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The Effects of Audible Sound for Enhancing the Growth Rate of Microalgae *Haematococcus pluvialis* in Vegetative Stage

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ABSTRACT

Physico-stimulant like audible sound is one of the new promising methods for enhancing microalgae growth rate. Here, microalgae *Haematococcus pluvialis* was cultivated with the addition of audible sound with titles "Blues for Elle" and "Far and Wide." The objective of this research was to evaluate the effect of audible sound to the growth and productivity of microalgae. The experiment has been conducted by exposing the audible sound for 8 h in 22 days to microalgae cultivation. The result showed that microalgae *H. pluvialis* treated by the music "Blues for Elle" shows the highest growth rate (0.03 per day), and 58% higher than the one without audible sound. The average number of cells in stationary phase is 0.76×10^4 cells/mL culture and the productivity is 3.467×10^2 cells/mL/day. The pH of microalgae medium slightly decreases because of proton production during photosynthesis process. The kinetic rate constant (k_{app}) is 0.078 per day, reaction half-life ($t_{1/2}$) is 8.89 days, and catalytic surface (K_{surf}) is 1.66×10^{-5}/day/cm^2. In conclusion, this audible sound is very useful to stimulate microalgae growth rate, especially *H. pluvialis*.

1. Introduction

Microalgae are unicellular microorganisms, which can be used as feedstock for producing biofuel or other value-added products (Choksi *et al.*, 2015; Hu *et al.*, 2008; Shah *et al.*, 2016) such as docosahexaenoic and eicosapentaenoic (Grima *et al.*, 2003), omega-3 (Barclay *et al.*, 1994), and protein (Hadiyanto *et al.*, 2012). Most of them contains protein, lipid, inorganic elements, polysaccharides, and also pigments such as chlorophyll, xanthophyll, zeaxanthin, canthaxanthin, astaxanthin, and β-carotene.

In food or pharmaceutical area, microalgae pigments, vitamin, and functional bioactive compound are collected as functional food or drugs because most of them has antioxidant properties, which is needed by living organisms, especially human and animals (Hadiyanto *et al.*, 2013). Microalgae also have been used as biofuel feedstock. Some microalgae can accumulate fatty acid which can be extracted to be bio-oil (Nur and Hadiyanto, 2015). In addition, microalgae have been used for wastewater treatment because they can capture carbon and nutrition from wastewater and also remove CO_2 from flue gas (Christenson and Sims, 2011; Olaizola, 2003). That is why microalgae are very useful for food, energy, and wastewater treatment area.

One of microalgae is *Haematococcus pluvialis*. These microalgae have four cycles of life; vegetative cell growth, encystment, maturation, and germination (Kobayashi *et al.*, 1997), but generally consist only of vegetative (green stage) and maturation (red stage) (Park *et al.*, 2014). *H. pluvialis* has a valuable pigment which can be used as an antioxidant, called astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione) (Lorenz and Cysewski, 2000; Ranga Rao *et al.*, 2010). For human body, astaxanthin is 54 times more powerful than β-carotene, 65 times more powerful than vitamin C, 100 times more effective than tocopherol (Borowitzka, 2013; Koller *et al.*, 2014; Miki, 1991; Pérez-López *et al.*, 2014), and good for eye health, central nervous system, immune system, anti-aging, and fertility. Astaxanthin is not only produced by *H. pluvialis*, but also by yeast, salmon, trout, krill, shrimp, crayfish, and crustaceans

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(Higuera-Ciapara *et al.*, 2006; Ranga Rao *et al.*, 2014). Astaxanthin is resulted from the secretion process of *H. pluvialis* during maturation stage, whereas chlorophyll was still produced in vegetative stage.

In previous research, effect of physico-stimulation such as addition of audible sound in plant growth has been performed by several researchers (Cai *et al.*, 2014; Creath and Schwartz, 2004; Gu *et al.*, 2013; Hassanien *et al.*, 2014; Hou and Mooneyham, 1999; Hou *et al.*, 2009). For microalgae experiment, Jiang *et al.* (2012) conducted the audible sound effect experiment on chlorella and Cai *et al.* (2016) conducted on *Picochlorum oklahomensis*. But, none exploring the type of sound which was used and only focusing on the frequency of sound.

Audible sound is very important in this experiment. Larsen and Gilbert (2013) have been arranged the music called "microbial bebop" from microbial deoxyribose nucleic acid (DNA) genes sequence. That music consists of "Blues for Elle," "Bloom," "Far and Wide," and "Fifty Degrees North, Four Degrees West," where each music has different function if it is used for microbe. Other experiment about arranging notation based on live creature's DNA genes sequences had been performed by Sousa *et al.* (2005).

In this experiment, we used specific songs called "Blues for Elle" and "Far and Wide" as audible sound because that music was created to stimulate photosynthesis, nutrient consumption, and temperature resistance for microbe or microalgae. On the other hand, "Bloom" and "Fifty Degrees North, Four Degrees West" were not used as audible sound because they were created especially for cyanobacteria and pseudomonas, which is avoided in *H. pluvialis* growth (Larsen and Gilbert, 2013). Microalgae growth rate would be determined by measuring optical density (OD) and manual cell counting method using hemocytometer.

