

# Antibiotics Addition as an Alternative Sterilization Method for Axenic Cultures in *Haematococcus pluvialis*

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**Abstract:** This study proposes antibiotics addition to the culture broth to explore an alternative method of sterilization for axenic cultivation of *Haematococcus pluvialis*. As a preliminary experiment, bubble column photobioreactors (400 mL working vol.) were operated in both open and closed modes, resulting in two times higher productivity in the closed systems. When different antibiotics were added to the open systems, the productivity of the open system was improved in some cases. For the cultures grown with griseofulvin or ampicillin, the maximum cell concentrations were comparable to that obtained for the sterilized closed culture, which was 10~30 % higher than that of the open unsterilized culture. Furthermore, the astaxanthin accumulation and the average cell size of the cultures grown under these antibiotics were much smaller than those under the open system, which clearly indicated that these antibiotics did not have any adverse effects on the growth of *H. pluvialis*. Although the productivities under these open cultures with griseofulvin or ampicillin were lower than that of the sterilized closed system with no antibiotics, the results reported here indicate that supplementation of suitable antibiotics can reduce the contamination to a certain degree without affecting growth or other metabolism; thus, it can be an alternative method of sterilization for axenic cultivation of *H. pluvialis*.

**Keywords:** antibiotics, *Haematococcus pluvialis*, alternative sterilization, carotenoid production, systemic approach

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## Introduction

The growing demand for natural products for health foods and beauty products has increased interest in microalgal biotechnology over the past two decades [1]. Algal biotechnology is a field where various high-value useful compounds have been found and isolated from numerous photosynthetic microorganisms [2].

When exposed under certain conditions, such as high-light stress, *Haematococcus pluvialis* produces and accumulates high amounts of astaxanthin (3,3'-dihydroxy- $\beta$ ,  $\beta$ -carotene-4,4'-dione) [3], a red xanthophyll of commercial interest in the food, feed, nutritional, pharmaceutical, and cosmetic industries [4,5]. As a model photobioreactor system, bubble column photobioreactors were chosen because *H. pluvialis* cultures are susceptible to easy contamination and are sensitive to shear stresses and cell settling [6].

Microalgae have been cultured in both open and closed systems for a long time. If a system requires a large culture volume and if there is no need for more sophisticated control of other environmental parameters, open culture systems are the systems of choice. The open systems can be categorized into large tanks, circular ponds, raceways (or oblong ponds), and cascade systems. The open systems have been used far more often than closed systems for current commercial production [7,8]. Open systems are those that have no boundary between the outside environment and inside contents, such as gas and nutrients [8]. Therefore, open systems have been successful only in culturing species exhibiting resistance to adverse culture environments [9]. Otherwise, open systems are susceptible to many sources of contamination, including other microorganisms. However, the most open culture systems, the productivity or the value of the product is not high enough to cover the expenses associated with sterilization of axenic culture [10]. The high capital cost required to build and maintain a closed system can hamper commercialization of closed culture systems in most

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cases [7].

As a result, there must be another way to alleviate the major drawbacks of the outdoor open culture systems: the low efficiency and low yield/productivity of the system. Since antibiotics were discovered and produced from the late 1940s, they have become among the most important compounds used in human medicine, livestock farming, and aquaculture [11]. They are often excreted intact as active compounds and can eliminate microbial ecology without doing much harm to the metabolism of the host cells [12,13]. This study focused on the possibility of using antibiotics, instead of conventional sterilization methods, by comparing the performance of cultures with various antibiotics with those obtained from open and closed cultures without antibiotics. Introducing antibiotics may keep the product from being used in applications such as feeding or as food, but the astaxanthin produced this way can be used as cosmeceuticals and in some pharmaceutical applications.

## Materials and Methods

### Strain Conditions

The unicellular green algae *Haematococcus pluvialis* UTEX 16 was obtained from UTEX, the Culture Collection of Algae at the University of Texas at Austin. *H. pluvialis* was cultivated photoautotrophically in modified Bold's Basal Medium (MBBM) [14,15]. A single colony grown on the agar was inoculated into 120 mL of MBBM medium in a 250-mL Erlenmeyer flask. These seed cultures were cultivated at 25 °C under continuous irradiation at a light intensity of 40  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at 175 rpm (agitation speed) [16].

### Bubble Column Photobioreactors

Bubble column photobioreactors (400 mL working volume) were made of Pyrex<sup>®</sup> glass tubes (650 mm height; 35 mm internal diameter). The bottom part of the column was tapered to have a gas inlet, while the top was blocked by a stopper having three ports: a gas outlet, a sampling port, and a medium port for supplying necessary components. Fluorescent lamps (FL20D, OSRAM, Korea) were used as light sources for photosynthesis.

