Optimization of Culture Conditions on Growth of *Chlorella* sp. Newly Isolated From Bagansiapiapi Waters Indonesia

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**Abstract.** *Chlorella* sp. is a type of micro-sized green algae obtained in fresh or marine waters. However, many factors need to evaluate the growth of *Chlorella* sp. mainly the culture conditions. This study aimed to evaluate the culture conditions in optimizing the growth of *Chlorella* sp. newly isolated from Bagansiapiapi marine waters. The experiment was carried out at temperature ±25°C, light intensity 2300 Lux using TL-D lamp (36 W). There were six treatments as culture conditions for the cultivation of *Chlorella* sp.: 1) Bean Sprouts (*Vigna radiata*) Extract Media (BSEM) with light continuously, 2. BSEM with a Photoperiod light:dark (10:14h), 3. Tofu waste media with light continuously, 4. Tofu waste media with a photoperiod light:dark (10:14h), 5. Fish pellet solutions media (25%), 6. Fish pellet solutions media (50%). The parameter observed was the growth, cell density and the specific growth rate of *Chlorella* sp. every 2 days for 14 days of cultivation. Based on the results shown that the marine *Chlorella* sp. from Bagansiapiapi waters could be adapted to all treatments. Nevertheless, the use of BSEM light continuously obtained the highest cell density (27.75 x 10^5 cell/ml) on 12 days and continues to increase until 14 days, while the other treatments had decreased. Furthermore, the *Chlorella* sp. obtained the specific growth rate was 0.42. Therefore, the marine *Chlorella* sp. from Bagansiapiapi could be cultivated in BSEM light continuously for further development as a functional food or bioactive source in pharmaceutical products.

1. **Introduction**

Bagansiapiapi is a city located at the Rokan River estuary, north coast of Rokan Hilir Regency, and is a strategic place due to it is close to the Malacca Strait which is international trade traffic, besides that the Malacca Strait has fish resources that have been utilized for world consumption. Therefore, the waters of Bagansiapiapi have considerable potential for the presence of *Chlorella* due to its rich in nutrients and good for growth. *Chlorella* is a green microalga containing chemical and bioactive compounds that can live in both fresh and marine waters [1] [2] and an estuarine coastal environment [3].

*Chlorella* is a microalga that has fast growth, wherein the highest population in 12 days of cultivation [4]. Moreover, *Chlorella* powder contains the highest protein about 61 g/100 g [5], and some bioactive compounds such as chlorophyll, carotenoids [6] [7], and phenolics [4]. Other than that, the pigment contained in *Chlorella* could be applied as a natural dyeing for cookies application [8]. The bioactive compounds have a role for potential applications such as pharmaceuticals, nutraceuticals, and cosmeceuticals that are yet to be fully explored. With the high nutritional content and the presence
of bioactive compounds that Chlorella plays a role as a physiological function in the body, Chlorella can be cultivated. Therefore, it is very necessary to develop microalgae.

To develop Chlorella, cultivation begins with the isolation process and continues with the purification process until the single cell alga is obtained. Many factors that influence the cultivation of Chlorella sp. among others are temperature, lighting, and the media used. Some of the media used are synthetic and some are natural. The amount of chemicals needed for Chlorella cultivation on synthetic media is very large e.g. basal medium [4], which makes it difficult to obtain these chemicals and requires high costs. For this reason, Chlorella cultivation was carried out with natural media namely bean sprouts extract media, tofu waste media, and fish pellet solution with different concentrations, lighting, and photoperiod.

Mung bean is a common food that has a high nutritional value (protein, vitamins, phytonutrients, and micronutrients) [9], consists of some minerals (mg/g) 132 Ca, 6.74 Fe, 189 Mg, 367 P, 1246 K, 15 Na, 2.68 Zn, 0.941 Cu, 1.035 Mn, 8.2 mcg of Se, and some of vitamins e.g. Vitamin C, Thiamin, Riboflavin, Vitamin A, B-6, B-12, E and K [10]. Tofu is a good source of protein, vitamins and minerals (calcium, magnesium, phosphorus, potassium, iron, manganese, zinc, copper, and selenium. Moreover, tofu is also rich in omega-3 and amino acids [11]. The content of some minerals and vitamins of tofu (mg/100gr) 105 Ca, 5.36 Fe, Zn, 0.08 thiamine, and 0.05 riboflavin [12], and wastewater of tofu contains some organic materials [11]. Fish Pellet contain some of protein, fat, vitamins, and minerals [13], wherein fat content 11.96%, Ca 984 mg/kg, Na 40 mg/kg, Fe 136 mg/kg, and mg 19 mg/kg [14].

