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Enhanced biomass production and nutrient removal capacity of duckweed via two-step cultivation process with a plant growth-promoting bacterium, *Acinetobacter calcoaceticus* P23

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HIGHLIGHTS

- A new duckweed cultivation process which enables effective PGPB use was proposed.
- The process reduces PGPB dose by separating colonization and cultivation steps.
- Remarkable improvements in duckweed growth and nutrient removal were attained.
- The growth-promotion by PGPB lasted for 5–10 days after the colonization step.
- Persistence of PGPB would be the key to maintain high performance of this process.

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ABSTRACT

Plant growth-promoting bacteria (PGPB) are considered a promising tool to improve biomass production and water remediation by the aquatic plant, duckweed; however, no effective methodology is available to utilize PGPB in large hydroponic systems. In this study, we proposed a two-step cultivation process, which comprised of a "colonization step" and a "mass cultivation step," and examined its efficacy in both bucket-scale and flask-scale cultivation experiments. We showed that in the outdoor bucket-scale experiments using three kinds of environmental water, plants cultured through the two-step cultivation method with the PGPB strain, *Acinetobacter calcoaceticus* P23, yielded 1.9 to 2.3 times more biomass than the control (without PGPB inoculation). The greater nitrogen and phosphorus removals compared to control were also attained, indicating that this strategy is useful for accelerating nutrient removal by duckweed. Flask-scale experiments using non-sterile pond water revealed that inoculation of strain P23 altered duckweed surface microbial community structures, and the beneficial effects of the inoculated strain P23 could last for 5–10 d. The loss of the duckweed growth-promoting effect was noticeable when the colonization of strain P23 decreased in the plant. These observations suggest that the stable colonization of the plant with PGPB is the key for maintaining the accelerated duckweed growth and nutrient removal in this cultivation method. Overall, our results suggest the possibility of an improved duckweed production using a two-step cultivation process with PGPB.

1. Introduction

Duckweeds (*Lemnaceae*) are free-floating aquatic plants that are globally distributed in eutrophic water bodies. They have a simple body comprised of a few or no roots and a small leaf-like structure

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called "frond," which propagates asexually through budding. The plants have recently attracted growing attention as biomass feedstock because they grow faster than most higher plants and do not require arable land for cultivation (Sree et al., 2015; Cui and Cheng, 2015). Furthermore, their high starch and protein contents, together with their low fiber content, make them valuable biomass for biofuel production, livestock feed and human food. Several recent studies reported the detailed biomass composition of the plants and their benefits in terms of nutrient balance and fuel conversion rates (Verma and Suthar, 2015; Appenroth et al., 2017; Chakrabarti et al., 2019).

Duckweed cultivation is often performed in wastewaters or environmental waters that can supply nutrient minerals, such as nitrogen and phosphorus, for plant growth, which enable the reduction of fertilization cost while simultaneously allowing water purification and biomass production. Many studies reported the successful production of starch- or protein-rich biomass through duckweed cultivation in wastewaters (Xu et al., 2011; Toyama et al., 2018; Chen et al., 2019). However, duckweed growth in wastewater is reportedly two to four times lower than that in synthetic medium (Ge et al., 2012; Yu et al., 2014), indicating that there is ample scope for improvement of duckweed cultivation in wastewater.

Recently, the use of plant growth-promoting bacteria (PGPB) has emerged as a promising way to improve duckweed growth. Although the study of PGPB has long been limited to terrestrial plants, *Acinetobacter calcoaceticus* P23 was isolated as the first PGPB strain from aquatic plants and could almost double the weekly yield of duckweed (Yamaga et al., 2010; Suzuki et al., 2014). The ubiquitous occurrence of PGPB in the duckweed phytosphere has also been reported in several studies (Tang et al., 2015; Ishizawa et al., 2017; Yamakawa et al., 2018). Furthermore, Toyama et al. (2017) showed that growth-promotion by *A. calcoaceticus* P23 is useful for a wide range of duckweed species and environmental conditions, even in non-sterile environmental waters. These studies suggest the prospects of enhanced duckweed yield by inoculating them with PGPB. However, it is also suggested that inoculating PGPB at a high cell density is required to effectively induce growth-promoting effects in the presence of indigenous environmental microbes (Toyama et al., 2017). This may not be feasible for full-scale duckweed cultivation because the large water volume and flow lead to the massive consumption of PGPB cells, resulting in environmental risks posed by PGPB discharge. Therefore, a reasonable methodology is highly desired to utilize PGPB for large hydroponic systems.

In this study, we introduced a two-step cultivation process as a novel strategy, which comprises of colonization and mass cultivation steps utilizing PGPB, to achieve a higher yield of duckweed biomass production (Fig. 1). In the colonization step, duckweeds are co-cultured with PGPB in a small and closed tank for a short period of time to enable PGPB colonization on duckweeds. The duckweeds colonized by PGPB are then used as the inoculum for a mass and open-pond cultivation step, in which they produce biomass and remove nutrients from wastewater. Since in this two-step method, PGPB are inoculated only in the small and closed tank at the colonization step, we can expect a reduction of the cost for PGPB cultivation and the ecological risk of PGPB outflow.