2. Materials and Methods

2.1. Materials

H. pluvialis strain (UTEX #2505) was used in this experiment and was cultured in 1 L flask with the addition of optimal haematococcus medium (OHM) (Fábregas *et al.*, 2000), which consists of (in g/L) KNO_3 0.41, Na_2HPO_4 0.03, $MgSO_4.7H_2O$ 0.246, $CaCl_2.2H_2O$ 0.11, (in mg/L) Fe(III)citrate.H_2O 2.62, $CoCl_2.6H_2O$ 0.011, $CuSO_4.5H_2O$ 0.012, Cr_2O_3 0.075, $MnCl_2.4H_2O$ 0.98, $Na_2MoO_4.2H_2O$ 0.12, SeO_2 0.005, and (in µg/L) biotin 25, thiamine 17.5, and B_{12} 15 (in g/L), then placed in a chamber. After 3 days or if the cell concentration was 0.4×10^4 cells/mL of culture, that culture was transferred into acrylic photobioreactor which contains 60 L distilled water (include OHM). The temperature of environment was set to 25°C and checked every day using Infrared Thermometer Q3 NUB8380H (China). Some cool white LED lamps were placed beside photobioreactor which resulted in photosynthetic photon flux of 20.3 µmol/m^2/s. Culture was left for 22 days with dark:light cycle was 12:12, and OHM would be injected into the culture every 2 days. Aeration with the rate of 100 mL/min was fed into the photobioreactor without any additional CO_2.

2.2. Audible sound treatment

Previously, Cai *et al.* (2015) reported that 2000 Hz is the major frequency component in most audible sound in nature. Based on that, music or audio with power 60 dB was set as audible sound and average frequency of the audible sound was measured by using Q4 Audacity software. Audible sound was played from music player HCB-811 (Hyundai, South Korea) and placed between the two photobioreactors as shown in Figure 1 (approximately 20 cm). The audible sound was continuously generated for microalgae growth, and played 8 h per day from 10 AM to 6 PM. The power of sound

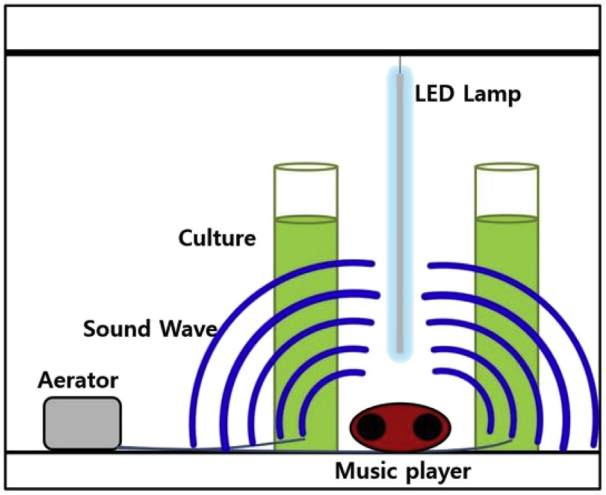

Figure 1. Schematic of *Haematococcus pluvialis* cultivation set-up.

was controlled by controller in music player. For control variable, culture with no addition of audible sound was set with the same parameter of temperature, light, aeration rate, and pH.

2.3. Biomass measurement

Most studies said that the maximum absorbance for microalgae was observed at 680 nm as the maximum peak of chlorophyll to estimate biomass concentration. Eq. 1 was followed to determine microalgae growth rate. The OD was monitored using bench spectrophotometer UV-1800 (Angstrom Advance, USA). Around Q5 20 mL of culture was collected into the sample bottle every day during growth period and used for growth rate and pH measurement. The pH of culture itself was checked by using pH meter Fisher Scientific S90525 (Fisher Scientific, USA).

$$\mu = \frac{OD_2 - OD_1}{t_2 - t_1} \quad (1)$$

where, µ is microalgae growth rate, OD is at 680 nm, and t is time (day).

The second method to measure growth rate was using hemocytometer. In this method, 1 mL of culture was injected to hemocytometer. *H. pluvialis* was observed by using microscope BA310 (Motic, USA), total cells number in eight areas was counted manually and then divided by eight. Then the resulted number was multiplied by 10^4. Eq. 2 was used to measure microalgae growth rate.

$$\mu = \frac{N_2 - N_1}{t_2 - t_1} \quad (2)$$

where, µ is microalgae growth rate, *N* is cells number (cells/mL), and t is time (day).

2.4. Kinetic model measurement

Profile of pseudo–first-order kinetics of *H. pluvialis* biomass during 22 days of cultivation following Eq. 3 (Rokhina *et al.*, 2010).

$$ln\frac{c_0}{c} = k_{app} \times t \quad (3)$$

where, C_0 is the initial number of *H. pluvialis* cells, C is the final number of *H. pluvialis* cells, k_{app} is apparent rate constant (per day), and t is time (day).

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