The effect of Aceh’s natural stone presence on the growth, produced biomass and primary metabolites production of *Chlorella vulgaris* LIPI12-AL042

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**Abstract.** This study sought to evaluate the influence of natural stone presence that was collected from Aceh Province, on the growth and the production of biomass, protein, total sugar and lipid of the microalgae *Chlorella vulgaris* LIPI12-AL042. Aceh’s natural stone used in this study were nephrite jade (NJ) and black jade (BJ) which were known to possess different minerals composition. The cellular growth of microalgae in terms of biomass yield and its productivity increased significantly (13.38%) under the presence of BJ stone compared to the control. Different results of reduced biomass yield and its productivity were demonstrated by the microalgae culture under the influence of NJ stone’s presence compared to the control. In terms of total sugar, both stones exhibited superior results over the control. The protein content and its yield of the *Chlorella vulgaris* LIPI12-AL042 cultivated under the influence of the stones demonstrated less quantity over the control.

1. **Introduction**

The growth of living organisms, among others, is affected by abiotic factors that include pH, salinity, temperature and moisture [1-4]. Abiotic factors are a non-living section in an ecosystem that make up the environment and significantly shape organism community. The stone is one of the abiotic factors composed by various minerals that might influence the life of organisms in an environment. The minerals content of the stone determines its characteristic in emanating such infrared radiation and, thus, could give such influences to living tissues existed in the environment [5].

Jade is one of the popular gemstones that were used for medical purposes, jewelry, ornaments, tools and religious artefacts. In Indonesia, major jade deposits are located in Aceh, North Sumatera and West Sumatera. Based on minerals composition, jades can be categorized as nephrite and jadeite. Nephrite jade (NJ) is a mineral belongs to the amphibole mineral group or often called the tremolite mineral group (Ca$_2$(Mg,Fe)$_5$Si$_8$O$_{22}$(OH)$_2$) [6,7]. NJ generally has green colour but it also has other variation colours like yellow, brown, white and grey [7]. Other stones considered as jade include serpentine, versuvianite or bio-solar gemstones from Aceh, etc [8]. Other famous stone from Aceh is black jade (BJ). By using X-Ray fluorescent, Ismail *et al.* [8] investigated the mineral composition of BJ stone. It is composed by SiO, Fe$_2$O$_3$, MgO as its predominant mineral compounds. Unlike NJ, BJ stone is not a jade, but a quartz mineral-hematite. NJ and BJ stone obtained from Aceh Province, Indonesia were used in this study.

NJ is a natural transmitter of far-infrared ray radiation that extends beyond the red color spectrum of visible light and have certain health benefits [5]. On the other hand, BJ transmits visible/near- and mid-
infrared ray radiation [9]. We hypothesize that the presence of nephrite and black jade stone in Chlorella vulgaris LIPI12-AL042 culture will have an influence on the growth and biochemical composition of the microalgae since both stones transmit infrared (IR) radiation that could interfere with the visible light spectrum. Therefore, this study was aiming at investigating the effect of the natural stones’ presence on the growth of microalgae and also on its primary metabolites content. The used of C. vulgaris LIPI12-AL042 is because their ability to grow under different cultivation conditions, high resistance against contaminants, and consist of beneficial compounds including protein, astaxanthin, β-carotene, and some polyunsaturated fatty acids (linoleic acid, linoleic acid, and eicosapentanoic acid) [10].

2. Methods

2.1. Microalgae, cultivation, and experiment setup
The microalgae strain used in this study was Chlorella vulgaris LIPI12-AL042, a collection of the Laboratory of Bioenergy and Bioprocess, Research Center for Biotechnology – Indonesian Institute of Sciences. The culture was maintained and grown in the AF6 medium. Microalgae cells were grown in a 600 mL bottle containing 500 mL microalgae culture with aeration and under continuous illumination (24 hours) with the light intensity of 25000 lux. A white LED flood-light (50 Watt) was used as a light source and was faced to the bottle wall. The experiment with 4 x 1.5 x 1.5 cm
natural stone size was conducted by placing the stone at the bottom of the bottle inside the microalgae culture during its cultivation for 7 days. Microalgae cultivation under the influence of natural stone existence was compared to the control treatment (the natural stone was absent). The cultivation of each treatment was conducted in triplicate.

