The Optimal Condition on the Growth of Green
*Haematococcus Pluvialis* as one of the Future Natural Resources

Judy Retti Witono*, Alvin Gunadi, Herry Santoso, Arry Miryanti, Angela J. Kumalaputri
Department of Chemical Engineering, Faculty of Industrial Technology, Parahyangan Catholic University, 40141, Bandung, Indonesia

*e-mail: judy@unpar.ac.id

Abstract. In the developing world nowadays, the need of antioxidants as counteracting free radicals becomes more and more. Astaxanthin is a carotenoid pigment with high antioxidant content. It can be obtained from natural sources or chemical synthesis. *Haematococcus pluvialis* is one of natural sources of astaxanthin which accumulates large amounts of astaxanthin under stressful condition. The growth of *H. pluvialis* in the green phase determines the amount of astaxanthin that can produced. It is influenced by various conditions such as amount and type of growing medium, light intensity, pH of medium, temperature, and initial inoculum concentration. The goal of this research is to obtain the optimum condition on the growth of *H. pluvialis* at the green stage using the experimental design i.e. Central Composite Design (CCD). The variables observed were the effect of the initial inoculum concentration (25-35% v/v), the concentration of nutrients (1-2 mL/L liquid water), and the types of growth medium (Walne’s and BG-11). Before the experiment, *H. pluvialis* was cultured until its optical density (OD) = 1 – 1.2. The responses observed were the cell density based on optical density (OD) using a spectrophotometer and cell number counted by using haemacytometer every day during 5 days. The results were examined using the Design Expert 7.0. It showed that the optimal growth conditions of *H. pluvialis* cells was in BG-11 medium with initial inoculum concentration and the number of nutrients were 37.07% and 1.5 mL/L respectively. The highest cell number was found 23,75 x 10^6 cells / mL on the 3rd day. Whereas by using Walne media, the optimum condition was obtained at the initial inoculum concentration and the amount of nutrients were 35% and 2 mL respectively. And the highest cell density number obtained was 17,5 x 10^6 cells / mL on the 3rd day.

Keywords: Astaxanthin, BG-11, Central Composite Design, Haematococcus pluvialis, Walne’s

1. Introduction
Our earth is abundant with natural resources which make us surviving. From time to time humans’ consumption is growing prodigiously. According to a new UN report, the amount of the planet’s natural...
resources extracted for human use has tripled in 40 years. Therefore, it should be explored the natural resources that can pursue those needs. Recently, microalgae have attracted considerable interest worldwide, due to their extensive application potential such as renewable energy, bioactive supplement, and functional food. In addition, a beneficial character of microalgae is renewable and sustainable.

Microalgae are categorized as a heterotrophic culture. This term appeared in 1946 in microbiology to define a microorganism which cannot produce its own food and need for exogenous supply of one or more essential metabolites (growth factors and vitamins) [1]. Furthermore, the heterotrophs are said as organism whose substrate and energy needs are derived from organic compounds synthesized by other organism [2-4]. According to the energy required for heterotrophic growth, it can be supplied by chemical energy (chemoheterotrophic) or light (photoheterotrophic).

Microalgae can be cultured by different methods and under different conditions [4-8], but hardly to find the cultivation method for H. pluvialis as the richest source of natural astaxanthin (anti-oxidant). Whereas, the amount of astaxanthin in H. pluvialis occurs in red cyst cells under unfavorable environmental conditions of their green vegetative cells. Therefore, the growth rate of cells in green stage phase should be maximized. Some variables are indicated affect the growth rate such as light [9], nutrition [9, 10], media [11] etc. So, the goal of this research is to determine the optimum condition on the growth of H. pluvialis at the green stage based on their initial inoculum concentration, the concentrations of nutrients and the types of growth medium.

2. Materials and Methods
A total of twenty-six 250 mL pyrex media bottles were used for this experiment. The ratio of Walne and BG-11 medium to algae in each bottle matched as in the table 1. Haematococcus pluvialis was obtained from Ugo Plankton Shop at kabupaten Purworejo, Jawa Tengah. The inoculum cells were cultivated up to OD = 1-1,2 prior to be used. The stock cultures were grown photoautotrophically at 28 ± 1°C under continuous light intensity of 3200 lux with dark: light cycle was 18:6 hours and aerated at 0.2 L/min. Culture density and cells number were monitored in 5 days consecutively. The composition of Walne and BG-11 medium used in this experiment can be seen in appendix.

