Two stage feeding evaluation of cattle waste medium use in chlorella vulgaris culture and chlorella pyrenoidosa culture for simultaneous production of biomass

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Abstract. Stress in microalgae through the metabolic pathway increases the value of lipids and carotenoids. In this study, two stage feeding in Chlorella pyerenoidosa and Chlorella vulgaris are evaluated. BBM for Chlorella vulgaris and Sorrokin/Krauss Chlorella pyrenoidosa are using as standard medium which is label as A0/B0, cow manure Medium with fluorescence light exposure labelled as A1/B1, with sunlight exposure labelled as A2/B2 plus urea 0.1 g / l [1] with fluorescence rays exposureas A3/B3. Two stage feeding is evaluated in A4/B4 that compose medium A3/B3 which is added to A1/B1 medium on reaching the stationary phase. Which is Chlorella pyrenoidosa is labeled as A and Chlorella vulgaris in label B. The highest accumulation of biomass in Chlorella species was seen with the addition of urea in A3 and A4, but it had the lowest lipid content. During the experiment with the addition of water and nutrients from cow manure, the lipid values visibly increased, but not optimally. In Chlorella pyrenoidosa and Chlorella vulgaris species which is the object of this study, the lipid values on A1 and B1 are higher than the control and treatment with sunlight exposure, as well as the addition of urea.

1. Introduction
Chlorella species have high resistance and adaptability to the wastewater tested on them, because of their high durability and adaptation they can be used for multi-product production joints. Cow waste is used as a nutrient source for Chlorella species to preserve the environment and to obtain optimal biosynthesis for microalgae growth. To optimize growth, good results, and to reduce production costs, environmental engineering can be applied to achieve this. It is directed to the strategic arrangement of integrated circle concepts with multi-product production joints [2]–[4]. The biotechnological process of microalgae culture is promising across several industrial applications[5]. For the production of biomass, optimal culture conditions need to be evaluated. High biomass production is achieved using high carbohydrate/lipid levels. If the lipid and carbohydrate levels are equally high, the biodiesel and bioethanol joint production can be performed [6]. There are two hypothesis obtained, Firstly, the addition of nutrients to biomass accumulation. Secondly, microalgae culture is diluted with water for lipid accumulation. Alternative biomass or residues are harvested after lipid extraction [7].

Stress in microalgae causes differences in biomass accumulation[8] Microalgae in sufficient nutrient conditions will direct their biosynthesis towards protein production, if available nitrogen is exhausted, the biosynthesis shifts to the formation of food reserves including carbohydrates and lipids. The direction of
the metabolic shift depends the type of microalgae. Some microalgae produce lipids more while others carbohydrates [9]

2. Methodology
2.1 Materials and Tools
Microalgae isolates obtained from the Brackish Water Aquaculture Center (BBAP) of Jepara, owned by the Ministry of Maritime Affairs and Fishery, Republic of Indonesia. Chlorella vulgaris is isolated from sea water and Chlorella pyrenoidosa from fresh water. The culture for standar medium the Chlorella vulgaris isolate was the Bold Bassal Medium with composition of NaNO₃ gL⁻¹ (0.25), KH₂PO₄.3H₂O (0.075), KH₃PO₄ (0.175), MgSO₄.7H₂O (0.075), CaCl₂.2H₂O (0.084), FeSO₄.7H₂O (0.00498), EDTA, 2 Na-Mg Salt (0.05), NaCl (0.025), KOH (0.031) and H₂BO₄ μg L⁻¹ (11.42), MnCl₂.4H₂O (1.44), ZnSO₄.7H₂O (8.82), CuSO₄.5H₂O (1.57), Co (NO₃)₂.6H₂O (0.49), MoO₃ (0.71) in 1 liter of purified water and Chlorella pyrenoidosa isolates were cultured with sorokine / krauss (Sorokin & Krauss 1956) which is a special medium for culturing Chlorella species, with composition, KNO₃ (1.25), KH₂PO₄ (1.25), MgSO₄.7H₂O (1.0),CaCl₂.2H₂O (0.04), FeSO₄.7H₂O (0.05), EDTA, 2 Na-Mg Salt (0.5), and H₂BO₄ μg L⁻¹(114), MnCl₂.4H₂O (14), ZnSO₄.7H₂O (88), CuSO₄.5H₂O (16), Co (NO₃)₂.6H₂O (5), MoO₃ (7), with a final pH of 6.8[10]. The dry cow feces commonly used by farmers as organic fertilizer was used in the procedure. It was obtained from local cattle farmers. Feces samples were taken from the cows on the same day and filter paper.

