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Colony formation of highly dispersed *Microcystis aeruginosa* by controlling extracellular polysaccharides and calcium ion concentrations in aquatic solution

Masahiro Sato[1] · Yoshimasa Amano[1,2] · Motoi Machida[1,2] · Fumio Imazeki[2]

Abstract This study isolated extracellular polysaccharides (EPS) as a powder material from cyanobacterial blooms and the powdered EPS was used to trigger colony formation of dispersed unicellular *M. aeruginosa* by controlling EPS concentration in culture medium. The effect of Ca^{2+} ions on the colony formation of *M. aeruginosa* was also investigated, then the interaction between EPS and Ca^{2+} ions on colony formation was discussed. The results showed that the addition of the powdered EPS into the medium did not cause morphological changes of *M. aeruginosa*, suggesting that EPS alone would not induce the colony formation of *M. aeruginosa*. On the other hand, a high concentration of calcium ions (1000 mg/l) caused colony formation. When EPS and Ca^{2+} ions in the culture medium were adjusted to 200 and 1000 mg/l, respectively, the colony density, the average cell number per colony and the particle size of *M. aeruginosa* showed ca. 1.7–2.0 times greater values than those in the Ca^{2+} added medium. Calcium ion contributed to the aggregation of *M. aeruginosa* via crosslinked

reaction with negatively charged *M. aeruginosa* cells, and the addition of EPS possessing negatively charged functional groups such as carboxy groups could enhance the reaction, promoting the crosslinked reaction between EPS and Ca^{2+} ions.

Keywords *Microcystis aeruginosa* · Colony formation · Cyanobacterial blooms · Extracellular polysaccharides (EPS) · Calcium ion

Introduction

Cyanobacterial blooms caused by eutrophication have recently plagued fresh and brackish water bodies in many countries (Yang and Kong 2013), and threaten aquatic organisms with shading of sunlight, as well as spoiling the landscape (Carpenter et al. 1998; Ou et al. 2006). They also produce toxins such as microcystin and anatoxin, which could cause a health hazard to birds, livestock, and even people (Matsunaga et al. 1999; Azevedo et al. 2002; Katırcıoğlu et al. 2004). For the prevention of such issues and conservation of the water environment, it is important to suppress the appearance of cyanobacterial blooms and clarify the mechanisms by which cyanobacterial blooms dominate in the phytoplankton community.

Microcystis aeruginosa is one of the representative algal bloom-forming cyanobacteria worldwide and often forms colonies aggregating several tens and hundreds of cells in transparent mucilaginous sheath materials (Ordorika et al. 2004; Yang and Kong 2013). The colony formation of *M. aeruginosa* provides great advantages for these organisms to dominate over other phytoplankton species, such as the promotion of floating speed, nutrient uptake and anti-predator defense (Fogg and Walsby 1971; Bolch and

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Blackburn 1996; Yang et al. 2008). *M. aeruginosa* possesses gas vesicles (Kromkamp et al. 1988; Brookes and Ganf 2001); thereby they can enhance buoyancy by developing and expanding the colony size (Stokes' law). There have been several attempts at on-site purification by sucking up algal blooms which have accumulated at the water surface (Kirke 2001; Hudnell et al. 2010), but only temporary effects have been obtained in most cases. It could be speculated that such negative outcomes could be due to *M. aeruginosa* with less ability for colony formation or buoyancy developing these abilities in the course of time and appearing as algal blooms again (Nakano et al. 2001).

It is widely recognized that, when the colony-forming *M. aeruginosa* isolated from cyanobacterial blooms is grown in culture medium under laboratory conditions, the colonial *M. aeruginosa* cells disaggregate into single cells or twin cells after multiple generations, and *M. aeruginosa* never forms a colony again (Stainer et al. 1971; Zhang et al. 2007). In addition, *M. aeruginosa* would not exhibit distinct buoyancy. In general, *M. aeruginosa* secretes extracellular polysaccharides (EPS) which help to maintain colonial morphology (Plude et al. 1991; Li et al. 2013), but the amount of EPS secreted by the disaggregated cells tends to decrease compared to the colonial cells (Yang et al. 2008). From these results, it might be expected that the disaggregated *M. aeruginosa* could form a colony if EPS production could be promoted under laboratory conditions, but ways and/or methods for artificially controlling EPS production of *M. aeruginosa* have not been suggested yet.

