H₂O₂ as a fungicide in the growth phase of green
*Haematococcus pluvialis*

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**Abstract.** One of the potential sources for bioenergy and biochemical products is *Haematococcus pluvialis*. However, these microalgae are vulnerable to chytrid infection when the cells are transitioning from the green flagellates’ stage to the resting stage. As a result, the productivity of their biomass is low. The objective of this research was to evaluate the effects of hydrogen peroxide as the fungicide, including those which were combined with fluazinam, on the growth of *H. pluvialis*. The variables used in this research were the dose of H₂O₂, the combination with fluazinam, and the frequency of H₂O₂ treatment. The initial cell number of *H. pluvialis* was 5 x 10⁴ cells/mL culture medium. The 5 different treatments were done, i.e., (1) 0.028 mL H₂O₂/L liquid culture dosed every day; (2) 0.028 mL H₂O₂/L liquid culture dosed every day for only 3 days and then every two days combined with single dose 0.5 ppm of fluazinam on the first day; (3) same as (2) but without combination with fluazinam; (4) 0.035 mL H₂O₂/L liquid culture dosed every day for 3 days then every two days combined with single dose 0.5 ppm of fluazinam on the first day; (5) same as (4) but without combination with fluazinam. As a comparison, there was also a culture without any treatment as a control. The culture which was treated with hydrogen peroxide 0.028 mL H₂O₂/L liquid culture dosed every day for only 3 days and then every two days at the 7th day showed the highest cell number (45 x 10⁴ cells mL⁻¹ ~ 9 fold from the initial cell number), even compared to the culture without any treatment. In conclusion, the treatment with hydrogen peroxide is effective against chytrid infection. The combination with fluazinam does not affect more to the effectiveness of H₂O₂ as a fungicide. It looks that by adding fluazinam, the *H. pluvialis* growth is depressed.

1. **Introduction**

The issue of limited petroleum resources in the world leads us to find a new material that can substitute it. One of the materials that can be sustainably produced in the future is microalgae. Microalgae contains macro- and micronutrients and bioactive components which are potential for human nutrition and promoting health. Furthermore, they are also a potential source for bioenergy and biochemical products.

The freshwater green microalga *Haematococcus pluvialis* has been receiving much attention nowadays because under stress condition it has the capacity to accumulate very high levels of the carotenoid astaxanthin [2, 3]. Therefore, it is a good source of ingredients for functional food. However, this microalga can be utilized more than just for that since it is rich of other bioactive components, as shown in Table 1.
The main problem on the green stage of *Haematococcus pluvialis* cultivation is parasites, which found growing epibiotically on algal cells and caused damage to the host cultures. The common microbial contaminants occurring in mass culture of *Haematococcus pluvialis* are fungal parasites, zooplankton predators (e.g., amoebas, ciliates, and rotifers), cyanobacteria, and several other eukaryotic microalgae. Of these contaminants, zoosporic fungi (chytrids) identified as the most dangerous one responsible for reduced astaxanthin productivity and frequent culture collapse [4-6].

Hydrogen peroxide is well known effective against all forms of microorganisms, including dormant forms with known high resistance such as bacterial spores and protozoal cysts [7]. However, the effective and safe use of hydrogen peroxide depending on the way it is used, in particular, the concentration [8, 9].

Therefore, the goal of this research is to determine the effectiveness of hydrogen peroxide concentration, which is combined with fluazinam as a micro biocide at the green stage.

### Table 1. Typical common components of *Haematococcus pluvialis* [1]

| Component          | Minimum  | Maximum  | Mean   |
|--------------------|----------|----------|--------|
| Protein (%)        | 17.30    | 27.16    | 23.62  |
| Carbohydrates (%)  | 36.9     | 40.0     | 38.0   |
| Fat (%)            | 7.14     | 21.22    | 13.80  |
| Iron (%)           | 0.14     | 1.0      | 0.73   |
| Moisture (%)       | 3.0      | 9.0      | 6.0    |
| Magnesium (%)      | 0.85     | 1.4      | 1.14   |
| Calcium (%)        | 0.93     | 3.3      | 1.58   |
| Biotin (mg/lb)     | 0.108    | 0.665    | 0.337  |
| L-carnitine (ug/g) | 7.0      | 12       | 7.5    |
| Folic acid (mg/100g)| 0.936 | 1.48     | 1.30   |
| Niacin (mg/lb)     | 20.2     | 35.2     | 29.8   |
| Pantothenic acid (mg/lb)| 2.80 | 10.57    | 6.14   |
| Vitamin B1 (mg/lb) | <0.050   | 4.81     | 2.17   |
| Vitamin B2 (mg/lb) | 5.17     | 9.36     | 7.67   |
| Vitamin B6 (mg/lb) | 0.659    | 4.5      | 1.63   |
| Vitamin B12 (mg/lb)| 0.381    | 0.912    | 0.549  |
| Vitamin C (mg/lb)  | 6.42     | 82.7     | 38.86  |
| Vitamin E (IU/lb)  | 58.4     | 333      | 186.1  |
| Ash (%)            | 11.07    | 24.47    | 17.71  |