The tool used consists of a 500mL container equipped with an aquarium pump and hose for aeration, filter paper, haemocytometer, microscope, android mobile.

2.2 Methods
This study consists of several stages
a. Dilution of cow manure and determination of dilution rate for microalgae growth, Chlorella pyrenoidosa was labeled A and Chlorella vulgaris was labeled B, after diluting 20X, the cow manure was soaked for 3 days before autoclaving. The results of dilution were added to purified water in a ratio of 1: 9 or 50ml of cow manure was added to 450ml of purified water. (obtained from previous research)
b. To find out the specific growth rate that existed during the research, a calculation was conducted to find the specific growth rate equation for biomass production in the logarithmic phase and the time needed for one cell division. Specific microalgae (k) growth rates were calculated by the formula Hirata et al (1981)

\[ \frac{\log[n_1/n_0]}{T_1-T_0} = K \]

This is the density of microalgae at time t, N₀ is the density of the initial microalgae, 3.222 is the constant, T₀ is the initial time and T₁ is the time of observation

Doubling time is calculated based on the formula \( \mu \max = 0.693 / \text{td} \)

Cell density is calculated with haemocytometer and calculations are done with cell calculator issued by Photonics Technology Lab version V2.2, an android application from the Google Play Store.

c. The results of the best test for specific growth rates are used for stage 2 of feeding. Different light sources are used for microalgae growth, biomass, and density of cow manure nutrient which was diluted using different light sources. The test results with lighting are also compared to the growth in urea fertilizer. Each treatment as follows:

a. A₀ / B₀ standar medium
b. A₁ / B₁: Cow manure medium with the best composition from previous study results with fluorescence light exposure
c. A2 / B2: Cow manure medium with the best composition from previous study with sunlight exposure
d. A1 / B1: Medium cow manure with the best composition from previous study plus urea 0.1 g / l (Yadavalli, S, & C.S.Rao, 2013) with fluorescence rays exposure.
e. A3 / B3: Medium A3 / B3 which is added to A1 / B1 medium on reaching the stationary phase.

1. Compound analysis, determination of protein content (Lowry et al, 1951), Determination of Lipid Levels with the Soxhlet Method, Determination of Carbohydrate Levels, Determination of Total Sugar Phenol H2SO4 Method (Dubios et al, 1956)

3. Result And Discussion

### Table 1. The Analyze of *Chlorella pyrenoidosa* compound

| Parameter          | A0     | A1     | A2     | A3     | A4     |
|--------------------|--------|--------|--------|--------|--------|
| Protein (gr/liter) | 14.49  | 14.09  | 17.68  | 43.97  | 41.96  |
| carbohydrate (%)  | 25.5   | 29.5   | 33.5   | 44     | 39     |
| Lipid              | 51     | 53.6   | 42.48  | 10.12  | 21     |
| Cl A               | 0.4772 | 0.3945 | 0.2038 | 0.4018 | 0.4    |
| Cl B               | 0.3538 | 0.5265 | 1.1937 | 1.0437 | 1.04   |
| Chl a+ Chl B       | 0.831  | 0.921  | 1.3975 | 1.4455 | 1.44   |
| SGR                | 0.215  | 0.291  | 0.307  | 0.177  | 0.160  |
| dt                 | 3.225  | 2.381  | 2.257  | 3.909  | 4.336466 |

