Effect of Air Nanobubble Water on the Growth and Metabolism of Haematococcus lacustris and Botryococcus braunii

Jiangyu Zhu and Minato Wakisaka*

Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, Kitakyushu 808–0196, Japan

Summary Miniature air bubbles with a diameter of less than 200 nm were generated by a nanobubble aerator, and nanobubble water (NBW) was eventually obtained using the gas-liquid mixing system with hydrodynamic function. As the air bubbles have long lifetime and high gas solubility in the liquids, NBW is stable in nature and inside contains sufficient dissolved oxygen. At present, there is no report on the use of NBW to replace ordinary water to cultivate microalgae. In this research, effect of NBW on the growth and metabolism of different microalgae, including Haematococcus lacustris and Botryococcus braunii was investigated. The result demonstrated that the growth of H. lacustris and B. braunii was increased by NBW and the highest promotion ratio was up to 44% and 26%, respectively. For H. lacustris, the astaxanthin content in the NBW treatment group was also improved compared to the control group. As the main product of B. braunii, lipid content in the dry matter was decreased after the treatment of NBW, but total lipid production was significantly higher than that of the control group. In general, NBW could serve as the potentially effective tool to promote the growth of microalgae in the future.

Key Words nanobubble water, Haematococcus lacustris, Botryococcus braunii, astaxanthin, lipid

Microalgae distributed in the fresh water, marine and sediments are the main producers in the water ecosystems. There are many species of microalgae and some of them have extremely high commercial values. For instance, Haematococcus lacustris can make high amounts of astaxanthin which is famous for the strong antioxidant activity; Botryococcus braunii was able to produce large amounts of lipids, which has been considered as a source of the new-generation bioenergy (1). In the recent decades with the development of modern biological separation technology, more and more application potentials of microalgae have been discovered. In addition to traditional applications such as food, feed, medicine and supplements (2–4), microalgae have also been exploited in some sunrise industries including cosmetics and biofuels (5, 6). Faced with the constantly increasing market demand, enhancing the yield of astaxanthin and lipids at a low cost and high efficiency has become a hot topic.

Recently, gene-editing of the strains (7) and supplementation of additives extracted from agro-waste during the culture (8) have been the commonly seen strategies to increase the microalgal yield. Generally, water, light, air and nutrients are essential to microalgae, however, there are few researches on the water used for microalgal cultivation. Replacing ordinary water with NBW seems to be an option. NBW contains a large number of miniature gas bubbles with less than 200 nm in diameter. Because of the negatively charged surface of the nanobubbles, they can remain stable in water for a long time. Furthermore, internal pressure of the bubbles in liquids is much higher than that of the external environment, accelerating the dissolution of the air into the liquids (9). Based on these unique properties and its security, NBW now is being applied in the fields of diagnostic aids and drug delivery. Recently, effect of NBW on the growth of lives has been investigated as well, including Brassica campestris, sweetfish, rainbow trout and male mice (10). In comparison with the normal water, the height and length of Brassica campestris, the weight of sweetfish, rainbow trout and male mice, were all notably promoted by NBW. Furthermore, previous reports demonstrated that hyperoxia could improve the growth of plants (11) and animals (12). It gives us a hint as to whether NBW can be used to replace normal water for increasing the production of microalgae.

This study was the first to assess the effect of NBW on the growth and metabolism of freshwater microalgae H. lacustris and B. braunii by analyzing the following indicators such as cell density, dry weight, astaxanthin content in H. lacustris, photosynthetic pigment and lipid content in B. braunii.

Materials and Methods Nanobubble water. Air nanobubble water was provided by Mishima Kosan Co., Ltd., Japan. Miniature air bubbles with a diameter of less than 200 nm were generated by splitting microbubbles with a strong shear force resulted from applying a negative pressure at the center and rotating the liquid at a high speed. NBW was eventually obtained using the gas-liquid mixing sys-
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**Microalgae and culture conditions.** *Haematococcus lacustris* (NIES-144) and *Botryococcus braunii* Race A (NIES-2199) were obtained from National Institute for Environmental Studies (NIES) of Japan. *H. lacustris* and *B. braunii* were cultured in NIES-C medium and BG-11 medium, respectively. In NBW treatment groups, the medium was prepared using NBW instead of distilled water (DW), with other mineral composition unchanged compared with the control group. Sterilization was performed by filtering the medium using Thermo Nalgene Filters (pore size <0.45 µm). Medium made of DW and medium made of NBW were added in the 300 mL Erlenmeyer flasks in different proportions and 10 mL of algal cells in the exponential phase were inoculated, with each flask containing 100 mL of medium. The NBW concentrations were 0%, 10%, 50%, and 90% (v/v). All groups were cultured in duplicate in an incubator at 25˚C and illuminated under the light intensity of 5,000 lx using cool-white fluorescent lamps (12:12 h light-dark cycle). Flasks were shaken by hand three times a day.