Thus, in this study, a series of cultivation experiments were conducted to test the ability of the proposed method for improving duckweed production. We first determined the effects of the pre-inoculation of the PGPB strain, *A. calcoaceticus* P23, on the growth and nutrient removal of duckweed (*Lemna minor*) in three non-sterile environmental waters. Because a sufficient growth-promotion effect was observed, we evaluated the duration of such growth-promoting effects through flask-scale experiments. Molecular analyses were also performed for a deeper understanding of the survival of the colonized strain P23 on the duckweed surface and the relationships between duckweed growth and its surface microbial community.

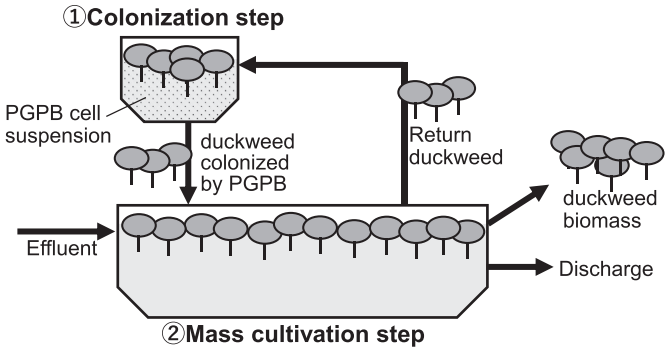

Fig. 1. Outline of the two-step cultivation process proposed in this study. The colonization of plant with PGPB was initiated by culturing duckweeds in a cell suspension containing a higher density of PGPB. In the next mass cultivation step, duckweeds colonized by PGPB are cultured in wastewater effluent for enhanced biomass production and nutrient removal. After a certain cultivation period, a part of duckweeds was returned to colonization step to again prepare PGPB-colonized duckweeds.

2. Materials and methods

2.1. Plant materials

Common duckweed, *Lemna minor* L. (RDSC clone #5512; http://www.ruduckweed.org/), was sterilized by washing with 0.5% sodium hypochlorite, and successively cultured in flasks containing sterile Hoagland medium with modifications by Toyama et al. (2006) (36.1 mg/L KNO_3, 293 mg/L K_2SO_4, 3.87 mg/L NaH_2PO_4, 103 mg/L $MgSO_4 \cdot 7H_2O$, 147 mg/L $CaCl_2 \cdot H_2O$, 3.33 mg/L $FeSO_4 \cdot 7H_2O$, 0.95 mg/L H_3BO_3, 0.39 mg/L $MnCl_2 \cdot 4H_2O$, 0.03 mg/L $CuSO_4 \cdot 5H_2O$, 0.08 mg/L $ZnSO_4 \cdot 7H_2O$, and 0.254 mg/L $H_2MoO_4 \cdot 4H_2O$; pH 7.0) in a growth chamber. The temperature, light intensity, and photoperiod in the growth chamber were adjusted to 28 °C, 80 μmol/m^2/s, and 16 h-light and 8 h-dark, respectively, unless otherwise noted.

2.2. Bacterial strain and preparation of cell suspensions

For cultivation of strain P23, a small loop of the bacterial colony was inoculated into a liquid LB medium (Lennox) and cultured overnight at 28 °C with shaking at 120 rpm or 150 rpm to the stationary phase. The cells were pelleted, washed with sterile Hoagland medium and resuspended in Hoagland medium or environmental water at the indicated cell densities calculated by measuring the optical density at 600 nm (OD_{600}).

2.3. Environmental water used for cultivation experiments

Secondary municipal wastewater effluent and river water were collected from the conventional activated sludge process of a municipal wastewater treatment plant and Kamata River, respectively, in Kofu City, Yamanashi, Japan. The half-strength secondary effluent was prepared by diluting the secondary effluent with tap water. All environmental water including pond water was passed through a filter (pore size, 10 μm) to remove coarse particles including microalgae.

Pond water used in flask-scale experiment was collected from Inukai pond in Suita City, Osaka, Japan (34° 82′ N, 135° 53′ E) on November 16th, 2014. Since the pond water contained only slight nitrogen and phosphorus, we added KNO_3 and NaH_2PO_4 before the

plant cultivation to be the final concentrations of 1.25 mg of N/L and 0.25 mg of P/L, respectively. In addition, native microbial populations in the pond water were collected by filtering with a 0.2 μm pore size filter, and DNA was extracted and analyzed as the procedures described below.

