Utilization of different nitrogen sources for the growth of microalgae isolated from mangrove leaves in Banda Aceh - Indonesia

S H Anwar1*, S Harzaki2, M I Sulaiman1, T Rinanda3

1Department of Agricultural Product Technology, University of Syiah Kuala, Banda Aceh 23111, Indonesia
2Alumni of Agricultural Product Technology Department, University of Syiah Kuala, Banda Aceh 23111, Indonesia
3Faculty of Medicine, University of Syiah Kuala, Banda Aceh 23111, Indonesia

Abstract. The aim of this research was to investigate utilization of different nitrogen sources used for the growth of microalgae and determined the optimum period of the growth. The microalgae had been isolated previously from mangrove leaves in Banda Aceh, Indonesia and has been identified morphologically. Three different nitrogen sources i.e., yeast extract, monosodium glutamate or MSG and sodium nitrate were used. The biomass production was measured by optical density using spectrophotometer. The results showed that MSG stimulated microalgae to produce the highest amount of biomass (0.38 g/L/day) followed by yeast extract with 0.33 g/L/day, but both were statistically not significant. Sodium nitrate developed the lowest OD value with the amount of dried biomass only 0.16 g/L/day. Correlation analysis indicated the yield of biomass was positively related, r = 0.953, with the optical density of the cultures. The OD values of yeast extract, MSG and sodium nitrate cultures were 1.20, 1.18 and 0.82, respectively. The optimum time for microalgae growth was at day 9 in which 4.41 g/L of dried biomass was accumulated in the culture where MSG was used. The maximum yield of dried biomass for yeast extract was 4.22 g/L followed by 2.58 g/L for sodium nitrate.

1. Introduction

Previous researches indicated that polyunsaturated fatty acids (PUFAs) have positive benefits to human health. PUFAs are effective to prevent diabetes during pregnancy, helping development of infant, lowering cholesterol and blood pressure as well as reducing joints inflammation and paint in rheumatoid arthritis [1]. Moreover, both important polyunsaturated fatty acids such as Eicosapentaenoic Acid and Docosahexaenoic Acid are actually precursors of some important compounds such as hormones. In human body, these compounds are needed for many essential biological processes [2].

Omega-3 fatty acids have attracted intensive studies since early 1900s and the term "Vitamin F" has been created for these fatty acids [3]. The most important PUFAs are ALA (α-linolenic acid, C18:3n-3), EPA (eicosapentaenoic acid, C20:5n-3) and DHA (docosahexaenoic acid, C22:6n-3) [4]. Many investigations related to PUFAs enrichment into food products are challenged by the sensitivity of PUFAs toward oxidation and the fishy odour because most of PUFAs are extracted from seafood,
particularly cold-water fish. In addition to these problems, overfishing, mercury contamination issue and fish sustainability have added the complexity of fish as the main source of omega-3 fatty acids [3].

Based on the facts that incorporation of fish PUFAs is difficult, alternative sources for omega-3 fatty acids must be found. Production of fatty acids in fish is actually originated from the microorganisms as the fish feed. Some microorganisms such as yeast, fungi and microalgae produce fatty acids in their cells. Some experts describe this alternative as microbial oils or Single Cell Oils (SCO) [5]. Microalgae that produce edible oil and the oil contains essential fatty acids as well as antioxidant (carotenoids and tocopherols) are Thraustochytrium, Ulkenia and Schizochytrium [6]. Moreover, thraustochytrids microalgae such as Cryptecodinium cohnii, Botryococcus braunii, and Schizochytrium sp. are informed to produce 20%, 25-75% and 50-77% oil, respectively [7].

Due to its flexibility for food application, investigation related to microbial oils particularly from microalgae has increases considerably. Studies to isolate microalgae and furthermore to identify them genetically are multiply every year worldwide. The outcomes were definite according to the area and environment (temperature and weather) where the microorganisms were isolated. In 2010, similar study had conducted in Indonesia to investigate production of PUFAs from microalgae by using glycerol. The authors claimed that seven strains of microalgae were able to produce docosahexaenoic acid in the range of 2.49-9.14 g/L biomass produced [8]. Previous research related to microalgae has also been done in Aceh Province. Two microalgae strains from mangrove area have been identified as potential PUFAs producers, morphologically. This investigation is still on going to identify these strains genetically [9].

Exploration to extract biodiesel from microorganisms are quite many but investigation to find new sources of omega-3 fatty acids particularly from microalgae are rare. Therefore this research was aimed to study the possibility of microalgae cultivation in laboratory scale from species that were isolated previously from mangrove leaves in Aceh Province, Indonesia. Two important factors investigated were nitrogen sources and the length of microalgae growth. In this research, three different nitrogen sources i.e., yeast extract, monosodium glutamate (MSG) and sodium nitrate were used for the growth of microalgae. The biomass produced were collected, dried and weight gravimetrically. The highest biomass production was an indicator for the optimum growth and measured by optical density using spectrophotometer.

