Impacts of different extracellular polysaccharides on colony formation and buoyancy of Microcystis aeruginosa

Kai Wei¹*, Yoshimasa Amano²,³ and Motoi Machida²,³

1 Graduate School of Science and Engineering, Chiba University, 1-33, Yayoi-cho, Inage-ku, Chiba 263-8522, Japan
2 Graduate School of Engineering, Chiba University, 1-33, Yayoi-cho Inage-ku, Chiba 263-8522, Japan
3 Safety and Health Organization, Chiba University, 1-33, Yayoi-cho Inage-ku, Chiba 263-8522, Japan

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Abstract – On the surface of Microcystis cells, there is a carbohydrate called extracellular polysaccharides (EPS) playing a significant role in the colony formation of Microcystis. EPS consists of tightly cell-bound EPS (TB-EPS), and both of these substances are considered to be strongly related to the colony formation and buoyancy of Microcystis. In this study, Microcystis aeruginosa (strain: NIES-843) was used to examine the effects of EPS, TB-EPS, and divalent metal cations such as calcium and magnesium on the buoyancy and colony formation of M. aeruginosa NIES-843. Under various light conditions, the addition of TB-EPS into the culture medium induced M. aeruginosa NIES-843 to obtain high buoyancy at concentrations of Ca²⁺ and Mg²⁺ concentrations of 10 mg/L and 30 mg/L, respectively. Under the absence of light, the addition of EPS could lead M. aeruginosa to form a colony and obtain buoyancy, and the addition of TB-EPS could not significantly change the buoyancy of M. aeruginosa NIES-843. The colony size analysis showed that at the same cationic concentration, the addition of TB-EPS could induce M. aeruginosa to form the largest colony and present strong buoyancy. This study suggested that temperature and illumination are conducive to colony formation and present higher buoyancy of M. aeruginosa.

Keywords: Microcystis / buoyancy / colony formation / extracellular polysaccharides / tightly cell-bound extracellular polysaccharides

1 Introduction

Microcystis bloom outbreaks often occur in eutrophic lakes in summer and have been found in many regions of the world (Otten et al., 2012; Park et al., 1993). The toxin microcystin produced by Microcystis has caused great harm to the survival of animals and plants in water (Nishiwaki et al., 1992). Microcystis overwinters at the lake bottom in winter after their growth season (Brunberg and Blomqvist, 2002; Preston et al., 1980). In the spring, Microcystis slowly rises to the water column with the increase of temperature and light intensity. During the summer, Microcystis floats to the water surface and multiplies. Microcystis gradually sinks to the bottom of the water as the temperature and light intensity decrease in autumn. The movement of Microcystis in lakes is reciprocating in water as an annual cycle (Sigee, 2005; Walsby, 1994). Two factors of Microcystis buoyancy have been proposed based on the previous studies; gas vesicle and extracellular polysaccharides (EPS). Microcystis has a particular structure called gas vesicle and can obtain buoyancy by synthesizing gas vesicles (Walsby, 1994). Under laboratory conditions, Microcystis aeruginosa NIES-843 synthesized gas vesicles and floated in a test tube, while none of the gas vesicles were detected at the bottom of the test tube (Wei et al., 2018).

It was reported that the buoyancy of Microcystis is also strictly related to colony formation (Wei et al., 2019). Wild Microcystis strain presents strong buoyancy, and the large size of the colony can be observed with a microscope (Liu et al., 2018; Wei et al., 2019). On the surface of Microcystis cells, there is a carbohydrate called extracellular polysaccharides (EPS) that play a significant role in the colony formation of Microcystis (Sato et al., 2017; Zhao et al., 2011). EPS contains polysaccharides, proteins, and DNA (De Philippis and Vincenzini, 1998; Flemming and Wingender, 2010). Carboxyl groups in polysaccharides can combine with divalent metal ions by the ionic attractive force, which induces M. aeruginosa colony formation in culture medium (Hahn et al., 2004; Sato et al., 2017; Wang et al., 2011). M. aeruginosa could combine with calcium or cadmium ions to form a colony (Bi et al., 2016; Pradhan and Rai, 2001; Wang et al., 2011; Zhao et al., 2011). With the EPS supplement, M. aeruginosa (strain: PCC7005) obtain buoyancy and form a bloom (Dervaux et al., 2015). The combination of cationic ions and EPS addition could make
M. aeruginosa NIES-843 form a similar colony as wild M. aeruginosa and possessed buoyancy in a measuring cylinder (Wei et al., 2019).