2.4. Bucket-scale two-step cultivation of Lemna minor with strain P23 in environmental water

Effects of the two-step cultivation system on the enhanced biomass productivity and the nutrient removal capacity of *L. minor* were evaluated in bucket-scale experiments using three environmental waters (1/2 secondary effluent, secondary effluent, and river water). Before the cultivation, *L. minor* plants were acclimated in 1 L of each environmental waters for 7 d. For the colonization step, 0.07 g dry weight of the plants were co-cultured with P23 cells ($OD_{600} = 0.3$, equivalent to 0.15 mg dry weight/mL) in 1 L of environmental water. After three days of the first colonization step, 0.07 g dry weight (equivalent to approximately 300–350 fronds) of the plants were used as the *L. minor* inoculum for the second mass cultivation step and transplanted to 1 L of fresh environmental water for 7 d. Dry weights of duckweed inoculum were calculated from the average weights of duckweed fronds grown in each culture condition. The biomass productivity was evaluated by measuring the dry weights after the 7 d mass cultivation step. A control experiment without inoculating strain P23 was also conducted. All plant cultivations in the above procedure were performed with natural sunlight in a plastic container (length 160 mm × width 125 mm × depth 80 mm) placed in an open-air greenhouse at the Kofu campus of University of Yamanashi, Japan (35° 68′ N, 138° 57′ E) in October 2012. The average maximum and minimum temperatures, and hours of sunlight during the cultivation were 21.2 ± 2.2 °C, 10.8 ± 2.1 °C, and 6.3 ± 4.3 h, respectively (refer to Table S1 for detailed weather conditions).

2.5. Repeated batch cultivation of Lemna minor inoculated with P23

A flask-scale repeated batch cultivation experiment was conducted to evaluate the durability of the enhanced growth of *L. minor* by strain P23 at the colonization step (Fig. 2). The pond water containing native microbes was used as the growth media as the representative of simple environmental waters with little adverse influence on the plant (Toyama et al., 2017). *L. minor* plants acclimated in the pond water were first co-cultured with P23 cells (inoculated with cells at $OD_{600} = 1.0$) in 100 mL of pond water for 3 d as the colonization step. The mass cultivation step was performed as a repeated batch mode. Twenty fronds of these plants co-cultured with strain P23 were transplanted into 100 mL of fresh pond water and incubated for 5 d, which was the first batch of mass cultivation step. After 5 d, 30 fronds were transplanted into 100 mL of fresh pond water and incubated for 5 d, which was the second batch. This process of a transfer of inoculated fronds and the 5 d batch cultivation was repeated for the third batch. Subsequently, the *L. minor* plants were again co-cultured with P23 ($OD_{600} = 1.0$) in 100 mL of fresh pond water for 4 d as "re-colonization step." The fourth and fifth batch cultivations were then performed in the same manner, but with 20 fronds and cultivation period for six and seven days, respectively. *L. minor* plants grown without P23 were used as control experiments. During the repeated batch cultivation experiments, the plant growth was evaluated by counting the plant frond numbers. Additionally, 20 to 30 fronds of the plants were collected after each batch cultivation and used for the microbial DNA analysis. All plant cultivation in the above procedure was performed in the growth chamber.

2.6. Water quality analyses

From the start and end of the bucket-scale cultivations, concentrations of dissolved NH_4-N, NO_2-N, NO_3-N, and PO_4-P in environmental waters were determined according to the standard methods (American Public Health Association, 1995). Indophenol method, N-(1-naphthyl) ethylenediamine method, the reduction–N-(1-naphthyl) ethylenediamine method, and molybdenum blue method were used, respectively for NH_4-N, NO_2-N, NO_3-N, and PO_4-P.

2.7. DNA extraction and purification

Microbial DNA was extracted from the filter-trapped samples (original pond water) and plant samples collected at the end of each cultivation step of the flask-scale experiment. The plant samples were rinsed with 50 mL of sterile sodium tripolyphosphate solution (5 mg/L) to remove the loosely attached microbes from the plant surface. Then, the plant or filter samples were immersed in 500 μL of Cica Geneus DNA Extraction Solution (Kanto Chemical, Tokyo, Japan), and DNA was extracted according to the manufacturer's protocol. The extracted DNA was diluted and then subjected to the real-time PCR analysis. For the terminal restriction fragment length polymorphism (T-RFLP) analysis, the DNA samples were further purified by Mag Extractor PCR & Gel clean-up kit (Toyobo, Tokyo, Japan) according to the manufacturer's protocol.

2.8. Primers and standard DNA fragments for real-time PCR

Real-time PCR was performed to determine the colonization density of strain P23 on the surface of *L. minor*. To this end, primers P23F (5′-TGGGTTGATGCAAGTGTAATTC-3′) and P23R (5′-AAGCCAACTTTCAATGACTGG-3′) were designed to specifically amplify the 141 bp-region of a hypothetical gene which occurs once in the draft genome of strain P23 (Sugawara et al., 2015). Standard DNA fragments for absolute quantification of strain P23 were prepared

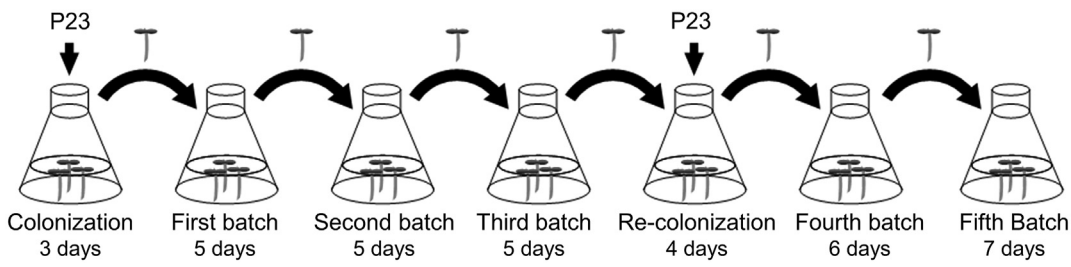

Fig. 2. The procedure of repeated batch cultivation experiments. Control experiments were performed without inoculation of strain P23 in "Colonization" and "Re-colonization" steps. The number of plant fronds was counted during each batch. DNA quantification and microbial community analysis were conducted after each cultivation step.