As addressed above, the disaggregation of *M. aeruginosa* colony into single cells or twin cells is an irreversible phenomenon under laboratory conditions, and the colony formation of *M. aeruginosa* never proceeds in a natural process as far as external factor such as predation by flagellate is added (Yang et al. 2008; Yang and Kong 2012). If colony formation of unicellular *M. aeruginosa* can be achieved through a culture experiment, the technique could be used with a wild *M. aeruginosa* strain (potential colony-forming strain) more effectively than the unicellular *Microcystis* strain. Then, *M. aeruginosa* cells in lake water could be collected at a high rate, and the development of such technology would contribute to establishing new measures against algal blooms including *M. aeruginosa* in eutrophic lakes as well as at water purification plants.

The objectives in this study were to isolate EPS as a powder material from cyanobacterial blooms, composed mainly of the colony-forming *M. aeruginosa* in eutrophic lakes, and then use the powdered EPS to trigger colony formation of dispersed unicellular *M. aeruginosa* by controlling EPS concentration in the culture medium. As reported by Zhao et al. (2011), colony formation of *M. aeruginosa* would also be related to bivalent cation ions,

such as calcium ions, and a high concentration of Ca^{2+} (100 mg/l) could contribute to the aggregation of *M. aeruginosa* due to the bridging effect of Ca^{2+} ions between *M. aeruginosa* cells and adherent EPS on the cells (Wang et al. 2011). Based on the studies mentioned above, we also examined the effect of Ca^{2+} concentration on colony formation of the dispersed unicellular *M. aeruginosa*. The interaction between EPS and Ca^{2+} ions on colony formation of *M. aeruginosa* is discussed. We expected that if it was possible to form colonies from dispersed unicellular *M. aeruginosa*, the technique could be effectively used with actual algal blooms. This study also has valuable and essential information that helps us to understand *M. aeruginosa* dominance in the phytoplankton community, and/or appearance of algal blooms in eutrophic lakes.

Materials and methods

Unicellular *M. aeruginosa*

Unicellular *M. aeruginosa* strain (UTEX LB 2061) was obtained from the Culture Collection of Algae of the University of Texas, USA. This strain exists as single cells or twin cells, and highly dispersed in the medium. Colony formation of this strain was investigated in this study.

M. aeruginosa was transferred to Wright's cryptophytes (WC) medium (Guillard and Lorenzen 1972), containing nitrogen and phosphorus concentrations of 14 mg/l and 1.55 mg/l, respectively, and cultured at 25 °C and 27 μmol photons/(m^2 s) under static conditions. The solution pH was adjusted to 8.0 by 0.5 M HCl. The cultivation was continued for about 2 weeks, then an aliquot of the medium was transferred to fresh WC medium to subculture the unicellular *M. aeruginosa*. All media and glassware used for cultivation were autoclaved at 121 °C for 30 min. Each flask was manually stirred several times a day, and randomly replaced to make sure that light irradiance was uniform. All procedures were conducted on a clean bench to minimize bacterial contamination.

Cyanobacterial blooms

The cyanobacterial bloom sample was obtained from Lake Senba, Japan (36°22′N, 140°27′E) in September, 2014. The dense cyanobacterial bloom sample was collected from the top 5 cm of the water column. The sample was immediately transferred to the laboratory, and stored at 4 °C in dark conditions before use. Optical microscopic observation confirmed that the main species in the cyanobacterial bloom was a colony-forming species of the genus *Microcystis*. EPS was extracted from this sample.

Extraction of EPS from cyanobacterial blooms

Extraction of EPS from cyanobacterial blooms was performed according to the method of Amemiya and Nakayama (1984), and Nishikawa and Kuriyama (1974) with some modifications. EPS on the cell surface of cyanobacterial blooms was solubilized with 2 % EDTA and 0.25 M NaOH and settled for 1 h at 25 °C. The bulk solution obtained in this process was centrifuged at 3000 rpm for 15 min, and the supernatant was collected. Approximately 1.5 times the volume of ice-cold ethanol was added to supernatant, then the mixture was stored overnight at −20 °C to precipitate EPS. After that, EPS was collected after centrifugation at 3000 rpm for 15 min and freeze-dried at −0.1 MPa, −20 °C for 3 h. The dried EPS was ground into a uniform powder and stored in a desiccator before use. As described below, the EPS sample possessed functional groups such as carboxy groups, which contribute to colony formation in *M. aeruginosa*. Thus, it is inferred that the activity and/or functionality of the EPS survives the chemical extraction processes used in this study.