### Cell Cultures

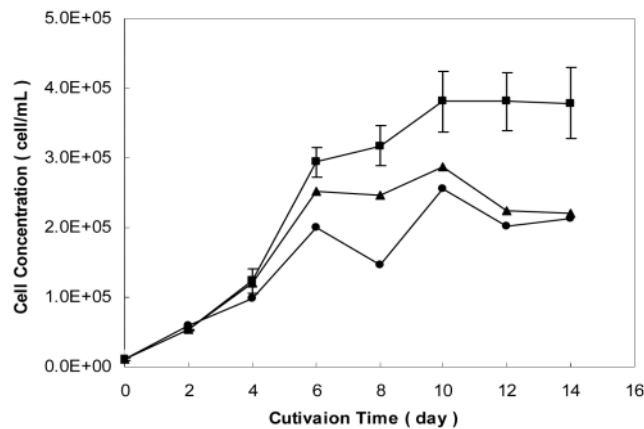
To find suitable antibiotics that reduce contamination without affecting the growth of *H. pluvialis*, a preliminary experiment was conducted in flasks containing 12 different antibiotics: cephalosporin, griseofulvin, bacitracin, polymyxin B, amphotericin B, erythromycin, neomycin, tetracycline, gentamicin, rifamycin, ampicillin, and kanamycin. *H. pluvialis* was found to be resistant to four antibiotics: cephalosporin, griseofulvin, amphotericin B, and ampicillin.

A more detailed experiment was conducted in bubble column photobioreactors using the four antibiotics above. Four bubble column photobioreactors were supplemented with the four antibiotics and cultured under non-sterilized open conditions. The recommended concentrations against antifungal contamination in the cell culture test were used for each antibiotic: cephalosporin (0.01 g/L), griseofulvin (0.1 g/L), amphotericin B (10 ml/L), and ampicillin (5 g/L). Two control cultures were also run in parallel: control 1 (designated as C1) was a closed culture system sterilized by autoclaving (121 °C, 15 min); the other two controls, C2 and C3, were open cultures used with and without sterilization, respectively. In other words, C2 was prepared exactly the same way as C1, but it was operated under open conditions after inoculation.

The surface light intensity of each bubble column photobioreactor was maintained at 40  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Bubble column photobioreactors were inoculated at a density of  $1.0\times 10^4$  cells/mL ( $\pm 0.1\times 10^4$ ) with seed cultures at their exponentially grown phase. All cultures were grown photoautotrophically with enough nitrate to prevent heterocyst formation. Aeration with containing 5 % CO<sub>2</sub> at 100 mL/min was introduced to the bottom of each column reactor to mix the culture medium while supplying a carbon atom source. The average duration of the culture was 18 days. The biomass and pigment content were sampled and analyzed at intervals of two days.

### Analytical Methods

The cell concentration, the average cell size, and the cell size distribution were measured using a Coulter Counter (model Z2, Beckman Coulter, Inc., Fullerton, CA, USA) and AccuComp<sup>®</sup> software (v. 2.01, Beckman Coulter, Inc.). The photosynthetic photon flux density was measured using a quantum sensor (model LI-190SA, LI-COR, Inc., Lincoln, NE, U.S.A.) equipped with a Datalogger (model LI-1400, LI-COR, Inc.). The light intensity at the surface of the photobioreactor was determined by averaging the values of light intensity around the perimeters of each bubble column photobioreactor. The total chlorophyll and carotenoid concentrations were analyzed using a spectrophotometer (model HP8453B, Hewlett Packard, Waldbronn, Germany) after diluting to suitable concentrations following acetone extraction. The astaxanthin concentration was calculated from a calibration curve using synthetic astaxanthin (A9335, Sigma Chemical Co., St Louis, MO, USA) as a standard. The nitrate concentration was also analyzed using a spectrophotometer, after treating the centrifuged sample with HCl, according to the standard method.



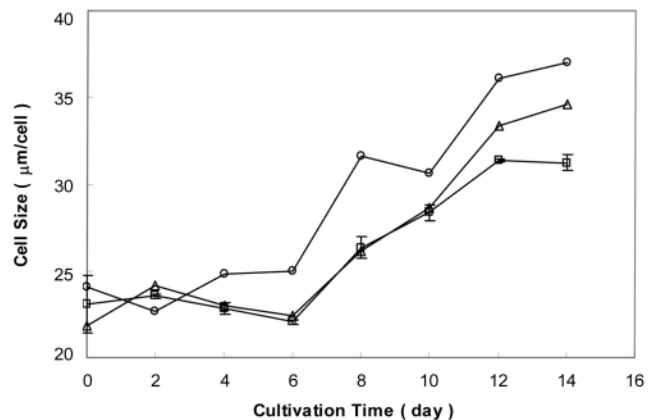
**Figure 1.** Growth curves in a sterilized closed culture (C1; ■), an open culture after sterilization (C2; ▲), and an open unsterilized culture (C3; ●).