by PCR amplification of the P23 genomic DNA with primers P23stF (5′-TGGGTTGATGCAAGTGTAATTC-3′) and P23stR (5′- TCAGCAGTCCTCATTAAAGCAA-3′), which were designed to generate the 1052 bp-DNA fragments including the target sequence amplified with P23F and P23R primers set. The amplicon was used as standard DNA after purification with NucleoSpin® Extract II (Macherey Nagel, Düren, Germany).

2.9. Quantification of DNA copies of strain P23

SYBR Green-based real-time PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The 20 μL of reaction mixture contained 10 μL of Power SYBR® Green PCR Master Mix (Applied Biosystems), 2 μL of DNA template, and each 0.3 μM of forward and reverse primers. The thermal cycle was set as follows: an initial incubation step at 50 °C for 2 min, denaturation at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A standard curve was prepared for every run with a 10-fold dilution series of the standard DNA fragments with known copy numbers. The range of amplification efficiency and correlation coefficient (r^2) of real-time PCR were 91.3%–95.2% and 0.993–0.999, respectively. The detection range of the real-time PCR was 9.31×10^2 to 9.31×10^9 copies per frond.

2.10. Microbial community analysis

In order to characterize the microbial communities during the flask-scale cultivations, T-RFLP analysis of 16S rRNA gene was performed as described previously (Matsuda et al., 2010). Briefly, the conserved region of eubacterial 16S rRNA gene was amplified with the forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), whose 5′-end was labeled with phosphoramidite fluorochrome 5-carboxyfluorescein, and reverse primer 1392R (5′-ACGGGCGGTGTGTACA-3′) as described previously (Amman et al., 1995). The PCR products were purified with NucleoSpin® Extract II and digested with the restriction enzyme *Hha*I. The DNA samples were then mixed with GeneScan 2500 ROX dye size standard (Applied Biosystems) and denatured at 95 °C for 3 min. Finally, the DNA samples were subjected to capillary electrophoresis using a genetic analyzer (ABI Prism 310; Applied Biosystems). The size and abundance of labeled T-RFs were determined using GeneScan ver. 3.7 (Applied Biosystems). The T-RFs of 776–778 bp were removed from the results because these fragments might be derived from *L. minor* chloroplast genome (GenBank; DQ400350.1). In addition, the length of strain P23-originated T-RF was determined by another run using DNA template extracted from a pure culture of strain P23.

2.11. Statistical analysis

The bucket-scale and flask-scale experiments were performed with duplicate and triplicate cultures, respectively. Statistical significance ($p < 0.05$) was analyzed by a Student's *t*-test in R version 3.5.1 (http://www.r-project.org). Ordination of microbial community structure was performed with non-metric multidimensional scaling (NMDS) using "metaMDS" function of the "vegan" package in R. In addition, permutational multivariate analysis of variance (PERMANOVA) was performed using "adonis" function of the "vegan" package in R, to test whether microbial communities formed on P23-inoculated and control plants were significantly different from each other. Bray-Cutis distance on the relative abundance of T-RFs was used for both NMDS and PERMANOVA.

3. Results and discussion

3.1. Enhanced biomass production and the nutrient removal capacity of Lemna minor in three environmental waters in two-step cultivation with strain P23

The two-step cultivation of *L. minor* was conducted in bucket-scale experiments. *L. minor* plants were cultured for 3 d with or without inoculating the strain P23 in the first step. The cultured plants in the first step were transplanted into the second mass cultivation step, in which plant growth and nutrient removal were monitored. Our results indicate that *L. minor* plants co-cultured with strain P23 apparently showed a rapid growth in the following 7 d cultivation (Fig. 3). The biomass yields of pre-inoculated plants in 7 d increased by 2.3, 1.9, and 2.3 times more in 1/2 secondary effluent, secondary effluent, and river water, respectively, when compared with control plants (Fig. 4). Our previous study reported 1.7–2.4 times increase of *L. minor* growth by direct co-cultivations with 0.15 mg dry weight/mL (equivalent to $OD_{600} = 0.3$) of strain P23 in pond water and secondary effluent (Toyama et al., 2017). Consistently, the present results indicate that similar extents of growth promotion can be achieved by the two-step cultivation process using the same PGPB strain.