Based on above, hence tofu waste, mung bean, and fish pellet potentially can be used as media to cultivation of Chlorella sp. newly isolated from Bagansiapi-api waters due to containing the macro and micronutrient to support the growth of Chlorella sp. Therefore, this study was aimed to optimization the culture conditions on the Growth of Chlorella sp. newly isolated from Bagansiapi-api waters Indonesia.

2. Materials and Methods

The sample of Chlorella from Bagansiapi-api waters was isolated, screened, and purified. After obtaining the pure of a single colony, the Chlorella cell was grown in 3 different media (Bean sprouts, Tofu waste, Fish pellet) with light continuously and photoperiod light-dark 10h:14h of each treatment. Therefore, there were 6 treatments: Bean sprout extract medium with lighting continuously (24 hours) /T1, Bean sprout extract medium with photoperiod (LD 10:14 hours)/T2, Tofu waste medium with lighting continuously (24 hours) /T3, Tofu waste medium with photoperiod (LD 10:14 hours) /T4, Fish pellet solution with concentration 25%/T5, Fish pellet solution with concentration 50%/T6. The parameter was measured for cell density and the specific growth of Chlorella sp.

2.1. Materials and Equipment

Chlorella sample was obtained from Bagansiapi-api waters in Rokan River estuary, Bangko District, Rokan Hilir Regency of Riau province, and the material used to collect the Chlorella were NaOH, KI, MnSO₄, H₂SO₄, amylum, Na₂SO₃, phenolphthalein, Na₂CO₃, with the equipment were plankton net, styrofoam, bottle, thermometer, pH meter, hand gloves, pipettes, label, refractometer, Secchi disc, and stationery. The materials used for isolation, purification and cultivation of Chlorella were agar, HCl, KOH, bean sprouts, tofu waste, fish pellet, and aquadest, with the equipment were petri dishes, Erlenmeyer, aerator, binocular microscope (Olympus CX21), bottle 1.5 L, autoclave, test tube, TL lamp, hemocytometer, porcelain cup, oven, digital scales, hand tally counter, and lux meter.

2.2. Sampling Water Condition

The Chlorella samples were obtained using plankton net 30 μm pore size. Samples with sampling point in the downstream area of Bagan waters with a distance of 9 km from the upstream, at latitude 2.258832 (2°15’31.8”N), longitude 100.751340 (100°45’04.8”E), and width 346.60 m. Samples were taken at a depth of 6 m, salinity 15 ppt, temperature 29 ⁰C, brightness 40.5 cm, current velocity...
1.56 m/s, pH 6.5, DO 5.20 mg/L and CO₂ 45.94 mg/L, the activity of fishermen near the sampling site is clam breeding.

![Sampling point of Chlorella in Bagansiapiapi waters](image1)

**Figure 1.** Sampling point of Chlorella in Bagansiapiapi waters

The sampling location of Chlorella in Bagansiapiapi waters showed with a point with the red arrow in figure 1, and the condition of waters can be seen clearly in figure 2, wherein the water is brown and muddy, however odorless waters.

![Bagansiapiapi waters](image2)

**Figure 2.** Bagansiapiapi waters

2.3. *Isolation and Identification of Chlorella* sp.
The samples that had been obtained were taken to the Microbiology and Biotechnology Laboratory of Fishery Products Technology Faculty of Fisheries and Marine Science Universitas Riau for morphological observations screening and isolation process. The collected pre-cultured sample was picked up 1 ml and streaked into agar medium and incubation at room temperature 25 °C. After incubation for 1 month, the Chlorella cell was grown on surface agar. The single colony on agar was picked up and observed under microscope and put on 25 ml liquid basal medium, after 7 days incubation transferred 1 ml into 50 ml, then 1 ml transferred into 100 ml, do this continuously for purification up to 250 ml Erlenmeyer flask, the purity was observed using a binocular microscope.

2.4. *Starter Preparation*
100 ml of Chlorella which was cultivated in 250 ml Erlenmeyer flask was transferred into 900 ml of basal medium, then as a starter, 200 ml of *Chlorella* sp. was prepared and inoculated into 1800 ml basal medium for 7 days cultivation. Furthermore, 100 ml was inoculated into 900 ml of each medium treatment (bean sprouts extract, tofu waste, fish pellet solution) with photoperiod (six treatments) with three replicated for 14 days cultivation. The bottles of Chlorella cultivation were placed on a rack with TL lamp 36 W (2300 lux), the space from lamp to bottles about 20 cm, temperature 25 °C, and humidity 59%.
2.5. Medium Preparation

**Bean sprouts extract.** 100 grams of bean sprouts were boiled with 500 mL of distilled water for 1 hour, filtered, and then the extract was taken 40 ml and added 960 ml of aquadest (4%). Furthermore 100 ml of this extraction with an addition 900 ml of aquadest as the medium at pH 7 [16].