2.1. The Cell Number Calculation Using Haemacytometer

| Table 1. The varied concentrations of inoculum and nutrient in this experiment |
|---------------------------------------------------------------|
| **Walne medium** | **BG-11 medium** |
| Run | [Inoculum] (%) | [Nutrient] (mL/L culture) | Run | [Inoculum] (%) | [Nutrient] (mL/L culture) |
| A1 | 37.07 | 1.5 | A2 | 37.07 | 1.5 |
| B1 | 30 | 0.79 | B2 | 30 | 0.79 |
| C1 | 35 | 2 | C2 | 35 | 2 |
| D1 | 30 | 1.5 | D2 | 30 | 1.5 |
| E1 | 22.93 | 1.5 | E2 | 22.93 | 1.5 |
| F1 | 30 | 2.21 | F2 | 30 | 2.21 |
| G1 | 35 | 1 | G2 | 35 | 1 |
| H1 | 30 | 1.5 | H2 | 30 | 1.5 |
| I1 | 25 | 1 | I2 | 25 | 1 |
| J1 | 25 | 2 | J2 | 25 | 2 |
| K1 | 30 | 1.5 | K2 | 30 | 1.5 |
| L1 | 30 | 1.5 | L2 | 30 | 1.5 |
| M1 | 30 | 1.5 | M2 | 30 | 1.5 |
The cell number was counted using the haemacytometer. It has 9 counting rooms, which each room divided into 25 boxes with volume=1x10^-4 mL. The counting was done 5 times in different boxes and then the result is an average number of it.

2.2. *The growth rate Measurement*

The mean growth rate (μ) was calculated on cell number basis according to the equation 1:

\[
\mu \ (\text{day}^{-1}) = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \ldots \ldots \ldots (1)
\]

Where \(X_1\) and \(X_2\) in the equation represent the cell number at the times \(t_1\) and \(t_2\), respectively.

3. *Results and Discussion*

The initial culture of *H. pluvialis* from Ugo Plankton has the OD value 0.7092 (using a wavelength 680 nm) and cell numbers 3.8 x 10^6 cells/mL (using haemacytometer). By plotting a graph of optical density (OD) on X-axis and absorbance value in the Y-axis, resulted equation of \(y = 43,936 \times \) with a correlation value \(R^2 = 0.9857\). *The H. pluvialis* was cultivated until OD = 1-1.2 prior to be distributed into 26 media bottles which contained nutrients at different concentration.

The figure 1 and figure 2 illustrates the growth of *H. pluvialis* during 5 days for every variation of conditions. From this bar graph it can be seen that without additional nutrients, the growth of *H. pluvialis* could not be maintained. It only reached 3-4 days and after that collapsed. Some researchers have observed that the nutrients not only contribute to the growth cycle of microalgae but also to the production of biomass and some certain bioactive components [12, 13].

From the observations, it can be seen that each sample gave a different *H. pluvialis* growth. In general, cultures that started a death phase on the 3rd day were cultures with an initial inoculum concentration of 30%. Samples with an initial inoculum concentration of 25% tended to grow until the 4th day. This could occur because the ratio of a number of cultures and a concentration of nutrient was not appropriate so that the nutrients provided were insufficient for the cell growth. Moreover, the competitive among of *H. pluvialis* cells to get nutrients was higher at initial inoculum concentration of 30% than 25%.

![Figure 1. The Growth rate of *H. pluvialis* during 5 days in Walne medium](image)

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In Walne media (figure 1), the number of *H. pluvialis* cells only reached average 2-folds without the addition of nutrients, whereas in BG-11 media was higher i.e. around 2.5-folds. But in term of cell density, the figure 3 showed a significant additional. At the 5th day, the cells were denser than at the 1st day. Figure 3 (a) showed the cell density at the 1st day and figure (b) showed the cell density at the 5th day. So it's acceptable that cells were difficult to develop in such media. In addition to nutrition competition, space competition also took place. It is thus important to consider also the initial concentration of the inoculum and also the amount of nutrition that is given.