Colony formation of unicellular *M. aeruginosa*

The unicellular *M. aeruginosa* was precultured in 350 ml WC medium ($N = 14$ mg/l and $P = 1.55$ mg/l) in a 500-ml Erlenmeyer flask at a light irradiance of 135 μmol photons/(m^2 s) with a light and dark cycle of 14:10 h, respectively, at 25 °C. The solution pH was adjusted to 8.0 by 0.5 M HCl. Each flask was manually stirred several times a day, and randomly replaced to make sure there was uniform light irradiance. The preculture was conducted for 7 days, during which *M. aeruginosa* reached an exponential growth phase. After preculture, the culture medium was centrifuged at 3000 rpm for 15 min and the supernatant was removed. The residual cells were dispersed in deionized water and centrifuged at 3000 rpm for 15 min to wash the cells. The washing process was repeated 3 times to remove ions from the medium.

The washed unicellular *M. aeruginosa* cells were inoculated into 50 ml deionized water in a 100-ml Erlenmeyer flask to give an initial cell density of 10^6 cells/ml. The deionized water medium was used in the experiment to eliminate the effect of dissolved ions contained in the WC medium. Calcium chloride (CaCl$_2$) was used to adjust the Ca^{2+} ion concentration and directly added to flasks, whereas the powdered EPS was first dissolved in deionized water and adjusted to 5000 mg/l, then the solution was filtered using Whatman GF/C filter paper. After that, the filtrate was added to each flask. A flask with no addition of EPS or Ca^{2+} ions was made and run as a control.

Concentrations of EPS and Ca^{2+} ions in the medium were determined in preliminary experiments. Briefly, EPS concentrations from 0.1 to 500 mg/l, or calcium concentrations from 0 to 750 mg/l did not affect the colony formation of *M. aeruginosa*. It was also revealed that a calcium concentration of 1000 mg/l led to colony formation, and that the EPS concentration inducing the promotion of colony formation was above 200 mg/l when the calcium concentration was adjusted to 1000 mg/l. From these results, three kinds of experimental medium were prepared to investigate the effects of EPS and calcium ions on colony formation: EPS (200 mg/l) added medium, Ca^{2+} (1000 mg/l) added medium and EPS (200 mg/l) + Ca^{2+} (1000 mg/l) added medium. The solution pH was adjusted to $6.0 ± 0.1$ by 0.5 M NaOH, because colony formation did not occur under higher pH conditions (data not shown). Each flask was cultured in an incubator at 25 °C, light irradiance of 135 μmol photons/(m^2 s), light and dark cycle of 14:10 h, respectively, and cultivated for 24 h under static conditions. After cultivation, each flask was agitated just for 3 s by a touch mixer (PMM-21, Yamato Scientific, Japan) to avoid colonial aggregation by sedimentation in the bottom of the flask. An aliquot of the culture medium was taken to measure cell and colony densities. The aggregation of more than 3 cells was defined as a colony in this study. Each sample was filtered through a Whatman GF/C filter paper and the filtrate was used for the determination of dissolved ions. The experiment was conducted in triplicate ($n = 3$) and the results were expressed as the mean value with a standard deviation (SD).

Analysis

Colony density, total cell density, and cell density except colonies, were measured by direct counting using an optical microscope with a digital camera system (AM-4023X, Dino-lite, Taiwan) and plankton counting chamber (MPC-200, Matsunami Glass, Japan). The average cell number per colony (ACN) was calculated by the following equation:

$$\text{ACN} = \frac{C_t - C_e}{c}, \tag{1}$$

where C_t is the total cell density, C_e is the cell density except colonies, and c is the colony density.

The zeta potential (ζ potential) of *M. aeruginosa* cells was determined by a zeta potential analyzer (ZC-3000, Microtec, Japan). Cell and colony size distribution were also determined by zeta potential analyzer, although particles larger than 30 μm could not be detected. In the particle size measurement, a particle diameter above 10 μm was regarded as a colony because twin cells of *M.*

aeruginosa were approximately 8–10 μm. In each experiment 300 particles were measured and the frequency distribution and frequency percentage were calculated. The solution pH was measured by pH meter (D-51, Horiba, Japan). Total sugar content was determined by the phenol–sulfuric acid method with glucose as the standard (Dubois et al. 1956). Nitrogen and phosphorus concentrations were analyzed by ion chromatography (ICS-1100, Dionex, USA) and molybdenum blue method (Murphy and Riley 1962) using a UV–Vis spectrophotometer (UV-2550, Shimadzu, Japan), respectively. Fourier transformed infrared (FT-IR) spectroscopy analysis was performed to characterize major functional groups on the EPS using an IR Affinity-1 instrument (Shimadzu, Japan). The powdered EPS was mixed with potassium bromide (KBr) in the ratio of 1:100 and a pellet with a diameter of 3 mm was prepared for the FT-IR analysis by pressing the mixture. The FT-IR spectra were recorded between 4000 and 400 cm^{-1} with a 4-cm^{-1} resolution in transmittance mode.