**Tofu waste.** Tofu liquid waste was sterilized by autoclaving at a temperature of 121°C with a pressure of 15lbs, and 5% tofu waste was diluted with aquadest [17].

**Fish pellet solution.** 2 kg of pellets are put in a sack, then the sack is soaked in 3 liters of distilled water and allowed to stand for 24 hours. After 24 hours the soaking water will turn cloudy, indicating that the existing pellet solution has come out. Then the soaked water was filtered so that there were no lumps of pellets produced. The next step was to take one liter of filtered fish pellet solution sample, take 25 ml for a concentration of 25% (25 ml + 75 ml aquadest) and 50 ml for a 50% concentration (50 ml + 50 ml aquadest) into a beaker then pasteurized with heated at 60°C for 30 minutes, cooled in the refrigerator for 30 minutes, and left at room temperature 23°C for 24 hours, repeated 3 times.

2.6. Determination on Growth of Chlorella sp.

Chlorella was cultivated with three different mediums (tofu waste, mung bean, and fish pellet) with photoperiod LD 10:14 (six treatments). The cell density of Chlorella was counted every 2 days for 14 days of cultivation, which was counted under a binocular light microscope using a haemacytometer and a hand tally counter. Determination of the amount of *Chlorella* sp. can be known by counting the number of *Chlorella* sp. contained in 4 large boxes (big blocks) that have a side of 1 millimeter on the hemacytometer. The density of *Chlorella* sp. $= \frac{N}{4} \times 10^4$

Furthermore, according to Hirata [18] the specific growth rate (k) of *Chlorella* sp. can be calculated by the following equation formula.

$$ k = \frac{Log \left( \frac{N_t}{N_0} \right)}{T_t - T_0} \times 3.22 $$

2.7. Data Analysis

The Chlorella growth data obtained were statistically analyzed by ANOVA using SPSS 19 for windows.

3. Results and Discussions

3.1. Isolation and Purification of Chlorella sp.

Contaminated Chlorella samples from Bagansiapiapi were isolated in agar medium first and were transferred to liquid medium continuously to obtain axenic Chlorella free of contaminant. The isolation and purification process can be seen in figure 3, and the media used to the cultivation of Chlorella can be seen in figure 4.

**Figure 3.** The isolation and purification process of *Chlorella* sp
Figure 4. Media which used to the cultivation of *Chlorella* sp.

- a) Bean sprout extract Medium (BSEM), b) Tofu waste
- c) Fish pellet solution (25%), d) Fish pellet solution (50%)

3.2. Morphological *Chlorella* sp.

*Chlorella* was isolated from Bagansiapiapi waters has a spherical shape, with a diameter of 8-10 µm, green color. It clearly can be seen in figure 5.

Figure 5. The morphological shape of *Chlorella* sp. isolated from Bagansiapiapi waters

3.3. The Cell density of *Chlorella* sp.

The growth of *Chlorella* sp. can be seen from the number of cell density (Table 1). The results have shown that *Chlorella* sp. isolated from Bagansiapiapi waters could be adapted to natural media, namely Bean sprouts, Tofu waste, and Fish pellet solution. *Chlorella* cultivated in the three media had different cell densities. This indicates that the treatment medium used affects the average cell density of *Chlorella* sp. The average cell density of *Chlorella* sp. on each medium and different culture conditions can be seen in Table 1. Effect of treatment media and culture condition on cell density of *Chlorella* sp. also proven by statistical tests, wherein has significantly different among treatment (p<0.05).