### 2. Materials and Methods

#### 2.1. Materials and culture conditions

*Haematococcus pluvialis* UTEX Collection Strain 2505 was purchased from Algae Analytics, US, and has been cultivated for almost one year in our laboratory. During that time, the inoculum was contaminated. Hydrogen peroxide 50% from Sigma Aldrich was standardized prior to use. Whereas fluazinam (Nando 500 SC) with active ingredient 500g/L was purchased from Nufarm, Indonesia. The stock cultures were grown photoautotrophically in Modified Bold’s Basal 1N-3P medium with thiamine 0.1 mg/L to substitute vitamin B12 and biotin. Every run was prepared from 50% stock culture, which contains 5 x 10⁴ cells/mL in 250 mL cylindrical glass bottles. The culture was illuminated with white-blue LED lamps at a light intensity of 3200 lux with dark:light cycle of 10:14 h. The cells were grown in batch cultures and aerated by bubbling air with the aeration rate of 2 L/min under the atmospheric temperature 25±1°C.
2.2. Investigating the effect of hydrogen peroxide

Five different treatments and concentrations of hydrogen peroxide were given to the contaminated culture with the initial cell numbers of 5 x 10⁴ cells/mL. The variations in each run can be seen in Table 2. The contaminated culture grown in the absence of hydrogen peroxide was used as a control. The cell growth based on cell number counting in every run was monitored for 11 days consecutively.

| Run | [H₂O₂] added | Fluazinam added | Frequency of H₂O₂ added |
|-----|-------------|----------------|-------------------------|
| control | -           | -              | -                       |
| 1    | 0.028 mL H₂O₂/L medium | -              | daily                   |
| 2    | 0.028 mL H₂O₂/L medium | 0.5 ppm       | daily for 3 days then every two day |
| 3    | 0.028 mL H₂O₂/L medium | -              | daily for 3 days then every two day |
| 4    | 0.035 mL H₂O₂/L medium | 0.5 ppm       | daily for 3 days then every two day |
| 5    | 0.035 mL H₂O₂/L medium | -              | daily for 3 days then every two day |

2.3. Growth measurement

Cell number was observed using an improved Neubauer hemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany) under a binocular microscope (Olympus, Tokyo, Japan) at 400X magnification. The cells growth rate constant (μ) was estimated based on the following first-order equation:

\[
\frac{dx}{dt} = \mu X
\]  

Where X and t represent the cell number (cells/mL) and time (days), respectively.

3. Results and Discussion

Figure 1 shows the growth of contaminated Haematococcus pluvialis cultures, either treated with or without hydrogen peroxide and fluazinam. In general, the cell number of microalgae in all variation decreased after 7 days. In the contaminated culture without micro biocides treatment (i.e., control run), the cell number of microalgae collapsed totally, mostly due to the fungus attack on the cell wall beside the lack of nutrients source for the biosynthesis of microalgae. It can be observed from Figure 2(a), (b) and (c). Figure 2(a) shows the healthy cell of Haematococcus pluvialis. Figure 2(b) shows a green palmella cell that starts to be infected by chytrid sporangia and lysis. Figure 2(c) shows the empty cell. Spores grow rapidly and evolve into sporangia, which caused cells regeneration does not happen.

Induction of 0.028 mL hydrogen peroxide /L to the cultivation medium can reduce the average chytrid infection without negatively impacting the growth rate of microalga. The cell number can increase by 9 times from 5x10⁴ to 45x 10⁴ on the 7th day (see Run 3). The same tendency occurs when fluazinam induced together with H₂O₂ into the medium culture (i.e., Run 2). But it seems that the use of H₂O₂ in combination with fluazinam suppresses not only the growth of fungi but also at the same time the growth of microalgae itself. The cell growth only reach 5 fold from 5x10⁴ to 25x10⁴ at the 7th day.