## Results and Discussion

### Open and Closed Systems

To determine the adverse effect of the open system, three control cultures were prepared and run as mentioned above: one using a conventional setup with thorough sterilization and running as a closed culture system (C1); one with a sterilized medium and photobioreactor, but running as an open system (C2); one cultured in an open system without proper sterilization (C3). All three systems were inoculated at a cell density of  $1.0 \times 10^4$  cells/mL ( $\pm 0.1 \times 10^4$ ) with cells from an exponentially growing period. The surface intensity of the bubble column photobioreactors was maintained at  $405 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

Figure 1 shows the growth profiles of the cultures under the three different conditions. The overall performance of the sterilized culture (C1; represented as ■ in Figure 1) was higher than those of the other two conditions, although the initial grow rates of all three cultures were almost identical. The maximal cell concentration in C1 was ca.  $3.8 \times 10^5$  cells/mL (ca. 6.1 g dry weight/L). The performance of the open cultures (regardless of the sterilization) was poorer than that of the sterilized closed culture, as can be seen in the figure. The final cell concentration of the unsterilized open culture (C3; represented as ● in Figure 1) was less than 40 % of that of C1. Initial sterility helped the performance of the cultures because the final cell concentration of the sterilized open culture (C2; represented as ▲ in Figure 1) was higher than that of C3. The growth profile of the sterilized open culture (C2) followed that of C1 longer (until Day 4 in Figure 1), while that of the unsterilized open culture followed the profile of C1 for only two days. The overall performance of C2 was higher than that of C3 by ca. 5 % on average.



**Figure 2.** Average cell size profiles obtained using different culture methods: a sterilized closed culture (C1; □), an open culture after sterilization (C2; △), and an open unsterilized culture (C3; ○).

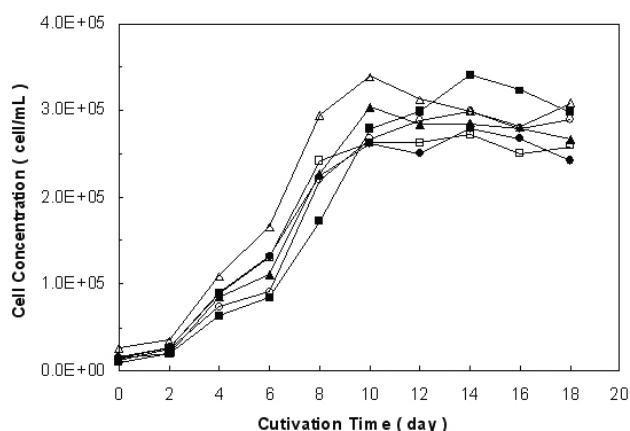
Figure 2 illustrates the average cell sizes for the same cultures as those in Figure 1. Because these cells were cultured without astaxanthin induction, the average cell sizes were relatively small. The average diameter for uninduced *H. pluvialis* was between 20 to 30  $\mu\text{m}$  from the everyday culture of the strain. The average diameter of the cells in C3 became larger before nitrate was depleted in the medium (refer ○ in Figure 2). In addition, the average cell size was ca. 15% bigger than that of C1 (cf. □ and ○ in Figure 2); the morphology of the C3 cells observed under an optical microscope showed that they formed coagulated masses, which represent cells formed under unfavorable conditions.

All of these results support the notion that maintenance of axenic culture is very important for the productivity and efficiency of photosynthetic microorganisms. Because maintaining axenic cultures for a prolonged period is extremely hard and expensive for fed-batch or repeated-batch cultures, an alternative sterilization method is clearly necessary. The possibility of using antibiotics as an alternative sterilization was tested.

### Antibiotics Addition

Most commercial microalgal mass productions are mainly performed in open systems, such as large tanks, ponds, and raceways; the commercial cultures of *H. pluvialis* are no exception. As a result, the productivity of open systems of *H. pluvialis* is relatively low compared to that from high-end closed photobioreactors. Antibiotics were tested to increase the productivity by reducing the contamination. In other words, antibiotics were used to extend axenic conditions to a certain degree.