2. Materials and methods

2.1. Materials

The materials to compose a so called YPGS media were yeast extract-peptone-glucose-seawater which then mixed in natural sea water (consist of yeast extract (Merck), mycological peptone (Himedia), glucose (Himedia), and bacto agar (Difco)). Other materials needed were ammonium nitrate (Merck), monosodium glutamate, antibiotics (penicillin and streptomycin), lugol staining and distilled water.

2.2. Methods

The experiments were divided into two stages: 1) the preliminary research, investigated the optimum growth of microalgae in liquid media to find the time (day) for microalgae to produce the highest biomass and 2) the main research, optimization of microalgae growth based on different nitrogen sources. Nitrogen sources tested were yeast extract, sodium nitrate and monosodium glutamate.

2.2.1. Inoculum preparation. Method for inoculum preparation is following the procedure developed previously [10] with modification. Briefly, one mL of the zoospore suspension of microalgae from previous investigation was transferred into 50 mL aliquot of liquid media in a 250 mL flask. The liquid media (YPGS media) was prepared in 1L natural seawater at a salinity of 15‰. The flask was covered with cotton wool and finally wrapped with aluminum foil. The mixture was then shaken at 150 rpm for
48 hours (at 25°C). The initial inoculum used was a 20 mL inoculum of the 48-hours old culture broth and was used for all subsequent growing time and nitrogen sources experiments.

2.2.2. Growth and nitrogen source experiments. The YPGS media was prepared like previously mentioned. The pH was adjusted to 6.0 using 2 N HCl. The culture flasks were shaken at 150 rpm at 25°C for 14-days period. Triplicate flasks harvested at 3 days intervals for biomass determination [10]. For finding the best nitrogen source, peptone was replaced by sodium nitrate (NaNO₃), yeast extract and MSG (C₅H₈NNaO₄).

2.2.3. Analysis of cell growth. Measurement of microalgal biomass was carried out by measuring the turbidity of microalgal suspension [11]. The culture medium (1.5 ml) was collected from each flask of growth from nitrogen source experiments every day during the incubation period (14 days) and measured the absorbance at 600 nm. The OD value accepted was ≤ 0.4 because if the OD value was too high, then it did not correlate well anymore with the cell densities. The values that were ≥ 0.4 (ODundil) must be diluted at first and the absorbances were red as ODdil. The value of ODdil was then divided by its dilution factor and written as ODcorr. The final OD of microalgae cells’ densities was recorded as the mean of the ODcorr.

2.3. Data analysis
All measurements were carried out in duplicate and from these results mean values and standard deviation are calculated. The quantification of microalgae oil was calculated gravimetrically.

3. Results and discussion

3.1. Preliminary research

3.1.1. Dried biomass. Determination of dried biomass was aimed to quantify the amount of biomass yielded from the growth of microalgae. The growth curve was built based on the biomass yield since the first day of growth until the 14th day of incubation time. The optimum growth of microalgae was the time when the biomass produced was at the highest amount. The result of the biomass achieved can be seen in Figure 1.

![Figure 1. The amount of biomass produced during incubation time.](image_url)

The highest amount of biomass produced by microalgae was at day 9 (4.18 g/L). At day 12, dried biomass started to decrease to become 4.16 g/L dan this declining continued until day 14. The exponential phase was begun on day 3 up until day 9, followed by the stationary phase and finally the falling growth period. The exponential phase is the phase where the biomass yield is increasing due to
the rising of photosynthesis activities. The growing number of microalgal cells with maximum and constant cells’ division are two important factors that determine the exponential phase [9].

3.1.2. Cultures’ optical density. In order to confirm the microalgal growth rate based on the biomass dry weight, it is important to measure the culture optical density. This measurement is actually to quantify the cells density during cultivation. In this study, the sample was taken every three day from the culture broth and the cells density was measured using Shimadzu UV-1700 spectrophotometer at wave length of 600 nm (OD600). The results can be seen in Figure 2 which showed that the highest cell density was achieved at day 9 of cultivation which was 27.58. The densities were declined afterward at day 12 and 14 which only accumulated for 22.80 and 22.02 respectively. However, these results were in agreements with the outcomes of dried biomass quantification.

![Figure 2](image)

**Figure 2.** The microalgal cell density during cultivation

3.2. The main research

3.2.1. Microalgae morphology. Microalgae used in this research was isolated from mangrove forest in Banda Aceh. The colonies were homogeneous and had been identified morphologically and the isolate is belong to *Thraustochytriaceae* (unpublished result). The colonies are round in shape and yellowish white in color. The colonies were growth densely and the surface is smooth but is not slippery.