**Cell growth assessment.** The growth of *H. lacustris* was assessed by determining the cell density periodically with a hemocytometer chamber, and the growth was assessed by determining the cell density periodically with a hemocytometer chamber, and the growth was assessed by determining the cell density periodically with a hemocytometer chamber, and the growth was assessed by determining the cell density periodically with a hemocytometer chamber, and the growth was assessed by determining the cell density periodically with a hemocytometer chamber, and the growth was assessed by determining the cell density periodically with a hemocytometer chamber. The growth of *B. braunii* was determined by the following method:

\[ \text{Wf} = \frac{\text{W} - \text{Wf}}{V} \]

\[ \text{W} \] is the dry weight of cell biomass (g/L); \( \text{Wf} \) is the final weight of the dried filter paper and algal cell biomass (g); \( \text{W} \) is the weight of the pre-dried filter paper (g), and \( V \) is the volume of collected algal fluid (L).

**Astaxanthin content in *H. lacustris*.** Astaxanthin content was measured at 490 nm using a spectrophotometer. Astaxanthin content (C<sub>a</sub>) was calculated as follows:

\[ C_a = 5.5 \times 10^{-2} \times A_{490} / V_a \]

Where the \( C_a \) is the astaxanthin content (mg/L), \( V_a \) is the volume of DMSO and acetic acid, and \( V_b \) is the volume of cell suspension, and \( A_{490} \) is the absorbance of the extract at 490 nm.

**Pigment content in *B. braunii*.** Pigment content was measured at 663 nm, 645 nm, and 470 nm, respectively. The content of chlorophyll a, chlorophyll b, and carotenoids were determined using a spectrophotometer (UV-vis 1200, Shimadzu, Japan) at the wavelength of 470 nm, 645 nm, and 663 nm, respectively. The ratio of chlorophyll a to b was calculated as well. Pigment content was calculated with the following formulas:

\[ C_a = 12.21 \times \text{A}_{663} - 2.81 \times \text{A}_{666} \]
\[ C_b = 20.13 \times \text{A}_{646} - 5.03 \times \text{A}_{653} \]
\[ C_{a+b+c} = (1000 \times A_{470} - 3.27 \times C_a - 104 \times C_b) / 229 \]

The above \( C_a \), \( C_b \), and \( C_{a+b+c} \) represent the content of chlorophyll a, chlorophyll b, and carotenoids (mg/L), respectively. \( \lambda_{663} \), \( \lambda_{645} \), and \( \lambda_{470} \) are the absorbances at the wavelength of 663 nm, 645 nm, and 470 nm, respectively.

**Lipid content in *B. braunii*.** Lipid content was determined by modified Fajardo’s method (15). For lipids extraction, 50 mL cell suspension was filtered and washed twice to remove the reagents. The cell biomass was then lyophilized and treated with 5 mL of 96% (v/v) ethanol with constant magnetic agitation at 500 rpm for 24 h at 25˚C. After the filtration the residues were extracted again with 5 mL of 96% (v/v) ethanol for 1 h. For further purification, the extract solution was combined and 6 mL of distilled water was added to obtain a hydroalcoholic solution. Then 3.2 mL hexane was added, forming a hexane-hydroalcoholic solution biphasic system. In this case lipids were transferred to the n-hexane phase. Total lipid content was obtained by evaporating n-hexane at 70˚C.

**Statistical analysis.** Data were processed by SPSS v. 19.0 (Armonk, NY: IBM Corp.) and presented in the form of mean±standard deviation. Kruskal–Wallis test at reliability level of 5% were used to test for differences in the growth and metabolism parameters between the target algae cultures treated with different concentrations of NBW and the control.

