Fluazinam Potential as a Fungicide in Liquid Culture System for the Growth of *Haematococcus pluvialis* Microalgae

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Abstract. All across biological system, fungal infection in freshwater systems is well known to obstruct the aquatic biomass population such as microalgae. However, there is a lack of extensive information on the suitable technique to control them. This research aims to assess the effects of the fungicide fluazinam as a single dose treatment on the *Haematococcus pluvialis* growth rate and the presence of chytrids fungus in a liquid culture system. The variables used in this experiment were the concentration of fluazinam given to the culture (i.e. 0.25; 0.5; 0.75; 1; 1.25 ppm) as a single dose treatment. The growth performance of *H. pluvialis* was observed in 6 days consecutively based on the cell density by a single direct absorbance measurement and the cell counting by haemocytometer. The best growth was achieved in a culture system which was given fluazinam at a concentration of 0.5 ppm (started from 1.0 x 10⁵ cells/mL to 5.5 x 10⁵ cells mL⁻¹ on the 5th day) and no chytrids fungus are found in the culture system either. So, in conclusion, the use of fluazinam is a promising treatment for removing contaminants that inhibit *H. pluvialis* growth.

1. Introduction

More than 7000 species of green microalgae are found growing in a variety of habitats. One of them is *Haematococcus pluvialis* which becomes a commercial interest due to its ability to produce the highest level among all known astaxanthin producing organisms. It may reach up to 5 % dry weight in the encysted aplanospore state [1] and can reach 95% of the total carotenoids [2].

Astaxanthin (3,3′-dihydroxy-ß-carotene-4,4′-dione) is a bright red secondary carotenoid from the same family as lycopene, lutein, and ß-carotene, synthesized by some microalgae, plants, yeast [3]. Astaxanthin has a wide range of applications in the food, feed, cosmetic, aquaculture, nutraceutical, and pharmaceutical industries because of its free radical scavenging capacity [4]. The natural astaxanthin exhibited higher bioactivity as compared to the synthesized astaxanthin [5, 6]. In general, astaxanthin is produced by Haematococcusis through a two-stage culture: vegetative (green) and aplanospore (red) stages [7, 8]. At first stage cultivation, *H. pluvialis* cells are green and vegetative under favourable environmental conditions (i.e. nitrogen sources, light intensities, aeration rates, temperature and nutrients), and once subjected to stress conditions like high light, cells begin to synthesize and accumulate astaxanthin, transforming green vegetative stage to red aplanosposes [9].
Due to the complex life-cycle of *H. pluvialis* it is important to maximize cell densities of alga at the “green stage” of cultivation by maximizing cell growth to obtain high biomass with high astaxanthin yield from the “red stage” [10]. However, vegetative cultivation of Haematococcus is the most problematic part due to the susceptibility to contamination which results in a slow growth rate or low cell concentration. Fungal contamination by chytrids has been recognized as one of the most serious hurdles for producing astaxanthin from the green algae *H. pluvialis* [11]. Moreover, the process of culturing a small volume *H. pluvialis* culture through the green swimmer stage to a large volume in the red cyst stage may take weeks due to the cultivation of Haematococcus involves growing, dividing, and accumulating carotenoids [12]. During this time the *H. pluvialis* cells are vulnerable, weaken the physical integrity of the cells (e.g., lysis) and easily attacked by contamination which reduces the chances of *H. pluvialis’s* survival in both the green swimmer and red cyst stages [13].

Fluazinam is known as a fungicide with broad-spectrum activities which was commercialized since the early 1990s. The good characteristics of this fungicide control every stage of a fungal life cycle, multi-site action, no cross-resistance to other fungicides, safe and beneficial. Its chemical structure can be seen in figure 1 and its IUPAC name is 3-Chloro-N-(3-chloro-5-trifluoromethyl-2-pyridyl)-α, α, α-trifluoro-2,6-dinitro-p-toluidine.

The species sensitivity of freshwater organisms such as invertebrates and algae to fluazinam has been assessed by Van Wyngarden et al. [14] and showed a good result. It has been tested as well in liquid culture systems for the growth of microalgae [15] combined with other fungicide. But until now it is hard to find research on the application of fluazinam as a single dose treatment. Therefore, the goal of this research is to study the application of fluazinam as a single dose treatment on the *Haematococcus pluvialis* growth rate and the presence of chytrids fungus in a liquid culture system.