All experimental data were represented by the mean ± standard deviation (SD). All data were subjected to one-way analysis of variance (ANOVA) followed by Tukey tests to examine significant differences between individual mean values. All statistical tests were carried out using Microsoft Excel Statistics 2008 (Social Survey Research Information Co., Ltd., Japan). Differences were considered statistically significant at $p < 0.05$.

Results

Optical microscopic observation

After 24 h of cultivation, *M. aeruginosa* was present as single or twin cells and highly dispersed in the culture medium in the control (Fig. 1a). The same result was obtained in the medium with EPS addition, which exhibited only single and twin cells, but the medium also contained sheath-like substances, which did not exist in the control (Fig. 1b). In contrast, several small colonies as well as single and twin cells were found in the Ca^{2+} added medium (Fig. 1c). This trend was similarly observed in the EPS + Ca^{2+} added medium, but this medium also allowed *M. aeruginosa* to form much larger colonies than in the Ca^{2+} added medium (Fig. 1d).

Colony and cell densities of *M. aeruginosa*

Although there were no colonies in the control and the EPS added medium, *M. aeruginosa* formed colonies in the Ca^{2+} added medium and the EPS + Ca^{2+} added medium (Table 1). The colony densities of the Ca^{2+} added medium and the EPS + Ca^{2+} added medium were
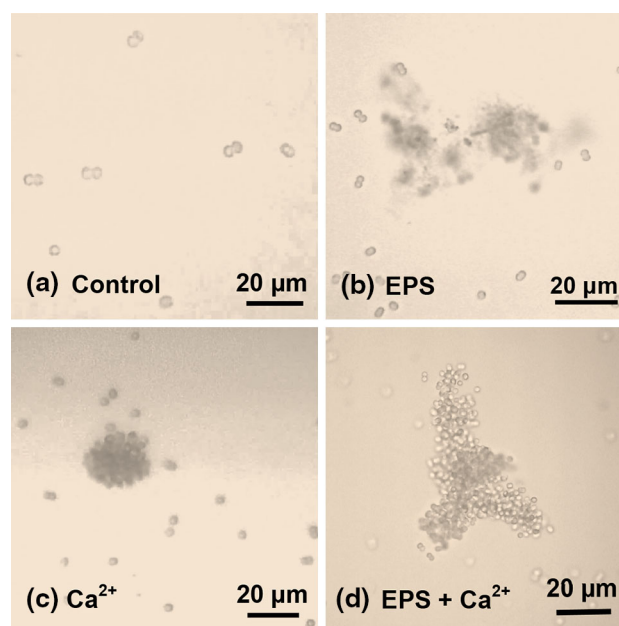

Fig. 1 Optical microscope images of *M. aeruginosa* in each medium

$3.44 \times 10^3 \pm 2.88 \times 10^2$ and $6.05 \times 10^3 \pm 2.72 \times 10^2$ colonies/ml, respectively, and the density for the latter medium was significantly higher than that for the former medium ($p < 0.05$).

The total cell density of the control was decreased from 1.00×10^6 to $9.00 \times 10^5 \pm 6.00 \times 10^4$ cells/ml after the cultivation time of 24 h (Table 1), whereas a significant increase in the total cell density compared with the control was observed for the EPS added medium ($p < 0.05$) ($1.12 \times 10^6 \pm 1.37 \times 10^5$ cells/ml). There was no remarkable difference in the total cell density between the control and the Ca^{2+} added medium ($p > 0.05$) ($9.80 \times 10^5 \pm 1.73 \times 10^4$ cells/ml), although ca. 1.4 times higher cell density of $1.39 \times 10^6 \pm 2.65 \times 10^4$ cells/ml than that for the control ($p < 0.05$) was obtained for the EPS + Ca^{2+} medium. The cell density except colonies showed the opposite trend against colony density, indicating that the density for the Ca^{2+} added medium ($5.67 \times 10^5 \pm 1.94 \times 10^4$ cells/ml) and the EPS + Ca^{2+} added medium ($1.25 \times 10^5 \pm 3.49 \times 10^3$ cells/ml) were much lower than the control, and a significant difference in the density was also observed for the two media ($p < 0.05$) (Table 1).