Based on table 1 shows that the cell density of Chlorella for 14 days of cultivation was higher in Bean Sprout Extract Medium with lighting continuously (24 hours)/T1 (128.36x10^5 cell/ml). It was significantly different among treatments. Moreover, the *Chlorella* sp. obtained the highest cell density on 12 days of cultivation. The previous research was shown that the cell density Chlorella obtained the highest number in 12 days of cultivation [4]. It means that the Chlorella could be harvested on 12 of cultivation day. T1 treatment there was still an increase in the number of cells up to 14 days of cultivation days, which means that the nutrients to support growth were still present. While the cell density of other treatments was decreased this may be due to the nutrients needed for the growth of Chlorella having started to run out. More details on the growth of Chlorella can be seen in figure 6.
Table 1. The cell density of *Chlorella* sp. with different media and culture conditions (x10^5 cell/ml)

| Time | Treatments | Day | T1 | T2 | T3 | T4 | T5 | T6 |
|------|------------|-----|----|----|----|----|----|----|
| 0    |            |     | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 |
| 2    |            |     | 3.75 | 3.20 | 1.57 | 2.57 | 2.37 | 3.50 |
| 4    |            |     | 7.03 | 3.07 | 1.85 | 3.27 | 2.47 | 2.70 |
| 6    |            |     | 13.60 | 4.07 | 2.77 | 7.02 | 3.32 | 2.75 |
| 8    |            |     | 19.55 | 5.60 | 6.72 | 5.55 | 7.57 | 4.95 |
| 10   |            |     | 23.55 | 6.07 | 7.52 | 6.30 | 9.80 | 5.65 |
| 12   |            |     | 27.75 | 15.80 | 12.00 | 8.77 | 10.32 | 4.37 |
| 14   |            |     | 31.45 | 9.30 | 3.92 | 7.85 | 8.55 | 2.12 |
| Total|            |     | 128.36^f | 48.67^g | 38.06^b | 43.36^c | 46.39^d | 27.83^a |

Information: Bean Sprout Extract Medium with lighting continuously (24 hours)/T1, Bean Sprout Extract Medium with photoperiod (LD 10:14 hours)/T2, Tofu waste medium with lighting continuously (24 hours)/T3, Tofu waste medium with photoperiod (LD 10:14 hours)/T4, Fish pellet solution with concentration 25%/T5, Fish pellet solution with concentration 50%/T6.

Figure 6 indicates that the T1 treatment was still in the logarithmic phase and continued to increase for 14 days of cultivation, moreover contained much higher cell density among other treatments. It also caused that in mung bean sprout contained vitamins (thiamine, riboflavin, pyridoxine, niacin, tryptophan, pantothenic acid, folacin, vitamins C and K). Vitamins act as growth factors in algae growth. Cobalamin is used for the synthesis of deoxyribose. Biotin functions in fatty acid synthesis, b-decarboxylation, and carbon dioxide fixation [19].

The specific growth of *Chlorella* sp. for 14 days of cultivation is shown in table 2. It is shown that the *Chlorella* sp. was cultivated in Bean Sprout Extract Medium with lighting continuously (24 hours).
hours)/T1) was obtained the highest specific growth rate (0.42). Based on statistical analysis it was significantly different among treatments (p<0.05).

| Treatments | Specific Growth Rate |
|------------|----------------------|
| T1         | 0.42±0.04\(^{d}\)    |
| T2         | 0.33±0.02\(^{a}\)    |
| T3         | 0.22±0.05\(^{b}\)    |
| T4         | 0.23±0.03\(^{c}\)    |
| T5         | 0.24±0.05\(^{d}\)    |
| T6         | 0.19±0.07\(^{a}\)    |

The specific growth rate T1 has similar with Hirata [18] was 0.42 which was cultured in temperature 23 °C, while this research was conducted in temperature 25 °C, while *Chlorella* sp was cultured in Bean Sprout extract Medium in T1 and T2 had the higher specific growth rate compared to Teluk Batik beach 0.26, Marina Core Resort 0.25, Titik Panjang 0.34, and microalga *N Oculata* 0.34 was cultured in F2 medium [20]. Nevertheless, the T6 treatment has the lowest specific growth rate, maybe this is due to the color of the media solution being darker than other media, so it is not suitable for the growth of Chlorella, it can inhibit the absorption of light during cultivation because the light produced plays a role in the photosynthesis process. It is also explained by Charlotte Ternetz (1912) in Anderson 2005 [21] that light has a role in cultivation, wherein the green color alga *Euglena viridis* becomes colorless when cultivated in the dark, however, become green again in the light.

### 4. Conclusion

The use of natural media for the cultivation of *Chlorella* sp. newly isolated from Bagansiapiapi waters could be conducted with bean sprouts extract, tofu waste, and fish pellet solution. However, the use of this media affects the growth of Chlorella cells. Based on these three treatments, the use of bean sprout extract media with light continuously was more optimal for the growth of *Chlorella* sp. on a laboratory scale. Therefore, this *Chlorella* sp. could be cultivated for further development as a bioactive source in nutraceutical or pharmaceutical products.

### 5. References

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