EPS consists of bound EPS (bEPS) and soluble EPS (sEPS) (Wingender et al., 1999). bEPS can be divided into loosely cell-bound EPS (LB-EPS) and tightly cell-bound EPS (TB-EPS) (Basuvaraj et al., 2015; Qu et al., 2012). TB-EPS is one of the main components of M. aeruginosa EPS. Chemical analysis shows that the protein content of TB-EPS exceeds that of EPS, and more enormous amounts of carboxy groups were observed in TB-EPS (Qu et al., 2012). Colony formation induced by TB-EPS is more efficient than EPS in wild Microcystis (Omori et al., 2019; Sakurai et al., 2019). The relationship between two types of bound EPS and M. aeruginosa colony formation has been investigated, and TB-EPS could influence the formation of M. aeruginosa aggregates, and LB-EPS contributed to the development from aggregates to mucilaginous colonies (Tan et al., 2019). However, environmental factors such as light and temperature on the effect of EPS and TB-EPS are unknown.

In this study, from the perspective of colony size, M. aeruginosa NIES-843 and wild Microcystis were cultivated to examine the relationship between colony formation and buoyancy. Based on the experimental data, the efficiency of EPS and TB-EPS to colony formation was investigated. Furthermore, the illumination and temperature conditions on the M. aeruginosa NIES-843 colony formation and buoyancy were discussed.

2 Materials and methods
2.1 Collection of test algae sample and culture conditions

The wild Microcystis strain was collected from Lake Senba, where Microcystis blooms often occur in summer (36°22′N; 140°27′E), Ibaraki, Japan, on September 2019 (Fig. 1). The collection of the sample was carried out at a depth of 5 cm from the water surface. The samples were brought to the laboratory and kept at 4 °C in the dark condition until they were used for microscopic observation and EPS isolation.

M. aeruginosa NIES-843 obtained from National Institute for Environmental Studies (NIES), Japan. This strain was cultured in 500 mL of modified Wright’s Cryptophytes (WC) medium in 1 L Erlenmeyer flasks at 25 °C for about 14 days under 4500 lx continuous illumination. The WC medium...
consisted of a mixture of CaCl₂ (36.76 mg), MgSO₄·7H₂O (36.97 mg), NaHCO₃ (12.60 mg), K₂HPO₄ (8.71 mg), NaNO₃ (85.01 mg), Na₂·EDTA (4.36 mg), FeCl₃·6H₂O (3.15 mg), CuSO₄·5H₂O (0.01 mg), ZnSO₄·7H₂O (0.022 mg), CoCl₂·6H₂O (0.01 mg), MnCl₂·4H₂O (0.18 mg), Na₂MoO₄·2H₂O (0.006 mg), H₂BO₃ (1.0 mg), thiamin·HCl (0.1 mg), biotin (0.005 mg), Vitamin B₁₂ (0.005 mg), ferric citrate (3 mg), citric acid (3 mg), 500 mg tris-(hydroxymethyl)-aminomethane (Tris buffer) in 1 L of distilled water (Guillard and Lorenzen, 1972). The pH of the medium was adjusted to 8.0 ± 0.1 by using 0.5 M HCl. The WC media used in the experiment were sterilized by autoclaving at 115 kPa for 20 min at 121 °C. Inoculation and sampling of the culture medium were conducted in a clean bench to minimize bacterial contamination.

2.2 Isolation of EPS and TB-EPS

The EPS extraction protocol was referred to as the previous studies (Amemiya and Nakayama, 1984; Nishikawa and Kuriyama, 1974; Sato et al., 2017). Under the room temperature condition, 0.25 M sodium hydroxide (NaOH) and 2% (w/v) ethylenediaminetetraacetic acid (Na₂·EDTA·2H₂O) were added to the wild Microcystis sample. After stirring the solution well, the sample was allowed to be left for 1 hour to dissolve the EPS from Microcystis cells. The sample solution was centrifuged at 3000 rpm for 15 minutes, and the supernatant was collected. The solution was further filtered with GF/C filter (Whatman, UK) to remove impurities, and ethanol was added into the filtrate to give a final concentration of 60% (v/v), and it was allowed to precipitate the EPS in the solution. Then, the mixture was stored at -20 °C for 16 hours. After that, the solution was centrifuged at 3000 rpm for 15 min, and the precipitate was collected. The collected sample was freeze-dried to −0.1 MPa, and the dried sample was ground with a mortar and a pestle.