The mean cell number per colony, calculated from values of the colony density, the total cell density and the cell density except colonies, for the Ca^{2+} added medium was $1.20 \times 10^2 \pm 1.05 \times 10^1$ cells/colony (Table 1), while a much higher value of cell numbers ($2.09 \times 10^2 \pm 1.36 \times 10^1$ cells/colony) was obtained for the EPS + Ca^{2+} added medium ($p < 0.05$).

Table 1 Effects of EPS and Ca^{2+} ions on colony formation by *M. aeruginosa*

Medium	Colony density (colonies/ml)	Total cell density (cells/ml)	Cell density except colonies (cells/ml)	Average cell number per colony (cells/colony)
Control	0	$9.00 \times 10^5 \pm 6.00 \times 10^4$	$9.00 \times 10^5 \pm 6.00 \times 10^4$	0
EPS	0	$1.12 \times 10^6 \pm 1.37 \times 10^5*$	$1.12 \times 10^6 \pm 1.37 \times 10^5*$	0
Ca^{2+}	$3.44 \times 10^3 \pm 2.88 \times 10^2*$	$9.80 \times 10^5 \pm 1.73 \times 10^4$	$5.67 \times 10^5 \pm 1.94 \times 10^4*$	$1.20 \times 10^2 \pm 1.05 \times 10^1*$
EPS + Ca^{2+}	$6.05 \times 10^3 \pm 2.72 \times 10^2*$	$1.39 \times 10^6 \pm 2.65 \times 10^4*$	$1.25 \times 10^5 \pm 3.49 \times 10^3*$	$2.09 \times 10^2 \pm 1.36 \times 10^1*$

Values are represented as mean $\pm$ standard deviation ($n = 3$)

* Significant difference compared with control ($p < 0.05$)

Cell and colony size distribution

The cell and/or colony size distribution in the control medium showed that ca. 98 ± 58 cells (33 ± 19 %) and 202 ± 58 cells (67 ± 19 %) of *M. aeruginosa* were in the range in 0–5 μm and 5–10 μm, respectively, and cells larger than 10 μm were not found in the medium (Table 2). In the EPS added medium, most of the cells had a particle diameter of less than 10 μm. On the other hand, the Ca^{2+} added medium included larger particles possessing 10- to 15- and 15- to 20-μm diameters with the proportions of 21 ± 3 and 5 ± 4 %, respectively. In the EPS + Ca^{2+} medium, particles with all ranges of diameters were present in the medium, and diameters ranging from 20 to 30 μm appeared only in this medium, at a proportion of ca. 4 ± 2 % of the total cell density. For both Ca^{2+} added and EPS + Ca^{2+} added medium, particles in the range 0–5 μm were apparently fewer than in the EPS added medium ($p < 0.05$).

Change in zeta potential

As shown in Fig. 2, the ζ potential for the control medium was -32.6 ± 0.8 mV, which was similar to the value of -31.0 ± 1.8 mV obtained for the EPS added medium. In the Ca^{2+} added medium, the ζ potential indicated a significantly lower value (-22.9 ± 4.1 mV) than that for the control ($p < 0.05$). A decrease in ζ potential was also observed for the EPS + Ca^{2+} added medium: this value of -17.0 ± 7.9 mV was the lowest in all media used in this study and was remarkably lower than that in the control medium ($p < 0.05$).

Total sugar content and nutrient concentration in each medium

The total sugar contents for the control and the Ca^{2+} added media were not detected either before ($t = 0$ h) or after ($t = 24$ h) the experiment (Fig. 3). When the powdered

Table 2 Particle size frequency distribution of *M. aeruginosa* cells and colonies for each medium

Cell and colony size distribution (cells or colonies)						
Medium (μm)	0–5	5–10	10–15	15–20	20–25	25–30
Control	98 ± 58 (33 ± 19 %)	202 ± 58 (67 ± 19 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
EPS	139 ± 22 (46 ± 7 %)	160 ± 22 (53 ± 7 %)	0 ± 1^a (0 ± 0 %)	0 (0 %)	0 (0 %)	0 (0 %)
Ca^{2+}	$36 \pm 3^\dagger$ (12 ± 1 %)	186 ± 10 (62 ± 3 %)	$64 \pm 8*$ (21 ± 3 %)	14 ± 16 (5 ± 4 %)	0 (0 %)	0 (0 %)
EPS + Ca^{2+}	$30 \pm 4^\dagger$ (10 ± 1 %)	155 ± 36 (52 ± 12 %)	$84 \pm 35*$ (28 ± 12 %)	$20 \pm 4*$ (7 ± 1 %)	$9 \pm 2*$ (3 ± 1 %)	$2 \pm 2*$ (1 ± 1 %)