### Table 2. The Analyze of *Chlorella vulgaris* compound

| Parameter          | B0     | B1     | B2     | B3     | B4     |
|--------------------|--------|--------|--------|--------|--------|
| Protein (gr/liter) | 12,898 | 16,08545817 | 15,687052 | 67,87829 | 65,06  |
| carbohydrate (%)  | 30     | 33.5   | 44.5   | 48.5   | 47.5   |
| Lipid              | 40.01  | 41.5   | 39.81  | 13.68  | 50.3   |
| Cl A               | 0.219  | 0.1793 | 0.9537 | 1.1443 | 1.150  |
| Cl B               | 0.711  | 1.0747 | 1.0933 | 0.4812 | 1.081  |
| Chl a+ Chl B       | 0.932  | 1.254  | 2.047  | 1.6255 | 2      |
| SGR                | 0.732  | 0.404  | 0.533  | 0.992  | 0.963  |
| dt                 | 0.946  | 1.716  | 1.300  | 0.699  | 0.482  |

3.1 Directions Shifts in Metabolism

In this study, standard medium A0 and B0 as well as microalgae, by giving cow manure A1 and B1 have smaller urea content compared to A3, A4, B3 and B4. The content difference in protein, lipid and carbohydrate content indicates a stress in the procedure. The difference is the addition of urea which increases the nitrogen content to a medium level. The dilution carried out on A0 and B0 decreases the turbidity levels, but has slightly lower nitrogen content compared to A3, A4, B3 and B4.

The highest accumulation of biomass in Chlorella species was seen with the addition of urea in A3 and A4, but it had the lowest lipid content. During the experiment with the addition of water and nutrients from cow manure, the lipid values visibly increased, but not optimally.

Generally, starch is the first polymer stored in the presence of excess organic carbon, and successively stored starch is gradually converted to bio oil and / or used for energy needs in biosynthetic activities. This transitional arrangement is very important. Bio production optimization is still an object of research which is not yet fully defined[5].

Stress in microalgae through the metabolic pathway increases the value of lipids and carotenoids. In *Chlorella pyrenoidosa* and *Chlorella vulgaris* species which is the object of this study, the lipid values on
A1 and B1 are higher than the standard medium and treatment with sunlight exposure, as well as the addition of urea. The difference in results on A1 and A2 with the same medium is due to the different responses to environmental factors from abiotic stress in the form of light and nutrients. In addition, some of the formed carbohydrates transformed to lipids. Furthermore, in accordance with the research of [5] as the concentration of carbohydrates metabolized in the medium decreases, the cell metabolism shifts and stored carbohydrates are converted to lipids (shifts of starch-lipids) [5]. Lipid content also increases because of the increase in alkalinity in *Chlorella vulgaris*, seen from changes in pH. Air bubbles can induce nitrogen levels related to changes in pH during culture. A lot of Nitrogen content increases cell concentration, but also reduces the lipid content. Cells are known to convert carbon like glucose to lipids as a metabolic reserve. In normal conditions, microalgae produces small amounts of TAGs, but can synthesize a ton of TAGs with significant fatty acids under stressful conditions. Addition of carbon sources (glucose) is carried out during the stationary phase. it increases the synthesis of lipid content and yield. [11]

4 Conclusion
Two stage feeding can increase biomass but decrease lipid or increase lipid but decrease biomass depend on treatment reaching medium in stationary phase, biotic and abiotic factor. During the experiment with the addition of water and nutrients from cow manure, the lipid values visibly increased, but not optimally. In *Chlorella pyrenoidosa* and *Chlorella vulgaris* species which is the object of this study, the lipid values on A1 and B1 are higher than the control and treatment with sunlight exposure, as well as the addition of urea.

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