2.2. Natural stones
Two natural stones (NJ and BJ) used in this study was acquired from Aceh’s Mountain area. BJ stone from Aceh contains SiO2, Fe2O3 and MgO as its predominant mineral compounds. BJ stone actually was not a jade, but a quartz mineral-hematite. NJ stone contains SiO2, MgO and CaO as its predominant mineral compounds. The composition of SiO2 minerals in NJ was higher compared to BJ stone [8].

2.3. Growth properties of microalgae
The growth of microalgae Chlorella vulgaris LIPI12-AL042 was determined by weighing microalgae dried biomass produced from every treatment of cultivation. The specific growth rate (μ) and doubling time (td) of Chlorella vulgaris LIPI12-AL042 in each treatment was calculated based on the equation as follows:

\[ \mu = \ln \frac{W_y}{W_x}/ (t_y - t_x) \]  

\[ t_d = \ln 2 / \mu \]

Wx : the dried weight of microalgae biomass at the earlier time (tx) of the logarithmic growth phase (3rd day), Wy : the dried weight of microalgae biomass at the end (ty) of the logarithmic growth phase (7th day).

Biomass productivity (Bp) was determined as the dried biomass produced during the exponential growth phase and was stated in milligram per liter per day (mg/L/day).

2.4. Microalgae protein extraction and analysis
For protein determination, an extraction of dried microalgae biomass was performed through incubation in NaOH 0.1 M at 70 ℃ for 45 minutes [11]. In the acquired extract, protein content was analyzed by
using the Bradford method [12], using bovine serum albumin as a standard. The analysis was performed in triplicate.

2.5. Microalgae total sugar extraction and analysis
For total sugar determination, an extraction of dried microalgae biomass was performed through incubation in the solution of H$_2$SO$_4$ 2.25% at 93°C for 70 minutes. In the acquired extract, total sugar was analyzed by adopting the Phenol-Sulphuric Acid method [13], using glucose as a standard. The analysis was performed in triplicate.

2.6. Microalgae lipid extraction
Microalgae lipid was extracted by adapting the modified method from Rykebosch et al. [14]. The extracted microalgae lipid was subsequently mentioned as lipid content and was reported as a percentage of the total biomass (in % dry weight). The extraction was performed in duplicate.

2.7. Productivity estimation of primary metabolites
Primary metabolites (Protein, Total Sugar, Lipid) productivity (Pp, Tp, and Lp) was calculated according to the equation (3, 4 and 5) as recommended by Nascimento et al. [15]:

\[
Pp = Bp \times Pc \tag{3}
\]
\[
Tp = Bp \times Tc \tag{4}
\]
\[
Lp = Bp \times Lc \tag{5}
\]

Pp/Tp/Lp : Protein/Total Sugar/Lipid productivity (mg/L/day) Bp : Biomass productivity (mg/L/day) Pc/Tc/Lc : Protein/Total Sugar/Lipid content (%).

2.8. Statistical analysis
To determine the significant difference among the groups (p<0.05), all average values of natural stone exposure treatments were analyzed against the control employing t-test analysis by using Microsoft Excel Software.

3. Results and Discussions
The measurement of growth kinetics, i.e., specific growth rate and doubling time, as seen in table 1, exhibited that the presence of the natural stone has negative effect on the growth rate compared to the control. T-test statistical analysis showed that the specific growth rate of control was significantly higher compared to BJ and NJ stone treatments. The slower growth of Chlorella vulgaris treated by the presence of BJ and NJ at the exponential growth phase might be caused by their spectral behaviour properties of both stones. According to Yoo et al [5], NJ stone radiates far-IR wave that extends beyond the red colour spectrum of visible light. On the other hand, according to Lane et al [9], BJ stone (hematite) emits mid- and near-IR wave. Both stones demonstrated to emits radiation below the visible light spectrum. Therefore, the emission energy from both stones was less than the energy required by the microalgae to make the photosynthesis performed properly. However, microalgae, such as Chlorella vulgaris, requires certain amount of energy at the range of visible light spectrum [16]. Thus, in this experiment it was shown that the IR wave emitted by the stones might destructively interfere with the visible light wave emitted by the light source causing the microalgae cell division at the exponential growth phase negatively affected.