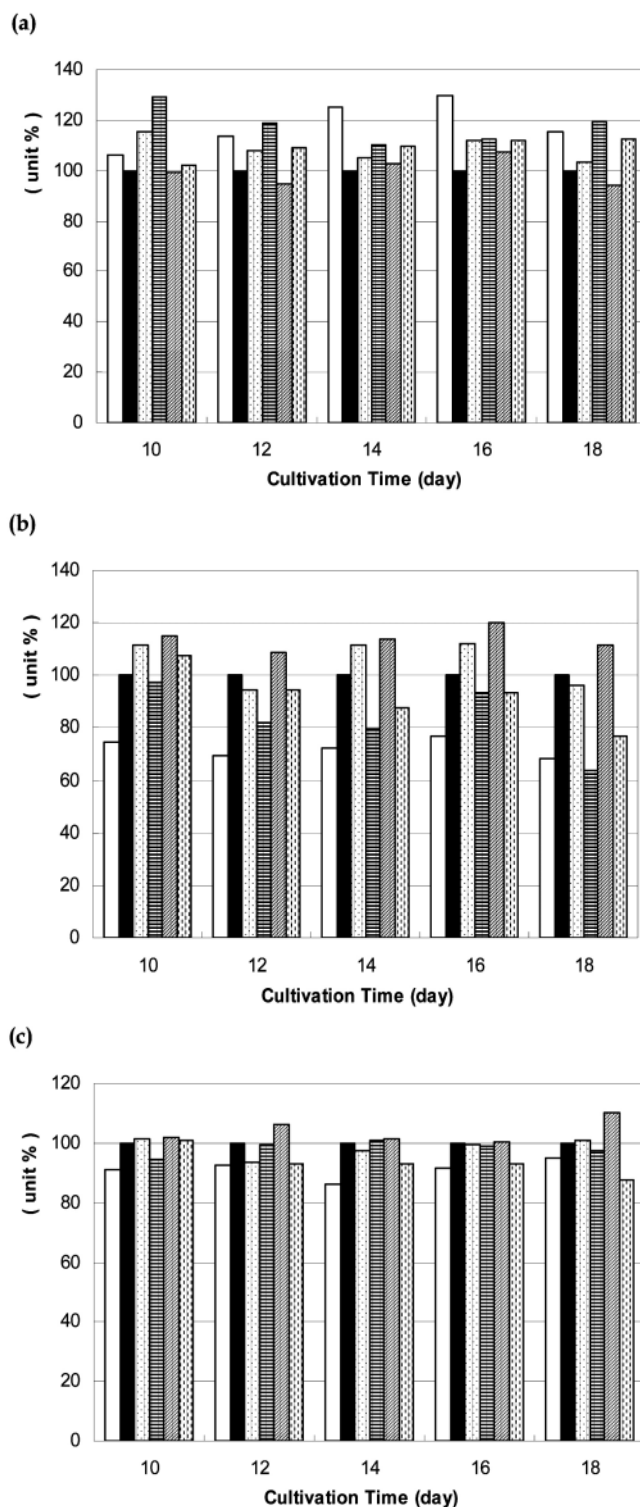
Bubble column photobioreactors (400 mL working volume) were used to determine the effect of antibiotics in open cultures in comparison with the performance of



**Figure 3.** Growth curve profiles under positive and negative controls and under various antibiotics. Two controls: closed sterilized culture (C1; ■), open unsterilized culture (C3; □); four open unsterilized cultures with different antibiotics: cephalosporin (A1; ▲), griseofulvin (A2; △), amphotericin B (A3; ●), and ampicillin (A4; ○).

controls: open and closed batch cultivations of *H. pluvialis*. Four cultures with the four antibiotics were prepared without sterilization of the equipment and were cultured in the open mode. Two controls without antibiotics ran in a parallel manner: the same setup as C1 and C3 in the previous experiment. Figure 3 shows the profiles of the cell concentrations for the six different conditions. The two controls mentioned above were one with the sterilized closed culture (C1; designated as ■ in Figure 3) and the other with the open culture without any sterilization (C3; designated as □). Four photobioreactors with open unsterilized setups incorporated the four different antibiotics: cephalosporin (A1; designated as ▲), griseofulvin (A2; △), amphotericin B (A3; ●), and ampicillin (A4; represented as ● in Figure 3). All columns were inoculated at an initial concentration of  $1.0 \times 10^4$  cells/mL. As can be seen in the cell concentration profiles, the maximum cell concentration reached  $3.4 \times 10^5$  cells/mL in C1 (refer to ■ in Figure 3), while the lowest maximum cell concentration was observed in C3 (refer to □ in Figure 3), ca.  $2.7 \times 10^5$  cells/mL. The growth profiles for most cultures with antibiotics were much improved over those of the unsterilized open cultures with no antibiotics.