Table 1 shows the nutrient removal capacity of the P23-inoculated and control plants in the bucket-scale cultivation. These environmental waters contained ammonium, nitrate, and negligible amounts of nitrite as the inorganic nitrogen. We observed that ammonium was completely removed in all cultures whereas not all nitrate was removed from the culture media, which is consistent with an earlier report that duckweed preferentially consumes ammonium to nitrate (Cedergreen and Madsen, 2002). Additionally, P23-inoculated plants almost completely removed nitrate and phosphate, while significant amounts of these nutrients remained in the culture media of control plants. These results indicate, for the first time, that PGPB can also enhance nutrient removal from environmental waters. On the other hand, the increment of nitrogen and phosphorus removal was not as high as the extent of growth-promotion by strain P23 (Fig. 4), probably due to the depletion of inorganic nitrogen and phosphorus in P23-inoculated cultures.

The two-step cultivation process proposed in this study (Fig. 1) was designed to improve duckweed production with minimal or reduced use of PGPB cells. To attain this, the scale, cultivation term,

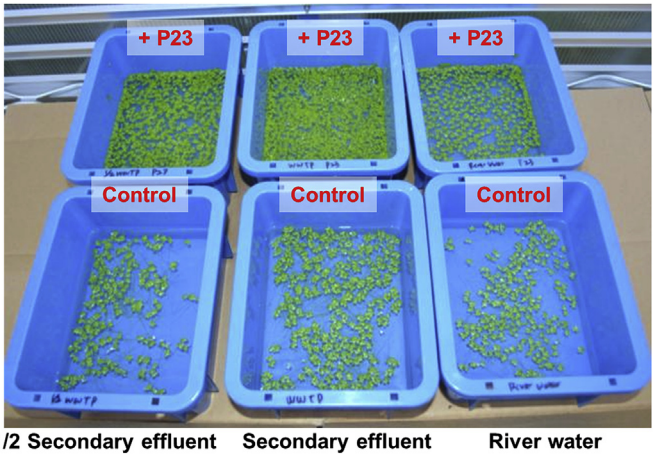

Fig. 3. The photograph of duckweed cultivation in three environmental waters with and without inoculation of strain P23. The image was taken on day 6 of mass cultivation step.

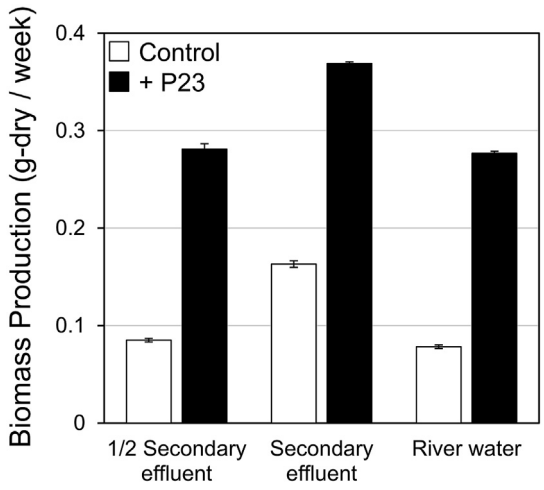

Fig. 4. The weekly yield of *Lemna minor* by the inoculation of strain P23. Error bars show the standard deviations (n = 2). The plants had 0.07 g dry biomass at the start of cultivation.

plants, respectively (Fig. 5). In contrast, such differences were not observed in the third batch, indicating the growth-promoting effect disappeared before the third batch. We, therefore, set up the re-colonization step between the third and fourth batches, which successfully restored growth-promoting effects in the fourth batch. However, the effect was again lost in the next fifth batch. Overall, these results indicate that the growth-promoting effect of strain P23 can last for up to one to two batches (5–10 days) and, therefore, the re-colonization step is requisite for the restoration of an accelerated duckweed growth.

3.3. Persistence of strain P23 on duckweed surface

The survival of inoculated bacteria in plant rhizosphere is one of the most significant concerns of PGPB application (Lugtenberg and Kamilova, 2009). We performed the real-time PCR to monitor the persistence of strain P23 on duckweed surface during the repeated batch cultivation experiments. Our results showed that each plant contained approximately 10^5 to 10^7 copies of strain P23 after the colonization and re-colonization steps (Fig. 6). These data are

Table 1
Removal of nutrient salts by *Lemna minor* with and without inoculation of strain P23. Concentrations in the unit of mg/L and percent removal are shown.

		1/2 Secondary effluent			Secondary effluent			River water		
		initial	7 d	% removal	initial	7 d	% removal	initial	7 d	% removal
NH_4-N	P23	3.04 ± 0.07	n.d.	100%	6.01 ± 0.13	n.d.	100%	1.77 ± 0.12	n.d.	100%
	Control	3.04 ± 0.07	n.d.	100%	6.01 ± 0.13	n.d.	100%	1.77 ± 0.12	n.d.	100%
NO_2-N	P23	n.d.	n.d.	–	0.07 ± 0.00	n.d.	100%	n.d.	n.d.	–
	Control	n.d.	n.d.	–	0.07 ± 0.00	n.d.	100%	n.d.	n.d.	–
NO_3-N	P23	4.82 ± 0.06	0.09 ± 0.02	98.2%	8.63 ± 0.10	0.10 ± 0.03	98.9%	4.47 ± 0.20	0.09 ± 0.03	98.1%
	Control	4.82 ± 0.06	2.78 ± 0.08	83.9%	8.63 ± 0.10	5.06 ± 0.12	41.3%	4.47 ± 0.20	2.33 ± 0.17	48.0%
PO_4-P	P23	1.01 ± 0.09	n.d.	100%	1.84 ± 0.09	n.d.	100%	0.52 ± 0.03	n.d.	100%
	Control	1.01 ± 0.09	0.17 ± 0.01	97%	1.84 ± 0.09	0.15 ± 0.05	92.0%	0.52 ± 0.03	0.15 ± 0.05	72.5%

n.d, not detected.