Different results were obtained for biomass yield and productivity of the microalgae Chlorella vulgaris. The biomass yield and productivity of the microalgae which was cultivated under the influence of the BJ stone was higher compared to the control and NJ treatment (table 2). In this particular study, the trade-off between growth rate and biomass yield of the control and BJ treatments sample were not
in linear relationship. According to Lipson [17], there is considerable theoretical and empirical support for a negative relationship between growth rate and yield in microorganism metabolism. Some authors [18,19] offer a thermodynamic explanation on the trade-off between growth rate and yield: for a reaction to be 100% efficient, the energy of the products would equal that of the reactants, so the rate would be zero; a decreased ATP yield with energy lost as heat could speed up the reaction at the expense of efficiency. Here Lipson mentioned that the trade-off between growth rate and yield tends to create two divergent ecological strategies: fast-growing but inefficient versus slow-growing but efficient. Therefore, in this study, according to Lipson [17], the microalgae cultivated under BJ treatment stone might be included under the category of slow-growing but efficient; while the control might be fast-growing but inefficient.

**Table 1.** Specific growth rate and doubling time of *Chlorella vulgaris* LIPI12-AL042 under the effect of natural stone presence in the microalgae culture

| Cultivation Treatment | Specific growth rate (day⁻¹) | Doubling time (day) |
|-----------------------|------------------------------|---------------------|
| Control               | 0.183 ± 0.006⁹ª             | 5.47 ± 0.17⁹ª      |
| BJ (black jade stone) | 0.153 ± 0.004ª              | 6.54 ± 0.18ª       |
| NJ (nephrite jade stone) | 0.117 ± 0.008ª              | 8.57 ± 0.55ª       |

⁹ª values within a column (stone treatment vs control) are significantly different at p<0.05 (t-test)

Table 2 summarizes *Chlorella vulgaris*’ biomass yield and productivity after 7-day-cultivation under BJ and NJ treatments compared to control. *Chlorella vulgaris* under BJ treatment produced higher biomass production or yield than NJ treatment and control. The emission of near- and mid-IR wave by BJ stone [9] could significantly increase the production of *Chlorella vulgaris* biomass compared to the control. The enhance mechanism of BJ radiated near- and mid-IR on the biomass production was presumably due to the IR-energy absorption by biological tissues triggered for physiological responses enhancing microalgae ability in the utilization of medium nutrients [20]. Meanwhile, the emission of far-IR wave of NJ stone [5] could negatively affected the microalgae biomass production compared to the control. The result presumably was caused due to the IR energy radiated by the NJ was not enough to trigger physiological response due to its shorter wavelength (lower energy) compared to the BJ’s IR radiation. The effect of NJ which emits far-IR wave was the same as the result of the research conducted by Politaeva et al. [21] which investigated the effect of IR lamp used in the microalgae cultivation. Therefore, the NJ stone was not suitable to be used in the microalgae cultivation system which had target to increase biomass production. Instead, BJ stone could create supportive condition to increase biomass production. The using of BJ stone inside the *Chlorella vulgaris*’ culture could be the practical method to increase the yield of microalgae biomass in such economical ways.

**Table 2.** Biomass yield and biomass productivity of *Chlorella vulgaris* LIPI12-AL042 under the effect of natural stone presence in the microalgae culture

| Cultivation Treatment | Biomass Yield (mg/L) | Biomass Productivity (mg/L/day) |
|-----------------------|----------------------|---------------------------------|
| Control               | 710.00 ± 15.00⁹ª     | 118.33 ± 2.50⁹ª                  |
| Black jade stone      | 805.00 ± 5.00ª       | 134.17 ± 0.83ª                   |
| Nephrite jade stone   | 678.33 ± 10.41ª      | 113.06 ± 1.74ª                   |

⁹ª values within a column (stone treatment vs control) are significantly different at p<0.05 (t-test)

The primary metabolites content in microalgae, including carbohydrates (total sugars), lipids, and proteins, depend on the strain and culture condition [22,23]. Since the microalgae in this study were cultured under the presence of NJ and BJ, some alterations on the primary metabolites composition compared to the control condition was predicted. As shown in figure 1, the presence of NJ and BJ did
not significantly affect the lipid content of the microalgae cells. Carbohydrates content of *Chlorella vulgaris* cultivated under the NJ treatment showed the highest percentage (23.36%). On the other hand, the control and the BJ treatment exhibited similar results in their carbohydrates content (20.44% and 20.52%) respectively. Different results were also seen in the protein content of *C. vulgaris* where the control showed the higher percentage (6.06%) compared to the BJ (4.66 %) and the NJ (5.92 %) treatments. However, in order to investigate the influence of the natural stones presence on the metabolism process of microalgae cells, further study in the genome level were certainly necessitated.