**Results**

**Growth promotion of *H. lacustris* and *B. braunii* by NBW**

Figure 1 shows the growth profiles of *H. lacustris* (A: Growth curve, a: dry weight) and *B. braunii* (B: Growth curve, b: dry weight) cultured with different concentra-
For *H. lacustris*, the growth of 10%, 50% and 90% NBW treatment groups were all promoted and the cell density at the concentration of 90% NBW was increased by 44% compared with the control group (*p* < 0.05). However, the difference in the effect of different concentrations of NBW (10%, 50% and 90%) was not significant (*p* > 0.05). Result of the final dry weight was basically consistent with the growth curve. The dry weight of the biomass in the control group was 0.56 ± 0.12 g/L, but it was upgraded to 0.81 ± 0.07, 0.84 ± 0.14, 0.76 ± 0.16 g/L respectively in the 10%, 50% and 90% NBW treatment groups, indicating a promotion effect of NBW on the growth of *H. lacustris*.

For *B. braunii*, growth promotion effect of NBW treatment was also observed and it was in a concentration-dependent manner. The maximum increase obtained at the 90% NBW treatment group, *B. braunii* cells increased by 26% compared with that of the control group (*p* < 0.05) for the optical density at 750 nm. Consistently, the dry weight of the biomass was also increased from 0.43 ± 0.06 g/L in the control group to 0.65 ± 0.02 g/L at the concentration of 90% NBW (*p* < 0.05).

**Effect of NBW on the astaxanthin content of *H. lacustris***

Figure 2 indicated the astaxanthin production in *H. lacustris* which cultured with different concentrations of NBW. Astaxanthin yield in 10% NBW treatment group was increased to 10.49 ± 1.60 mg/L, which almost doubled compared with 5.40 ± 2.58 mg/L of the control group. In addition, astaxanthin content in dry matter was calculated, and it also showed an increasing trend in NBW treatment groups, while the difference was not significant (*p* > 0.05).

**Effect of the NBW on the photosynthetic pigment content of *B. braunii***

Figure 3 indicated the photosynthetic pigment content of *B. braunii* which cultured with different concentrations of NBW.
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Charge of the surface of microbubbles could attract the positively charged ions of metal ions (16). Compared with microbubbles, nanobubbles have a larger specific surface area, which can attract more metal ions to benefit the growth. On the other hand, in our experiment, the initial oxygen concentration in the NBW was higher than that of the normal water. It was reported that, within a certain range, high oxygen partial pressure and hyperoxia could promote the growth of plants (11) and animals (12), as the low demand of respiration and elevated metabolism would result in the greater proportion of metabolized energy was used for the growth.

For *H. lacustris*, the increase of astaxanthin content in NBW treatment groups was observed. It is reasonable because as the major antioxidant in *H. lacustris* cells, intracellular astaxanthin content would vary in response to the redox status. In hyperoxia condition, algal cells will generate a lot of reactive oxygen species (ROS) unavoidably (17). The increase of astaxanthin content might be the adaptive response of algal cells (18). Moreover, due to the increase of cell density, the total production of astaxanthin will increase as well.

Cells of *B. braunii* could immobilize inorganic carbon sources through photosynthesis and then converted them into lipids by some induced reactions (19). In our experiment, we found the decrease in the content of total chlorophylls, which indicated that the ability of absorbing light power was also decreased. Besides, a slight decreasing trend in lipid content in the dry matter was observed, which was consistent with the decrease in total chlorophyll content. It is likely that the hyperoxia condition would weaken the photosynthetic activity (17) and the photosynthate would be consumed for the photosynthesis (20). Although the lipid content in the dry matter was slightly decreased, due to the increase of the cell number, lipid production of *B. braunii* has also been increased, indicating the feasibility of enhancing the lipid yield by NBW.

To summary it, the growth of *H. lacustris* and *B. braunii* could be promoted by simply replacing ordinary water with NBW. In view of the increase in the number of cells, the yield of valuable metabolites such as astaxanthin and lipid has thus been increased. Effect of NBW on other different specces of microalgae remains to be studied. In addition, we will further verify the effect of DO content and oxygen partial pressure values on the growth of microalgae.

**Disclosure of State of COI**

No conflicts of interest to be declared.

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