The TB-EPS extraction protocol was referred to as the previous studies (Sakurai et al., 2019; Xu et al., 2013). Sodium chloride (NaCl) was added to the wild Microcystis sample to concentrate of 0.05% (w/v). The supernatant of the sample was then removed by centrifugation at 3000 rpm for 15 min. After that, distilled water was added into the Microcystis residue and filled to the same original volume. NaCl (0.05% (w/v)) was added into the solution and heated at 60 °C for 30 min. After the sample was cooled to room temperature, it was centrifuged at 3000 rpm for 15 min, and the supernatant was collected. The sample was filtrated by GF/C filter (Whatman, UK) and mixed with 1.5 times the amount of ice-cold ethanol to precipitate EPS. The subsequent procedure was the same as mentioned above, and TB-EPS was obtained. The powdered EPS and TB-EPS sample were stored in a desiccator until use.

2.3 Colony formation and buoyancy experiment of M. aeruginosa NIES-843

*M. aeruginosa* NIES-843 was precultured for 14 days. The cations (Ca²⁺ and Mg²⁺) concentration were analyzed by atomic absorption spectrometer (novaAA 300, Analytik Jena AG, Jena, Germany). Then, 50 mL of the sample was poured into a graduated cylinder, and then calcium chloride (CaCl₂), magnesium chloride hexahydrate (MgCl₂·6H₂O), EPS, and TB-EPS were added. Four experimental groups were set up in the experiment, “control”, “Ca²⁺ + Mg²⁺ group”, “EPS added group”, and “TB-EPS added group”. The concentration of EPS and TB-EPS was 200 mg/L in EPS and TB-EPS added group. The control medium was prepared without any addition of Ca²⁺, Mg²⁺, EPS, or TB-EPS. In the previous study, the influences of cationic ions and extracellular polysaccharides (EPS) on colony formation of Microcystis buoyancy were investigated (Sakurai et al., 2019; Wei et al., 2019). With different proportional addition of magnesium and calcium concentration, *M. aeruginosa* exhibited the strongest buoyancy at the mass ratio of 3 (Wei et al., 2019). Therefore, the mass ratio of Ca²⁺ and Mg²⁺ was kept constant at 3 in all experiments. The Ca²⁺ concentration (w/v) used in the buoyancy experiments was controlled from 0 mg/L to 50 mg/L, while Mg²⁺ (w/v) was from 0 mg/L to 150 mg/L.

The prepared medium was cultivated at 25 °C for 24 hours at 10,000 lx and 0 lx in the light-controlled experiment. The prepared medium was cultivated at 25 °C and 30 °C for 24 hours at 10,000 lx in the temperature-controlled experiment. The experiment was conducted in triplicate (n = 3), and the results were expressed as the mean value ± standard deviation (SD).

2.4 Microscopic observation and measurement of colony size

The microscope (Eclipse E100, Nikon, Japan) was used to observe the colonial morphology of *M. aeruginosa*. In this experiment, more than three cells in the aggregation were regarded as a colony. Optical microscopic images were taken with a digital camera system (AM-4023X, AnMo Electronics Corp, Taiwan). Since the *M. aeruginosa* NIES-843 colony always presents irregular morphologies, the following method was used to measure and calculate the diameter. The length and width of colonies were measured directly from the longest axis (length, μm) and the shortest axis (width, μm, aligned perpendicular to the longest axis) (Li et al., 2014). The following equation calculated the diameter (μm) of the colony.

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\text{Diameter} = \sqrt{\text{length} \times \text{width}}
\]

The measurement of colony size was conducted in triplicate (n = 3), and the results were expressed as the mean value ± SD.