The frequency of H₂O₂ induction also affects the microalgae growth. From the experiment Run 1 and Run 3, it was observed that the induction of H₂O₂ every day gave a lower cell number on the 7th day compared to the sample with the induction of H₂O₂ given only every two days after every day for 3 days. Hydrogen peroxide as an oxidizing agent, in the cultivation system, oxidizes biological macromolecules, namely carbohydrates, lipids, proteins, and nucleic acids. Cells become stressed which can cause DNA damage after such a long exposure to hydrogen peroxide. Furthermore, as the consequences, the growth and multiplication of the targeted microalgae is inhibited [7].
In the matter of the amount of hydrogen peroxide concentration used (Run 3 and Run 5), the culture treated with 0.028 mL/L produces higher cell numbers than the culture treated with 0.035 mL/L H₂O₂ for the same treatment frequency. The high concentrations of hydrogen peroxide might be effective for reducing chytrid infections; nevertheless, the cell lysis was also high.

Compare to our previous study [10], it is shown that the effect of H₂O₂ as a fungicide in the medium culture of H. pluvialis is more effective than fluazinam. In the use of fluazinam as a single fungicide, the H. pluvialis growth only reaches 5 times from the initial cell number. It is lower compared to the H. pluvialis growth in the use of H₂O₂ as a single fungicide.

The cell growth rate constant μ (see Equation 1) for the first seven days was estimated using a nonlinear regression method that minimizes the sum of square error (SSE) of cell number X. The results are shown in Table 3.
The higher the value of the cell growth rate constant $\mu$ is the higher the cell growth rate of the culture in the system. The values of the cell growth rate constants $\mu$ estimated using the nonlinear regression method are consistent with the explanation given in the discussion above.

4. Conclusion

Hydrogen peroxide is a good fungicide for *Haematococcus pluvialis* cultivation. A combination of hydrogen peroxide and fluazinam as a fungicide is not necessary because it can suppress not only the growth of fungi but also the growth of microalga. Furthermore, the use of a higher concentration of $\text{H}_2\text{O}_2$ as well as the more frequent introduction of $\text{H}_2\text{O}_2$ can inhibit the microalga growth.

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References

[1] Dr. Lorenz R T 1999 A technical review of *Haematococcus* algae *Naturose*TM Technical Bulletin #060

[2] Aflalo C, Meshulam Y, Zarka A and Boussiba S 2007 On the relative efficiency of two- vs. one-stage production of astaxanthin by the green alga *Haematococcus pluvialis* *Biotechnology and Bioengineering* 98(1) 300–5

[3] Borowitzka M A 2018 *Biology of Microalgae* in *Microalgae in Health and Disease Prevention* I. A. Levine and J. Fleurence Eds. pp 23–72

[4] Carney L T and Lane T W 2014 Parasites in algae mass culture *Frontiers in Microbiology* 5 278

[5] Gutman J, Zarka A and Boussiba S 2009 The host-range of *Paraphysoderma sedebokerensis*, a chytrid that infects *Haematococcus pluvialis* *European Journal of Phycology* 44(4) 509–14

[6] Han D, Li Y and Hu Q 2013 *Biology and Commercial Aspects of Haematococcus pluvialis* in *Handbook of Microagal Culture: Applied Phycology and Biotechnology* Richmond A and Hu Q Eds. (USA: John Wiley & Sons) pp 388-405

[7] McDonnell G 2014 The use of hydrogen peroxide for disinfection and sterilization applications *PATAI’S Chemistry of Functional Groups*

[8] Carney L and Sorensen K 2016 Methods for preventing lysis in a culture of *Haematococcus pluvialis* using hydrogen peroxide *US Patent* 9,347,034 B1

[9] Carney L T and Sorensen K 2015 Methods for treating a culture of *Haematococcus pluvialis* for contamination using hydrogen peroxide *US Patent* 9,113,607 B1

### Table 3. The cell growth rate

| Run | $\mu$ (day$^{-1}$) | SSE   |
|-----|-------------------|-------|
| control | 0.296            | 97.562 |
| 1   | 0.307             | 24.527 |
| 2   | 0.270             | 6.602  |
| 3   | 0.384             | 113.050|
| 4   | 0.327             | 9.653  |
| 5   | 0.327             | 15.328 |
[10] Witono J R, Novianty V, Santoso H, Miryanti A and Kumalaputri A J 2019 Fluazinam potential as a fungicide in liquid culture system for the growth of Haematococcus pluvialis microalgae

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