### Figure 1.

The percentage of primary metabolites content in *Chlorella vulgaris* LIP112-AL042 cultivated under control (C), addition of black jade (BJ), and addition of nephrite jade (NJ) conditions.

Table 3 summarized the yield and primary metabolites (protein, total sugar and lipid) productivity *Chlorella vulgaris* LIP112-AL042. It can be seen that BJ and NJ affected the primary metabolites composition and its production rate compared to the control. The different influence of the stones on the biochemical composition can be seen in protein yield and productivity results (table 3). Both stones demonstrated negative influence over protein production compared to the control. IR emissions, whether it is near-, mid- or far-, radiated by the BJ and NJ stone significantly decreased the protein production by *Chlorella vulgaris* LIP112-AL042. Thus, under the exposure of BJ and NJ stones, microalgae fixed carbon as a product of photosynthesis process was not utilized for protein synthesis, but was transferred for carbohydrate synthesis. This result was quite different with the research conducted by Politaeva *et al.* [21] which demonstrated the positive influence of infrared radiation (using infrared lamp) on the protein production by the microalgae of *Chlorella sorokiniana*. According to Politaeva *et al.* [20], the inhibited growth of *C. sorokiniana* under the exposure of IR lamp provided more nitrogen for microalgae to be utilized in order to accumulate in the form of protein metabolite.

IR radiation (near-, mid- and far-) can be absorbed by biological tissues to be converted into oscillating energy of endogenous oxygen that will trigger physiological response activation at a bio-tissue level [20]. This particular response could be observed in this study at which the far-IR radiation
emitted by NJ stone could transfer photo-synthetically fixed carbon for carbohydrate synthesis. On the other hand, near- and mid-IR emitted by the hematite mineral contained in the BJ stone might trigger the microalgae to harness the photosynthetic-energy for biomass production in general. Therefore, the biomass of *Chlorella vulgaris* LPI12-AL042 produced under BJ treatment exhibited relatively higher productivity of total sugar and lipid compared to the other two treatments. This may be occurred due to the regulation of intracellular carbon influenced by the constructive interference of near- and mid-IR radiation with the visible light spectrum used in the microalgae cultivation. This particular result showed that the energy storage (carbohydrate and lipid) was a response to the environmental stress of IR spectral behaviour [21] of BJ, and it was higher compared to the control and the NJ stone treatment. Thus, the BJ stone could be used in the microalgae cultivation that is conducted with the intention to produce microalgae biomass for bioenergy production purposes.

**Table 3.** Primary metabolites yield and productivity of *Chlorella vulgaris* LPI12-AL042 with natural stone presence in the microalgae culture

| Cultivation Treatment | Protein Yield (mg/L) | Protein Productivity (mg/L/day) | Total Sugar Yield (mg/L) | Total Sugar Productivity (mg/L/day) | Lipid Yield (mg/L) | Lipid Productivity (mg/L/day) |
|-----------------------|----------------------|---------------------------------|--------------------------|------------------------------------|-------------------|-------------------------------|
| Control               | 43.14±0.28<sup>a,b</sup> | 7.19±0.05<sup>c</sup>       | 145.10±7.74<sup>e</sup>  | 24.18±1.29<sup>c</sup>             | 222.5±10.61       | 37.08±1.77                  |
| Black Jade stone      | 37.55±0.98<sup>d</sup>  | 6.26±0.16<sup>e</sup>       | 165.23±2.16<sup>e</sup>  | 27.54±0.36<sup>d</sup>             | 275±28.28         | 45.83±4.71                  |
| Nephrite Jade stone   | 40.20±0.15<sup>b</sup>  | 6.70±0.03<sup>d</sup>       | 158.46±3.30               | 26.41±0.47                        | 225±14.14         | 37.50±2.36                  |

<sup>a, b, c, d, e, f</sup> values within a column (stone treatment vs control) are significantly different at p<0.05 (t-test)

4. Conclusions
The addition of BJ natural stones on microalgae culture *Chlorella vulgaris* LPI12-AL042 could significantly enhance the biomass production and biomass productivity, which presumably was due to the absorption of near- and mid-IR energy enhancing the utilization of medium nutrients. The carbohydrates content and its production rate was significantly increase under BJ and NJ treatments that might be caused by constructive interference of IR radiation with visible light spectrum in microalgae cultivation system. On the other hand, the presence of NJ and BJ stones reduced protein content. Lipid content was not significantly different among all treatments.

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