and PGPB cell density in the colonization step should be minimized as far as the PGPB can sufficiently colonize the plant and exert its growth-promoting effect. Although we co-cultured duckweed with strain P23 (OD_{600} = 0.3) for 3 d for the colonization step in the present study, the parameters of the colonization step need to be optimized toward minimizing the cost. In this regard, it was recently found that the kinetics of bacterial duckweed colonization differs depending on bacterial strains and inoculation densities, and some bacterial strains can fully colonize on duckweed within 3–24 h even with smaller cell densities (OD_{600} < 0.02) (Ishizawa et al., 2019). The detailed conditions of colonization step should be determined based on the colonization behavior of each PGPB strain used for the two-step cultivation process.

3.2. Duration of plant growth-promoting effects after pre-inoculation of strain P23

Although the bucket-scale experiments successfully demonstrated the efficiency of the proposed two-step cultivation strategy, the duration of such growth-promoting effect is quite important for the application in full-scale duckweed cultures. We examined this by a repeated batch cultivation experiment using non-sterile pond water according to the procedure depicted in Fig. 2. Although the extent of plant growth promotion was restricted unlike in the bucket-scale experiments as shown in Fig. 4, in the first and second batches after the colonization step, P23-colonized plants showed significantly faster growth, and average frond numbers after 5-day cultivations were 27.7% and 16.9% higher than those of control

similar to that of strain P23 inoculated with *L. minor* in sterilized pond water (10^5 to 10^6 copies per frond; data not shown) and to those reported for three other duckweed-associated bacteria in axenic culture media (10^5 to 10^7 cells per frond) (Ishizawa et al., 2019). Therefore, our data confirmed that maximum colonization of strain P23 could be achieved by each colonization and re-colonization steps. However, no colonization was detected at the subsequent cultivation steps, except for the end of the first batch. These data strongly suggest that strain P23 was excluded from the plant surface through the competition with indigenous microbes, as reported in the previous studies (Liu et al., 2014; Qiao et al., 2017). Since a significant rapid plant growth was observed in the first, second, and fourth batches (Fig. 5), it is likely that a better plant growth-promotion is possible only when more than 10^3 copies/frond of strain P23 bacteria remain on the plant surface at the start of each batch. Therefore, maintaining a certain density of strain P23 on the plant surface would be essential to extend the duration of growth-promoting effects, and rotating duckweeds between colonization step and mass cultivation step, as shown in Fig. 1, is useful for maintaining high growth of duckweeds.

3.4. Shifts in duckweed-associated microbial community

In order to understand the dynamics of the whole microbial community formed on *L. minor*, culture-independent microbial community profiling was performed by T-RFLP during the repeated batch cultivation experiments. Similar to the results of real-time PCR (Fig. 6), the T-RF representing strain P23 (205-bp) was