### 2. Materials and Method

#### 2.1. Materials and culture conditions

*Haematococcus pluvialis* UTEX Collection Strain 2505 was obtained from Algae Analytics, US, and has been cultivated for 1 (one year) in our laboratory. During that time, the inoculum was contaminated by fungus. Indeed, fluazinam (Nando 500 SC) with active ingredient 500g/L was purchased from Nufarm, Indonesia. The stock cultures were grown photoautotrophically in Modified Bolds 1N-3P medium without soil water, vitamin B12, and biotin (only thiamine 0.1 mg/L). The culture cell concentration was 1 x 10^5 cells/mL when 200mL *H. pluvialis* starter cultures were carried out into 250 mL erlenmeyer flask. The cultures were illuminated with white-blue LED lamps at light intensity of 3200 lux with dark: light cycle was 10:14 hours. The cells were grown in batch cultures and aerated at 0.2 L/min. under the temperature of the environment at 25.7±1°C without any additional CO2 (see figure 2 below).

#### 2.2. Investigating the effect of fluazinam

Five different concentration of fluazinam (i.e. 0.25; 0.5; 0.75; 1; and 1.25 ppm) were given to the contaminated culture (initial cell numbers 1 x 10^5 cells/mL) as a single dose. The contaminated culture grown in the absence of fluazinam was used as a comparison. Culture density and cells number were monitored in 6 days consecutively.

![Figure 1. Fluazinam – phenyl pyridinamin](image-url)
2.3. The growth rate measurement

The optical density was measured using a spectrophotometer UV-6100PC Double Beam (Mapada, Shanghai, China). The absorption peak of green *H. pluvialis* cells was obtained at 680 nm. Cells number was quantified using an improved Neubauer haemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany) at 400x magnification with a binocular microscope (Olympus, Tokyo, Japan).

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

$$
\mu \ (\text{day}^{-1}) = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (\text{eq. 1})
$$

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 presence of fungus in the liquid culture medium is not just a significant biological problem but furthermore is an economic challenge to the commercial cultivation of algae in industrial scale [16]. Han et.al [3] indicated that the fungal contamination by chytrids has been recognized as one of the most serious hurdles for producing astaxanthin from the green algae *Haematococcus pluvialis*. By applying fluazinam into the medium it was expected that the presence of fungus would be reduced. But as fungicide action has side effects on nonfungal microorganisms as well, therefore, it might affect the *H. pluvialis* growth rate [17]. It can be seen from figure 3, although the cell density (≈ optical density of the cell) represents the absorbance of both living and dead cells, whereas the cell number only measures the living cells, both of the graphs illustrate the same pattern.

3.1. The effect of fluazinam on the growth rate of *H. pluvialis*

From figure 3 can be seen that *H. pluvialis* in all cultures after being treated by fluazinam on the first day, did not grow, even some of the cell died especially at the culture with high concentration of fluazinam. The cells number of *H. pluvialis* in a contaminated culture in the absence of fluazinam and the presence of 0.25 ppm fluazinam just really collapsed on the 4th day. These were most likely due to the lack of nutrients source for the de novo protein biosynthesis of microalgae as filamentous fungus wrapped and bound from

![Figure 2. Set up of the experiments](image)

![Figure 3. The Growth of *H. pluvialis* during treated by various concentrations of fluazinam (a) based on cell number (b) based on culture density](image)
one to another alga. Besides, in the contaminated culture with 0.5 ppm or higher concentrations of fluazinam, more fungus could be reduced. Therefore, they were still increasing on the 4th day and so forth.

On the 6th day, the culture in the presence of 0.5 ppm of fluazinam resulted the highest cell number (5.5 x 10^5 cells mL^-1), it was about 5 times from the initial number. The cell number in the culture with 0.75 and 1 ppm of fluazinam resulted in 4 x 10^5 cells mL^-1 and 2.5 x 10^5 cells mL^-1 respectively. They had a similar trend when viewed from the culture density side. The culture with fluazinam at a concentration of 0.5 ppm resulted in microalgae density at 0.59 ODU which was about 3 times the density of microalgae grown in the absence of fluazinam. It was also observed that the culture with fluazinam at concentrations 0.5 ppm, 0.75 ppm and 1 ppm were still growing on the 6th day.