Figures 4a, 4b, and 4c represent the normalized values of cell concentration, astaxanthin concentration, and average cell size, respectively, with respect to the values of the negative control (C3; □ in Figure 4a). The normalized values were calculated for the period between Days 10 and 18. Again, the highest cell performance was observed in C1 at Day 16 (□ in Figure 4a); interestingly, the open unsterilized culture with griseofulvin (■) was able to support almost the same growth as did the pos-



**Figure 4.** Normalized values of (a) cell number, (b) astaxanthin content per cell, and (c) average cell size, with respect to those values of the negative control (C3). Two controls: closed sterilized culture (C1; □), open unsterilized culture (C3; ■); four open unsterilized cultures with different antibiotics: cephalosporin (A1; □), griseofulvin (A2; ■), amphotericin B (A3; □), and ampicillin (A4; ■).

itive control (C1; ■ in Figure 4a). This result is very promising because it means that the growth in an open unsterilized system when supplemented with proper antibiotic(s) can be comparable to that in a sterilized closed system. The relative cell concentration of A2 was at least 9.9 % higher (at Day 14) than that of C3 and was as high as 129.1 % of C3, proving the possibility of the usage of antibiotics instead of conventional sterilization.

The bars in Figure 4b show the relative concentrations of astaxanthin per cell in all six cases. Because these cultures were not grown under astaxanthin induction conditions, the higher the astaxanthin concentration, the less favorable the culture conditions. In almost every case, the astaxanthin concentration in C1 was dramatically lower than it was in any of the other cases, showing that this positive control (C1) was the best condition among those tested in this experiment. On the contrary, the maximum astaxanthin concentration attained per cell of C3 was ca.  $1.0 \times 10^{-4}$  mg/cell, which was 30 % higher than that of C1 ( $7.3 \times 10^{-5}$  mg/cell). The astaxanthin concentrations per cell of A1 and A3 (□ and ▨ in Figure 4b, respectively) were almost always higher than the astaxanthin concentration per cell of the negative control, which means that cephalosporin (A1) and amphotericin B (A3) are not adequate antibiotics for use as alternatives to conventional sterilization. However, the per-cell astaxanthin concentrations of A2 and A4 (▩ and ▪ in Figure 4b, respectively) were significantly lower than that of the negative control (C3) and only slightly higher than that of the positive control (C1). Moreover, when the culture broths were streaked on rich medium plates, the number of contaminated colonies of A2 and A4 was smaller than that of C3. Thus, these two antibiotics, griseofulvin and ampicillin, are good candidates for application as an alternative sterilization method (or at least extending the period of axenic culture) with comparable growth rates (refer Figure 4a) without exposing the system to unfavorable conditions (Figure 4b). A similar and confirming result can be found in the profiles of the average cell size (Figure 4c). The data indicate that the average cell size of the cells grown with griseofulvin or ampicillin was similar to that of the cells grown under sterile conditions in a closed system. Visual inspection of the cell morphology under an optical microscope also showed that the two antibiotics, griseofulvin and ampicillin, did not affect the cell growth nor any other metabolism of the cell.

Consequently, the proper use of suitable antibiotics can protect the culture from contamination to a certain degree without affecting the growth rate and exposing it to unfavorable conditions, resulting in a higher productivity than that of open unsterilized cultivation. Maintaining an axenic culture for an extended period in fed-batch or repeated-batch cultures is extremely difficult and very

expensive. Thus, an alternative sterilization method that can replace an autoclave is necessary. The results reported here clearly demonstrate that the addition of specific antibiotics can be used as an efficient alternative sterilization method for the axenic cultivation of *H. pluvialis*.

## Conclusion

This study demonstrates that the addition of specific antibiotics into an open system can enhance the productivity of the unsterilized culture by preventing contamination to a certain degree without affecting cell growth. Among the 12 antibiotics tested, *H. pluvialis* was found to be resistant to four antibiotics: cephalosporin, griseofulvin, amphotericin B, and ampicillin. However, cephalosporin and amphotericin B affected the growth of the culture and induced astaxanthin formation, which are typical symptoms of unfavorable conditions. On the other hand, griseofulvin and ampicillin did not affect the cell metabolism and, thus, the cultures containing griseofulvin or ampicillin showed comparable growth rates to that of the sterilized closed culture. Though the overall productivity obtained under the influence of antibiotics did not overtake that of the closed system, addition of these antibiotics can serve as an alternative to using autoclaves. Because this alternative sterilization method can be cheaper than conventional thermal sterilization, introducing antibiotics in lieu of using an autoclave can be economical and, thus, practical.

## Acknowledgement

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