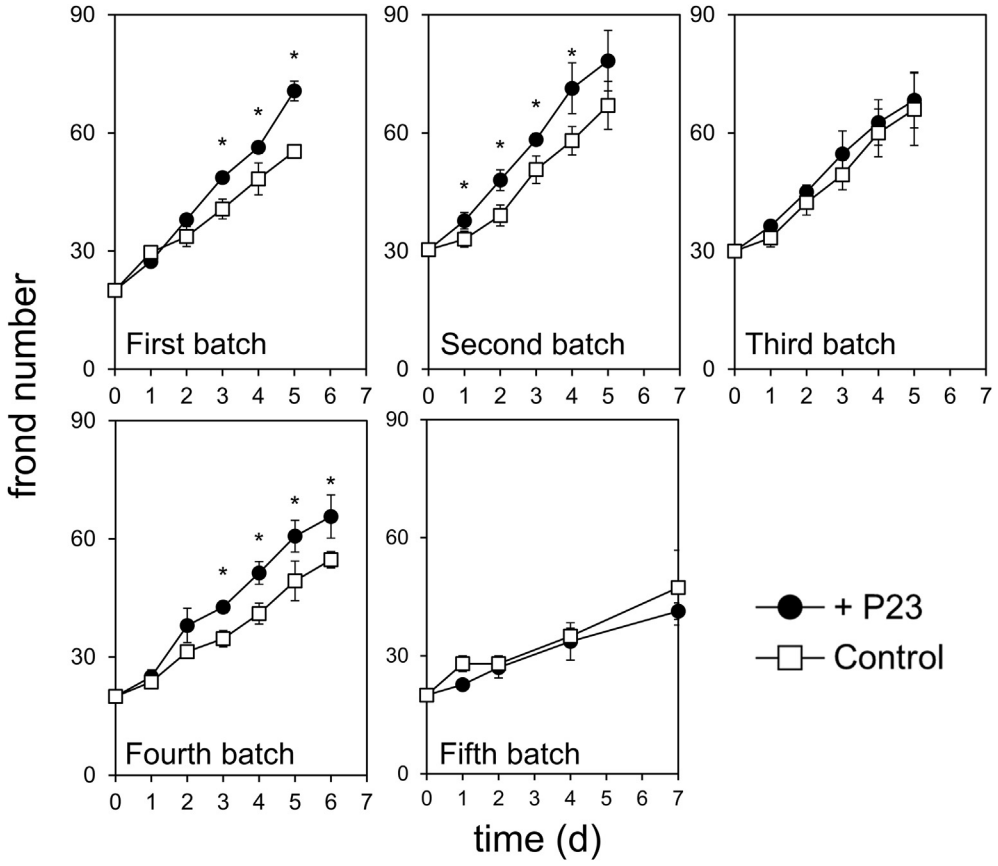

Fig. 5. Increase of duckweed frond number in the repeated batch cultivation experiments. Error bars show the standard deviations (n = 3). Asterisks indicate a significant difference ($p < 0.05$) among P23-treated and control plants.

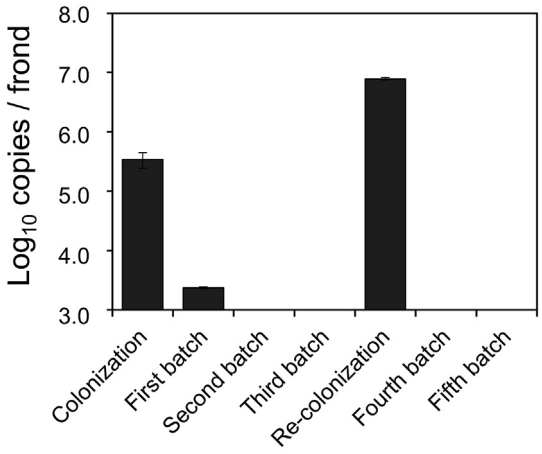

Fig. 6. The abundance of strain P23 on the plant surface by estimating the copy numbers of P23-specific gene after each cultivation step. Error bars show the standard deviations (n = 3).

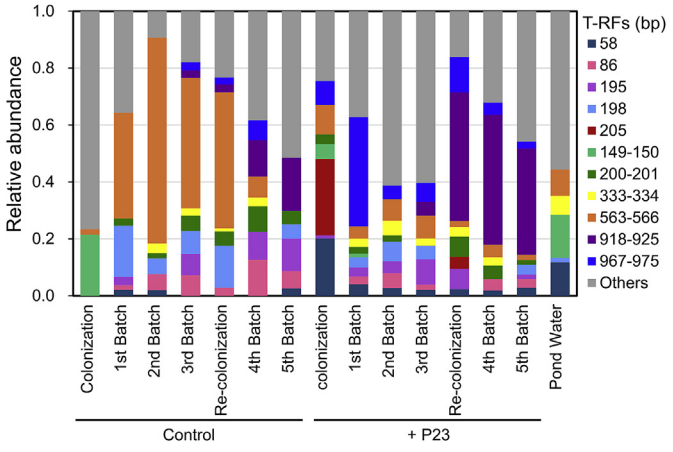

Fig. 7. Microbial communities on the plant surface of *Lemna minor* after each cultivation step, evaluated by terminal restriction fragment length polymorphism (T-RFLP).

detected after the colonization and re-colonization steps, but not in the subsequent cultivation steps (Fig. 7). Among the T-RFs detected, many were commonly observed in duckweed-associated microbial communities, irrespective of P23 inoculation, suggesting the strong selectivity of the host plant. Nevertheless, overall microbial communities were significantly different among P23-inoculated and control plants even in the batch cultivation steps, as revealed by PERMANOVA (Figs. 7 and 8; $r^2 = 0.17$, $p < 0.01$, all samples; $r^2 = 0.21$, $p < 0.05$, excluding colonization and re-colonization samples). These data indicate that inoculation of strain P23 strongly impacted the duckweed-associated microbial community, and the effect persisted even after the disappearance of strain P23 from the plant surface. Additionally, we found that the relative abundances of the most dominant T-RFs (563–566 bp, 918–925 bp, and 967–975 bp) were extremely different among different batch plants (Fig. 7). Similarly, a previous study that conducted high-throughput sequencing analyses of duckweed-associated bacterial