Values are represented as mean $\pm$ standard deviation ($n = 3$). Values in parentheses are the proportion of individual particles to total particles

* Siginificant difference compared with the control medium ($p < 0.05$)

[†] Significant difference compared with the EPS added medium ($p < 0.05$)
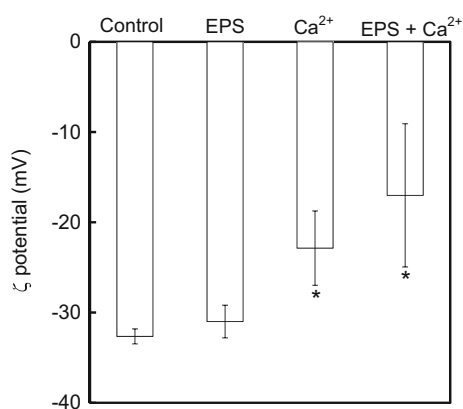
[a] Flocculation of EPS interacted with each other

Fig. 2 Zeta potential of *M. aeruginosa* cells and colonies in each medium. Values are represented as mean ± standard deviation ($n = 3$). Asterisk (*) indicates a significant difference compared with control ($p < 0.05$)
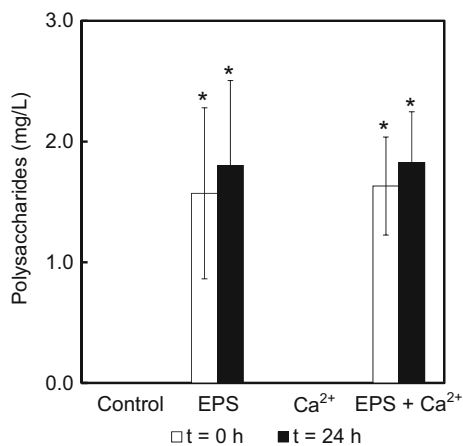

Fig. 3 Changes in total sugar contents before and after culture experiment in each medium. Values are represented as mean ± standard deviation ($n = 3$). Asterisk (*) indicates a significant difference compared with control ($p < 0.05$)

EPS was added into the medium, the total sugar contents for both EPS added and EPS + Ca^{2+} added media showed ca. 1.57 ± 0.71 and 1.63 ± 0.41 mg/l at $t = 0$ h, and 1.80 ± 0.70 and 1.83 ± 0.42 mg/l at $t = 24$ h, respectively. Although total sugar contents were slightly increased during the experiment, a significant change in total sugar content before and after the experiment was not observed for either media ($p > 0.05$).

During the culture experiment of *M. aeruginosa*, a nitrogen concentration was not detected in any of the media, whereas the opposite result with phosphorus was observed when the powdered EPS was added to the medium (Fig. 4). Phosphorus concentrations for the EPS added and the EPS + Ca^{2+} added media ranged from 3.01 ± 0.05 to 3.25 ± 0.02 mg-P/l during the experiment. Although phosphorus concentration for the EPS added
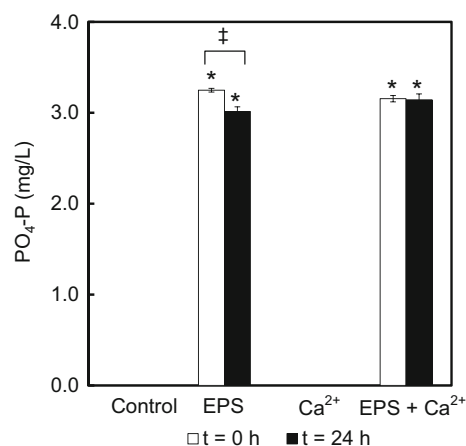

Fig. 4 Changes in phosphorus concentration before and after culture experiment in each medium. Values are represented as mean ± standard deviation ($n = 3$). Asterisk (*) indicates a significant difference compared with control ($p < 0.05$). *Double dagger* (‡) represents a significant difference between media at $t = 0$ and 24 h

medium was remarkably decreased after 24 h ($p < 0.05$), little difference in the concentration before and after the experiment was observed for the EPS + Ca^{2+} added medium ($p > 0.05$).