2.5 Evaluation of the buoyant ability of Microcystis

The ability of buoyancy can be reflected by calculating the buoyancy rate (Wang et al., 2011). Wild *Microcystis* was cultivated for 24 hours and exhibited strong buoyancy floating to the upper 10 mL of the water surface, as mentioned in detail below (Fig. 2). From these trends, wild *Microcystis* in the upper 10 mL layer (V₁, mL) of the graduated cylinder was considered to possess strong buoyant ability, and the cell density was recorded as C₁ (cells/mL). On the other hand, wild *Microcystis* in the lower 40 mL (V₂, mL) of the medium was supposed to present weak buoyant ability, and the density of
this layer was recorded as $C_2$ (cells/mL). To evaluate buoyancy of $M. \text{aeruginosa}$, the relative buoyancy ($RB_{20}\%$) was calculated from the cell density ($C_1$, $C_2$) and the solution volume ($V_1$, $V_2$) by the following equation (Wang et al., 2011).

$$RB_{20}\% = \frac{C_1V_1}{C_1V_1 + C_2V_2} \times 100$$

When $M. \text{aeruginosa}$ was uniformly distributed in the graduated cylinder, the RB value was calculated to be 20%.

3 Results and discussion

3.1 Buoyancy of wild Microcystis

The sample of wild Microcystis obtained from Lake Senba was cultivated for 24 h in a cylinder (Fig. 2A). Wild Microcystis floated to the water surface after 24 hours of cultivation and presented strong buoyancy (Fig. 2B). The relative buoyancy value of wild Microcystis was calculated to be nearly 100%. Microscopic observation showed that wild Microcystis formed a large colony, indicating that the colony size was about 100 μm (Fig. 3).

3.2 Buoyancy regulation of $M. \text{aeruginosa}$ NIES-843 under the light-limited condition

Under the light condition, the buoyancy of $M. \text{aeruginosa}$ NIES-843 increased in all groups except control. In the TB-EPS added group, $M. \text{aeruginosa}$ NIES-843 presented high buoyancy when concentrations of $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ were more than 10 mg/L and 30 mg/L, respectively. In the EPS added group, the RB value of $M. \text{aeruginosa}$ NIES-843 was close to 80%, with the $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ concentrations of 50 mg/L and 150 mg/L, respectively. At the same concentration of $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$, the buoyancy of EPS added group buoyancy was lower than the TB-EPS added groups (Fig. 4).

Under the dark condition, the buoyancy of $M. \text{aeruginosa}$ NIES-843 increased in the EPS added group. In the EPS added group, the RB value of $M. \text{aeruginosa}$ NIES-843 was close to 75%, with the $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ concentrations of 50 mg/L and 150 mg/L, respectively (Fig. 5). In the TB-EPS added group, the buoyancy of $M. \text{aeruginosa}$ NIES-843 changed slightly, and RB values were nearly 30% with the $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ concentrations of 50 mg/L and 150 mg/L, respectively. The buoyancy of $M. \text{aeruginosa}$ NIES-843 did not change obviously with the addition of $\text{Ca}^{2+}$ + $\text{Mg}^{2+}$ (Fig. 5). $M. \text{aeruginosa}$ NIES-843 presented higher buoyancy in light conditions than dark conditions under the same concentration (Figs. 4 and 5). In the northern rivers of Lake Taihu (China), where Microcystis blooms often occur, the $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ concentrations were reported to be about 58 mg/L and 15 mg/L, respectively (Ye et al., 2010). The cationic ion concentrations in our study are same level in the field study.
The TB-EPS addition can change the buoyancy more efficiently than EPS addition in the case of lighting conditions. The colony size analysis showed that the colony size in Ca²⁺ + Mg²⁺ added group was about 35 μm, while that of the EPS added group was about 70 μm. The largest colony size was about 110 μm observed in the TB-EPS added group, which was similar to the wild Microcystis strain is about 100 μm (Fig. 6). With the same concentration of addition, TB-EPS can induce M. aeruginosa NIES-843 to form a more massive colony than EPS. TB-EPS contains more carboxyl groups than EPS (Omori et al., 2019). In conclusion, M. aeruginosa can combine with more cationic ions such as Ca²⁺ and Mg²⁺ and obtain higher buoyancy with TB-EPS addition.

