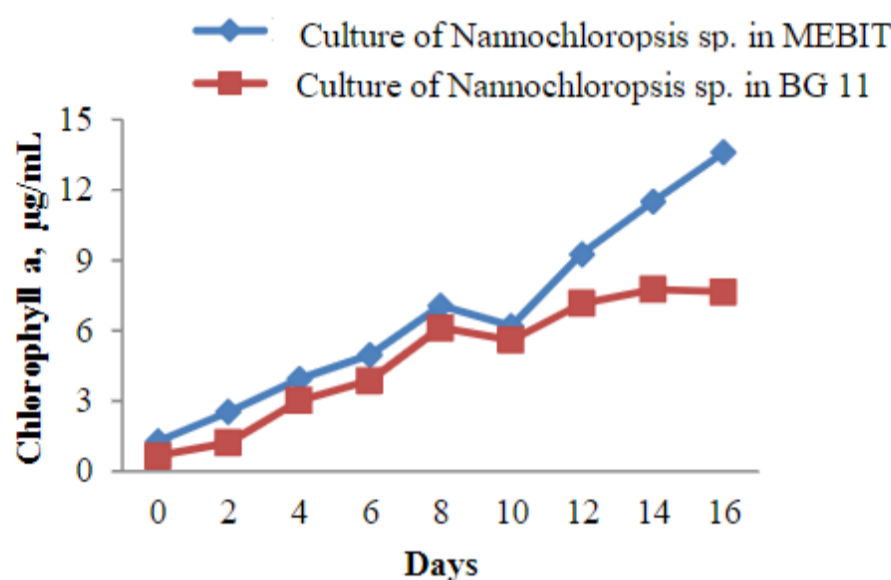


Figure 4. Curve of *Nannochloropsis sp.* growth in MEBIT and BG11 media based on chlorophyll a concentration.

3.7 Protein Content

Measurement of protein content is conducted by the Lowry method. Protein concentration in *Nannochloropsis sp.* was determined based on the curve Bovine Serum Albumine (BSA) standard solution measured at wavelength of 650 nm. The curve of BSA standard can be seen in figure 5. Based on the curve of BSA standard, the regression equation is $y = 0.0014x + 0.0016$ where "x" indicates concentration and "y" indicates absorbance with coefficient correlation obtained of 0.9985. Protein content of *Nannochloropsis sp.* in BG11 is 0.364 g ($36.41 \pm 0.47\%$) and in MEBIT is 0.306 g ($30.65 \pm 0.471\%$), as previous study that the average protein content in *Nannochloropsis sp.* between 30-45% of biomass [18].

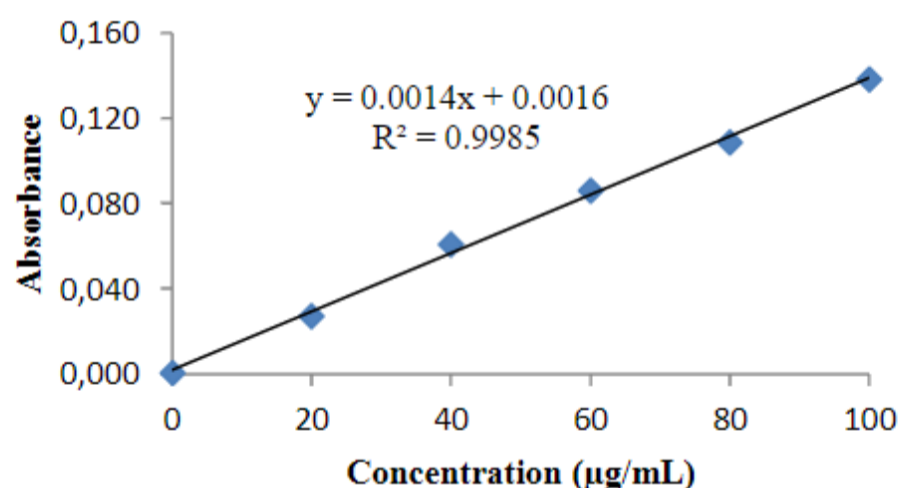


Figure 5. The curve of BSA standard

4. Conclusion

The *Nannochloropsis sp.* is able to grow in MEBIT 6% v/v with OD 750 nm about 0.14-1.48 and chlorophyll a concentration of 1.27-13.51 µg/mL as well as *Nannochloropsis sp.* that grow in BG11 media. Biomass production when it was cultivated in MEBIT was higher than cultivated in BG11, it is 0.311 g / L and the productivity of $0.019 \text{ g L}^{-1} \text{ d}^{-1}$ whereas when it was cultivated in BG11 the biomass concentration is 0.256 g / L the productivity is $0.016 \text{ g L}^{-1} \text{ d}^{-1}$. The protein content of *Nannochloropsis sp.* in MEBIT was lower ($30.65\% \pm 0.471$) than when it was cultivated in BG11 ($36.41\% \pm 0.47$).

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