FT-IR analysis of the EPS extracted from cyanobacterial blooms

The results of the FT-IR spectra of the EPS extracted by cyanobacterial blooms are presented in Fig. 5. The results showed that the EPS had a broad band at 3429 cm^{-1}, implying the presence of hydroxyl groups (Xu et al. 2013). The band around 1647 cm^{-1} could be attributed to the C=O adsorption of uronic acids (Chakraborty et al. 2015). The presence of C=O and C–O bonds from carboxylate might be exhibited by the adsorption at 1456 cm^{-1} (Bramhachari et al. 2007). A peak at 1020 cm^{-1} was attributed to the C–O in alcohols, suggesting the hydroxyl
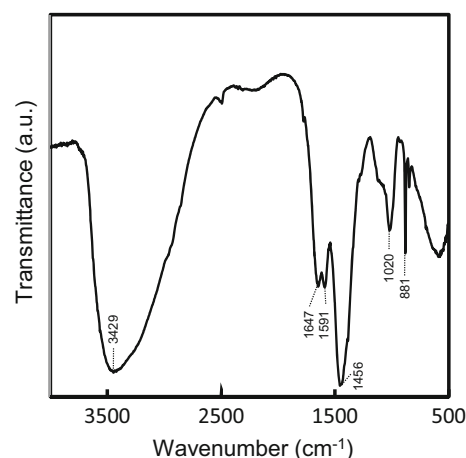

Fig. 5 FT-IR spectrum of EPS extracted from cyanobacterial blooms

groups in the EPS (Abd-El-Haleem et al. 2008). The linkage between monosaccharides was observed as the band at 820 cm^{-1} (Ismail 2013).

Discussion

It is widely recognized that the colony-forming wild strain of *M. aeruginosa* loses its colony formation ability and becomes single cells and/or twin cells by cultivation under laboratory conditions. Although a reasonable explanation has not been suggested, several studies imply that colony formation could be induced by the presence of zooplankton (Burkert et al. 2001; Yang et al. 2008), infochemicals secreted by *Daphnia* (Lampert et al. 1994), extracellular microcystins produced by *M. aeruginosa* (Sedmak and Eleršek 2005), and fluid motion (Li et al. 2013). Among these parameters, one of the influential factors causing colony formation in *Microcystis* is extracellular polysaccharides (EPS). In this study, we investigated colony formation in *M. aeruginosa* by using powdered EPS to control the EPS concentration in the culture medium. Since EPS strongly interacts with cationic ions (Iijima et al. 2002; Wang et al. 2011), colony formation may be affected by the interaction of these factors. The effects of EPS and calcium concentration as well as nutrients (nitrogen and phosphorus) and total EPS sugar content on colony formation are discussed in detail below.

The addition of powdered EPS to the culture medium did not cause morphological changes in *M. aeruginosa*, suggesting that EPS alone would not induce colony formation in *M. aeruginosa*. The phosphorus concentration in the media with EPS addition was measured to be about 3.0 mg-P/l, although nitrogen was not detected in any media. As shown in Table 1, the total cell density in the EPS added medium increased after the 24-h cultivation compared with the control ($p < 0.05$), while a significant decrease in the phosphorus concentration during the experiment was also observed ($p < 0.05$) (Fig. 4), indicating that the increase in *M. aeruginosa* cells correlated with the presence of phosphorus. The same trend of increase in total cell density was obtained in the EPS + Ca^{2+} added medium, although the phosphorus concentration in the medium was not largely reduced after 24 h ($p > 0.05$). Shi et al. (2013) examined the effect of calcium ions on the growth of *M. aeruginosa* and revealed that a calcium concentration of 60 mg/l increased the growth of *M. aeruginosa*. In this study, the total cell density in the EPS + Ca^{2+} medium remarkably increased compared with the control ($p < 0.05$), but such trends were not obtained in the Ca^{2+} added medium. These results imply that the higher cell density in the EPS + Ca^{2+} medium than in the same medium without EPS addition

could not be attributed to calcium ions or phosphorus. Amemiya and Nakayama (1984) investigated metal content in the EPS obtained from algal blooms (mainly the genus *Microcystis*) and indicated that EPS contained several metals (Na,K,Ca,Mg,Fe,Mn,Zn,Ni,Cu), and the highest content of the metals was Fe (ca. 25 % in total metal contents). As suggested by Nagai et al. (2007), iron limitation significantly affects the growth of *M. aeruginosa*. From these results, it is suggested that metals, especially Fe, contained in EPS could affect the total cell density of *M. aeruginosa* in the media with EPS addition in this study. Despite the higher phosphorus concentration, which was ca. 3 times greater than the phosphorus level (1.2 mg/l) in an extremely hypereutrophic lake (Xu et al. 2003), *M. aeruginosa* never formed colonies in the EPS added medium, implying that phosphorus was not involved in the development of *M. aeruginosa* colony formation.