3.2.2. Dried biomass. Quantification of microalgal dried biomass was conducted every three days during 9 days of growing time. The usage of different nitrogen sources during cultivation resulted different amount of dried biomass, as can be seen from Figure 3. Yeast extract and MSG yielded higher amount of dried biomass compared to sodium nitrate. At day 9, dried biomass produced with yeast as nitrogen source was 4.22 g/L, while when MSG and sodium nitrate as nitrogen sources used resulted 4.41 g/L and 2.58 g/L dried biomass, respectively. These values were lower than the results published elsewhere [12] in which yeast extract produced 6.108 g/L, MSG yielded 6.585 g/L and 3.15 g/L of dried biomass if sodium nitrate was used [11].
Higher yield of dried biomass if MSG was used as nitrogen source can be caused by the ability of microalgae to absorb MSG was faster than to absorb yeast or sodium nitrate. Microalgae needs nitrogen to form chlorophyll [11]. When the nitrogen decreases to the lowest level, microalgae start to convert this rich nitrogen substance into protein, nucleic acids, the cells’ wall and other important substances for the cells’ growth. Therefore, the faster the nitrogen absorb, the better the growth of microalgae.

MSG and sodium nitrate are two sources of simple nitrogen which can be easily utilized by microalgae for its growth [12]. Complex nitrogen such as yeast extract also provides protein, amino acids, sugar, fatty acids and vitamins beside its main function as nitrogen source. The influence of nitrogen source to growth of microalgae is varied among the species. For Thraustochytrid in particular, MSG and yeast extract are the best nitrogen sources for microalgae to produce high yield of dried biomass [13]. Lipid production in microalgae cells is correlated well with nutrient availability particularly nitrogen and glucose which contained in medium growth. Figure 4 explains how do the cells stimulated to produce lipid [14].
Figure 4 shows the relationship between nutrient availability in culture media with formation of biomass and lipid produced during cultivation time (hours). Nitrogen is absorbed fast and diminished in the first 20 hours followed by glucose in 70 hours during cultivation. Biomass and lipid production increase significantly after 20 hours and lipid accumulation in the cells start to begin when nitrogen (commonly in form of ammonium salt) in cultivation media is finished. At the same time, carbon supply (in the form of glucose) start to decrease. The nutrients depletion causes the cells growth and division to discontinue although the cells are able to absorb glucose. These conditions accelerate lipid biosynthesis in microalgae cells which is actually the conversion of glucose into lipid. When the external nitrogen in media was ceased then biomass concentration increases and nitrogen rich substances converted into other important substances such as protein, nucleic acids and cells wall which can help the growth process [11].

3.2.3. Cultures’ optical density. The optical density (OD) of the culture is measured to predict the metabolic activity and the growth of cells microorganism. The principle of OD measurement by spectrophotometer is based on light absorption or radiation energy by a solution or liquid in a cuvette. The greater solution concentration in a sample, the higher the absorbance reading will be.

Measurement of optical density from microalgae cultures varying in nitrogen source resulted the density in the range of 0.82 – 1.20. Statistical analysis indicated that optical densities were influenced by different nitrogen source used, significantly (P≤0.05). As can be seen from Figure 5, yeast extract resulted the highest OD value (1.20) which was significantly different with OD value of sodium nitrate (0.82). The OD values were directly proportional to the biomass yielded. Yeast extract and monosodium glutamate developed the highest OD values as well as the amount of dried biomass. These values consistently higher than that produced by cultures which used sodium nitrate as nitrogen source.

![Figure 5. Influence of nitrogen sources to the cultures optical densities](image)

3.2.4. Correlation analysis between dried biomass and cultures optical density. Analysis of correlation was performed to study the degree of linear relationship between two or more variables. In this research, the analysis was conducted for dried biomass and optical density of the culture from microalgae cultivation. The relationship value achieved was 0.953 which mean that the correlation is positive with high degree of relationship. This also means that increasing the amount of dried biomass give rise to higher optical density of the culture which was in accordance with Figure 6. Figure 6 demonstrates comparison of dried biomass with optical density of cultures from three nitrogen sources used in this research. The trend is similar where intensifying the density of cultures optically giving impact to accumulation of dried biomass.
Figure 6. Comparison of dried biomass yield and cultures optical density from different nitrogen sources: (a) yeast extract, (b) sodium nitrate and (c) monosodium glutamate.
4. Summary
Nitrogen sources affected the growth of microalgae cultures. Monosodium glutamate stimulated microalgae to produce the highest amount of biomass during cultivation as proved by yield of dried biomass (0.38 g/L/day). Yeast extract gave results slightly differ with MSG but statistically not significant (0.33 g/L/day). Sodium nitrate on the other hand, developed the lowest OD value and the amount of dried biomass (0.16 g/L/day). Correlation analysis indicated that the yield of biomass was positively related with the optical density of the cultures (r = 0.953). The yeast extract culture had the highest OD value of 1.20 which was significantly not different with the OD value of MSG culture (1.18). Sodium nitrate had the lowest OD value which was only 0.82. The optimum time for microalgae growth was at day 9 in which 4.41 g/L of dried biomass was accumulated in the culture where MSG was used. The maximum yield of dried biomass when yeast extract was as nitrogen source was 4.22 g/L and followed by 2.58 g/L of dried biomass produced by culture added with sodium nitrate.

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