From figure 1 and figure 2 can be seen as well that at the same variation of concentration of initial inoculum and nutrients it was obtained slightly different results of the growth cells of *H. pluvialis*. It might be, there were another variable which affect the growth cells which was not observed but maintained constant during the experiment such as light and aeration rate.

Another problem that cannot be avoided was the growth of fungi and microbes around *H. pluvialis* cells especially when cells were getting bigger and their movements were slowing down. As Lee and Zhang [12] stated that the vegetative cultivation of *Haematococcus* is the most challenging task owing to its slow growth rate, low cell density and susceptibility to contamination. Therefore, optimization
of the culture conditions becomes essential to support the growth cell of \textit{H. pluvialis}, which further the amount of bioactive components obtained.

4. Conclusions

- In \textit{H. pluvialis} cultivation, the used of BG-11 medium is better than Walne medium in term of the growth rate, although the folding number of cells in both medium has not been as expected.
- During its growth, microalgae needs a constant supply of nutrients.
- There are still some variables which should be observed during the cultivation and also the interaction between variables should be considered as well.

Acknowledgement

The authors would like to express their thanks to the Director General of Research and Development Reinforcement (Ministry of Research, Technology and Higher Education) - Direktorat Riset dan Pengabdian Masyarakat, Direktorat Jenderal Penguatan Riset dan Pengembangan RISTEKDIKTI, which finances this research and also Research Institutions and Community Development - Parahyangan Catholic University (LPPM – UNPAR) who supported the authors in the preparation of this research. Special thanks are addressed to our research group team who made this research can be carried out.

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### Appendix

#### A. The composition of Walne medium

| Component                                      | Concentration |
|-----------------------------------------------|---------------|
| **(1) Trace Metal Solution (TMS)** per 100 mL |               |
| ZnCl₂                                         | 2.1 g         |
| CoCl₂·6H₂O                                    | 2.0 g         |
| (NH₄)₆Mo₇O₂₄·4H₂O                             | 0.9 g         |
| CuSO₄·5H₂O                                    | 2.0 g         |
| **(2) Vitamin solution** per 100 mL           |               |
| Vitamin B12 (Cyanocobalamin)                  | 10 mg         |
| Vitamin B1 (Tiamine HCl)                      | 10 mg         |
| Vitamin H (Biotin)                            | 200.0 µg      |
| **(3) Nutrient solution** per liter            |               |
| FeCl₃·6H₂O                                    | 1.3 g         |
| MnCl₂·4H₂O                                    | 0.36 g        |
| H₃BO₃                                         | 33.6 g        |
| EDTA (Disodium salt)                          | 45 g          |
| NaH₂PO₄·2H₂O                                  | 20 g          |
| NaNO₃                                         | 100 g         |
| TMS                                           | 1 ml          |

**Medium** per liter
- Nutrient solution 1.0 ml
- Vitamin solution 0.1 ml
- Sterilised seawater 1.0 liter

#### B. The composition of BG-11 medium

| Component                                      | Concentration |
|-----------------------------------------------|---------------|
| **(1) Trace Metal Solution (TMS)** per 100 mL |               |
| H₃BO₃                                         | 2.86 g        |
| MnCl₂·4H₂O                                    | 1.81 g        |
| ZnSO₄·7H₂O                                    | 0.222 g       |
| NaMoO₄·2H₂O                                   | 0.39 g        |
| CuSO₄·5H₂O                                    | 0.079 g       |
| Co(NO₃)₂·6H₂O                                 | 49.4 mg       |
| **(2) Distilled water**                       | 1.0 L         |
| NaNO₃                                         | 1.5 g         |
| K₂HPO₄                                         | 0.04 g        |
| MgSO₄·7H₂O                                    | 0.075 g       |
| CaCl₂·2H₂O                                    | 0.036 g       |
| Citric acid                                   | 0.006 g       |
| Ferric ammonium citrate                       | 0.006 g       |
| EDTA (disodium salt)                          | 0.001 g       |
| Na₂CO₃                                         | 0.02 g        |

**Medium** per liter
- Trace Metal Mix (TMM) 1.0 mL
- Distilled water 1.0 L