The culture grown in 1.25 ppm of fluazinam had the same number of cells (2 x 10^5 cells mL^-1) on the 4th and 5th day. However, there was a slight increase in culture density between 4 and 5 days of the culture grown in 1.25 ppm of fluazinam. That might happen because the cell density represents both living and dead cells as explained above. It means the rate of cell reproduced and collapsed were almost similar at that time.

This shows that the higher concentration of fluazinam might cause a decrease in microalgae growth. However, an effective concentration of fungicide may cause a decrease in microalgae growth but causes a greater reduction in the growth of a chytrid as well (can be seen in figure 5). Many of the fungicides used in agriculture inhibit respiration, and many are effective against a wide range of plant-pathogenic fungi. Fluazinam belongs to the secondary amine group, is cited as an uncoupler of oxidative phosphorylation, this compound inhibits reactions most likely on some proton-coupled uptake process necessary for fermentative growth within the cell [18]. Whereas some fungicides affect fungal respiration at the level of the enzyme complex system, other fungicides may impact respiration through other targets. Fluazinam sparks very unusual uncoupling activity in target cells. The metabolic state of their mitochondria was found to be inhibited after exposure to fluazinam. Consequently, ATP production is inhibited and downstream cellular metabolism is interrupted [17]. Low concentrations of the uncoupler fluazinam inhibited conidial germination and germ tube elongation in fungus [19].

The culture with 0.5 ppm fluazinam gave the maximum growth rate (on cell basis) of *H. pluvialis* with the value 0.568 day^-1 starting on the 4th day. Whereas before the 4th day, the culture without fluazinam and treated by 0.25 ppm of fluazinam supported the high growth rate of *H. pluvialis* (i.e. 0.805 day^-1 and 0.973 day^-1). This condition is in line with the observation by some researchers, instead of killing the fungi, fluazinam also inhibits the growth rate of *H. pluvialis* by weakening cell walls [17].

After the 4th day, the cell number of *H. pluvialis* in the system with 0.25 ppm fluazinam and without fluazinam collapsed. This showed that the development of contaminants is more dominant than the cell resistance.

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**3.2. The effect of fluazinam on the fungus**

Figure 4 (a) shows *H. pluvialis* cells that collapsed because of fungus in the absence of fluazinam on the 5th day whereas figure 4 (b) shows the dead and living cells of *H. pluvialis* in 0.25 ppm of fluazinam on the 5th day. Different microalgae may have different ranges of toxicity that determined by the growth of a microalga in the presence of certain concentrations of a fungicide. An effective concentration of a fungicide

![Figure 4. *H. pluvialis* cells that collapsed because of fungus (a) in the absence of fluazinam and (b) in culture with 0.25 ppm of fluazinam at the 5th day](image-url)
should not be toxic to the microalgae being cultured in the liquid system.

Figure 5 displays a macrozooid cell, green palmella cells, a sporocyst that was infected with chytrids, respectively (before the culture treated with fluazinam). As shown in figure 3, the growth of *H. pluvialis* treated by 0.5 ppm or higher concentration of fluazinam continued to increase from the first day being given a fungicide. This indicated that 0.5 ppm or higher of fluazinam in culture can inhibit the growth of chytrids (showed by no visible filament), even though the greater concentration of fluazinam has a different effect on the growth of microalgae. Figure 5 (b) displays healthy green motile cells, green palmella cell and a healthy cyst and sporocyst, respectively.

**Figure 5.** A macrozooid cell (a), green palmella cells (b), sporocyst (c) of *H. pluvialis* before treated with fluazinam and after treating with fluazinam (d, e)

4. Conclusion
Fluazinam as a single dose fungicide at a certain concentration (0.5 ppm) in the culture of the *H. pluvialis* can be an effective toxic to the growth of chytrids but do not turn off the growth of microalgae. It can be concluded that fluazinam at a certain concentration has a potential as a fungicide even as a single dose treatment.

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