Under dark conditions, the colony induced by the Ca²⁺ + Mg²⁺ added group and TB-EPS added group was smaller than under light conditions. The largest colony size was observed about 80 μm in the EPS added group (Fig. 6). M. aeruginosa cannot obtain energy without photosynthesis (Takamura et al., 1985). EPS is mainly composed of polysaccharides, and the carboxyl groups combined with Ca²⁺ and Mg²⁺ do not require energy, while TB-EPS is mainly composed of protein (Basuvaraj et al., 2015; Liu et al., 2010). Protein hydrolysis requires energy, and some of the carboxyl groups exist as peptide bonds in protein molecules. These carboxyl groups need hydrolysis to combine with cationic ions (Liu et al., 2008; Mimmack et al., 1989). Therefore, the addition of TB-EPS cannot change the buoyancy of M. aeruginosa NIES-843 in the absence of light. In the previous study, the addition of powdered TB-EPS sample and the adjustment of cationic ion concentrations were promoted effective the colony formation and enlarged the colony size of wild Microcystis (Sakurai et al., 2019). Consequently, Microcystis buoyancy was enhanced by enlarging the colony size, indicating that the control of EPS and cationic ion concentrations would be one of the options for the removal of Microcystis blooms from the viewpoint of cost-effective, low-energy, environmentally-friendly.

M. aeruginosa NIES-843 induces higher buoyancy than dark conditions even at the same concentration of addition, indicating that light affects the buoyancy of M. aeruginosa NIES-843 (Fig. 7). In the present study, M. aeruginosa NIES-843 generated many bubbles in the graduated cylinder with TB-EPS under the light condition (Fig. 7A). Under the dark condition, no bubbles were observed in the graduated cylinder, and the buoyancy of M. aeruginosa NIES-843 was lower than the light condition (Fig. 7B). EPS enhanced the photosynthesis of M. aeruginosa, M. aeruginosa generated many bubbles via photosynthesis after the addition of EPS (Dervaux et al., 2015; Wei et al., 2019). These bubbles have a particular effect on buoyancy reconformed in this experiment. In this study, the concentration of calcium and magnesium greatly enhanced the colony formation of M. aeruginosa NIES-843. Compared with the chemical composition in WC (Guillard and Lorenzen, 1972) and BG-11 (Waterbury and Stanier, 1981) medium,
their concentrations are not significantly different. Therefore, the same experiment could be carried out in BG-11 medium or other media.

3.3 Buoyancy regulation of *M. aeruginosa* NIES-843 under the temperature controlled condition

The specified concentration conditions (Ca$^{2+}$ and Mg$^{2+}$ of 30 mg/L and 90 mg/L, EPS and TB-EPS of 200 mg/L, respectively) were set in the experiment to confirm temperature effect on buoyancy and colony formation. In the control group, the buoyancy of *M. aeruginosa* NIES-843 did not change significantly under different temperature conditions. With the temperature increased from 20°C to 30°C, the buoyancy of the Ca$^{2+}$ + Mg$^{2+}$ added group increased from 50% to 53%, the EPS added group increased from 70% to 73%, and the TB-EPS added group increased from 90% to 94% (Fig. 8). The colony size of Ca$^{2+}$ + Mg$^{2+}$ added group increased to 51 µm, the colony size of the EPS addition group increased to 94 µm, and the colony size of the TP-EPS added group increased to 134 µm (Fig. 9). Under the same concentration conditions, the increase in temperature is conducive to colony formation and presents higher buoyancy of *M. aeruginosa* NIES-843.

Wild *Microcystis* strain isolated from Lake Nieuwe Meer in Holland, the percentage of total colonies that were sinking increased a few days to 100% after the temperature shifted from 20°C to 10.5°C (Visser et al., 1995). Lake Taihu is the third-largest freshwater lake in China, and seasonal *Microcystis* blooms have regularly formed in the lake for the past three decades. With global warming, *Microcystis* growing season has advanced by approximately 20 days over the last two decades (Deng et al., 2014; Otten and Paerl, 2011). Therefore, monitoring the lakes’ temperature where *Microcystis* bloom often occurs is incredibly essential to protect the water environment.

4 Conclusions

In this study, we induced *M. aeruginosa* NIES-843 to form colony by using EPS and TB-EPS extracted from *Microcystis* blooms. The change of the *M. aeruginosa* buoyancy was examined, and principal conclusions were summarized as follows:

- The light influenced the colony formation and buoyancy of *M. aeruginosa* NIES-843. Under light conditions, the addition of TB-EPS colud induce *M. aeruginosa* to obtain high buoyancy at low Ca$^{2+}$ and Mg$^{2+}$ concentration.
- The colony size analysis showed that at the same cationic concentration, the addition of TB-EPS could induce *M. aeruginosa* to form the largest colony and present strong buoyancy.
The increase in temperature is conducive to the colony formation of *M. aeruginosa* and presents higher buoyancy.

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