The total sugar content in the media with EPS addition was approximately 1.5–2.0 mg/l larger than those without EPS addition at the beginning of the experiment ($p < 0.05$), and the content tended to increase after 24 h (Fig. 3), although a significant difference before and after the experiment was not observed ($p > 0.05$). Several studies examined the effects of nutrient concentration (nitrogen and phosphorus) on the amount of EPS production for *M. aeruginosa*, and indicated that *M. aeruginosa* enhanced EPS synthesis when the concentration in the medium was limited (Wang et al. 2010a, b). It has also been suggested that EPS secretion by strains of the cyanobacterium *Nostoc* was promoted under nitrogen limitation (Otero and Vincenzini 2003). In this study, nitrogen was not present in all media, and phosphorus was not detected in the media without EPS addition, but the EPS was not observed. The results in this study clearly indicate that the EPS production of *M. aeruginosa* was not related to nutriment limitation. It is also implied that the increase in phosphorus concentration or other substances such as trace metal ions accompanying the addition of the powered EPS may contribute to EPS production, although a significant difference between the media at the beginning and the end of the experiment was not observed.

The values of ζ potential in the Ca^{2+} added media were ca. 10–20 mV lower than those without calcium addition. Qu et al. (2015) examined the effect of calcium ions on the ζ potential of colonies and cells of *M. aeruginosa* in an EPS solution originating from *M. aeruginosa* and clarified that the ζ potential value was gradually lowered by increasing the amount of calcium ions added. In the present study, the addition of calcium ions to each medium also led to a decrease in the ζ potential value of *M. aeruginosa* cells/colonies, implying that the reduction of negative surface charge of EPS may promote the aggregation of cells and colonies.

Despite EPS (200 mg/l) alone having no effect on colony formation in *M. aeruginosa*, calcium ions (1000 mg/l) did contribute to the morphological change. Wang et al. (2011) investigated the effects of calcium and magnesium concentrations on the colony formation of *Microcystis*. Their results clearly indicated that colony formation was concerned only with calcium ions, and that the concentration inducing colonies was required to be above 100 mg/l. In our preliminary experiments, calcium concentrations from 0 mg/l to 750 mg/l at pH 6.0 in aqueous solution did not cause colony formation in *M. aeruginosa* (data not shown). This disagreement was due to the differences in *M. aeruginosa* strain, the presence of nutrients/other trace ions as well as culture conditions such as light and temperature. When calcium ions and EPS concentrations were simultaneously adjusted to 1000 and 200 mg/l, respectively, colony formation was promoted more than in the Ca^{2+} added medium. Previous studies clarified that EPS contained large portions of protein and polysaccharide, and the latter consisted of amino acids and several monosaccharides possessing surface functional groups such as carboxy groups (Amemiya and Nakayama 1984, Plude et al. 1991). According to the study of Amemiya and Nakayama (1984), EPS was composed of 12.2–14.8 % uronic acids. Plude et al. (1991) revealed the presence of pectin as uronic polymers in the EPS of *M. flos-aque*. In the present study, carboxy groups on the EPS surface were detected by FT-IR analysis (Fig. 5), which implies the presence of uronic acids including pectin. Although we were concerned that the chemical processes for the extraction of EPS from cyanobacterial blooms in this study may alter the properties of EPS, the addition of the powdered EPS as well as Ca^{2+} ions induced colony formation in *M. aeruginosa*. This indicates that the powdered EPS may be different from that in situ, but the activity or functionality of the EPS was fundamentally kept after the chemical processes. Iijima et al. (2002) indicated that carboxy groups on uronic acid were crosslinked by polyvalent metal cations such as calcium ions. Wang et al. (2011) also suggested the possibility that divalent cations crosslinked with negatively charged functional groups within bacterial EPS could contribute to cell–cell aggregation. In the present study, a slight amount of EPS surrounding *M. aeruginosa* cells allowed them to aggregate with each other via crosslinked reactions induced by the addition of Ca^{2+} ions, causing colony formation in the Ca^{2+} added medium. Similarly, *M. aeruginosa* colonies in the Ca^{2+} + EPS added medium were formed in the same way as the Ca^{2+} added medium, but due to the presence of a large amount of EPS added, the crosslink reaction between EPS and Ca^{2+} ions was promoted. This would be the mechanism by which the large colonies of *M. aeruginosa* formed in the Ca^{2+} + EPS added medium.

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