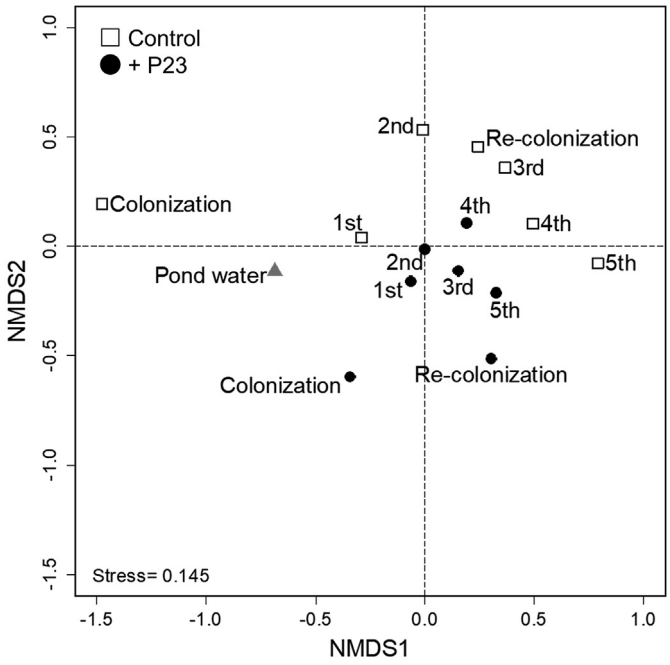

Fig. 8. Non-metric multidimensional scaling (NMDS) showing the structure of pond water and the plant surface microbial communities.

communities reported the shifts of dominant bacterial taxa depending on the environmental context (Xie et al., 2015). These observations suggest the possible multistability of duckweed microbiome, whose mechanisms and implications on culturing systems are yet to be proved. Since the different microbial communities can affect the plant growth (Anderson and Habiger, 2012; Ishizawa et al., 2017), such dynamics of duckweed-associated bacterial communities should be further analyzed in detail.

4. Conclusions

In this study, we demonstrated that significant increase in duckweed production could be achieved by two-step cultivation with a PGPB strain, *Acinetobacter calcoaceticus* P23. The remarkable improvements in biomass yield and nutrient removal in bucket-scale cultivations indicate the prospect of the two-step cultivation process as an effective strategy for maximizing duckweed yield without extensive use of PGPB and ecological risks associated with PGPB discharge into the environment. Repeated batch cultivation experiments revealed that strain P23 influenced overall microbial community structures of the plant surface, and exerted growth-promoting effects until no colonization of strain P23 was detected. These results indicate that the effect of the two-step cultivation process is quite dependent on the persistence of PGPB in the duckweed-associated microbial community. The rotation of duckweed between the colonization step and mass cultivation step, as proposed, would facilitate the maintenance of a high growth rate of duckweed.

Conflicts of interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.124682.

References

Amman, R.I., Ludwing, W., Schleifer, K.H., 1995. Phylogenic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Mol. Biol. Rev. 59, 143–169.
Appenroth, K.J., Sree, K.S., Böhm, V., Hamman, S., Vetter, W., Leiterer, M., Jahreis, G., 2017. Nutritional value of duckweeds (Lemnaceae) as human food. Food Chem. 217, 266–273.
Anderson, M., Habiger, J., 2012. Characterization and identification of productivity-associated rhizobacteria in wheat. Appl. Environ. Microbiol. 78 (12), 4434–4446.
Cedergreen, N., Madsen, T.V., 2002. Nitrogen uptake by the floating macrophyte *Lemna minor*. New Phytol. 155 (2), 285–292.
Chakrabarti, R., Clark, W.D., Sharma, J.G., Goswami, R.K., Shrivastav, A.K., Tocher, D.R., 2018. Mass production of *Lemna minor* and its amino acid and fatty acid profiles. Front. Chem. 6, 479.
Chen, G., Huang, J., Fang, Y., Zhao, Y., Tian, X., Jin, Y., Zhao, H., 2019. Microbial community succession and pollutants removal of a novel carriers enhanced duckweed treatment system for rural wastewater in Dianchi Lake basin. Bioresour. Technol. 276, 8–17.
Cui, W., Cheng, J.J., 2015. Growing duckweed for biofuel production: a review. Plant Biol. 17 (s1), 16–23.
Ge, X., Zhang, N., Phillips, G.C., Xu, J., 2012. Growing *Lemna minor* in agricultural wastewater and converting the duckweed biomass to ethanol. Bioresour. Technol. 124, 485–488.
Ishizawa, H., Kuroda, M., Morikawa, M., Ike, M., 2017. Evaluation of environmental bacterial communities as a factor affecting the growth of duckweed *Lemna minor*. Biotechnol. Biofuels 10 (1), 62.
Ishizawa, H., Kuroda, M., Inoue, K., Inoue, D., Morikawa, M., Ike, M., 2019. Colonization and competition dynamics of plant growth-promoting/inhibiting bacteria in the phytosphere of the duckweed *Lemna minor*. Microb. Ecol. 77 (2), 440–450.
Liu, W., Yang, C., Shi, S., Shu, W., 2014. Effects of plant growth-promoting bacteria isolated from copper tailings on plants in sterilized and non-sterilized tailings. Chemosphere 97, 47–53.
Lugtenberg, B., Kamilova, F., 2009. Plant-growth-promoting rhizobacteria. Annu. Rev. Microbiol. 63, 541–556.
Matsuda, M., Inoue, D., Anami, Y., Tsutsui, H., Sei, K., Soda, S., Ike, M., 2010. Comparative analysis of DNA-based microbial community composition and substrate utilization patterns of activated sludge microorganisms from wastewater treatment plants operated under different conditions. Water Sci. Technol. 61 (11), 2843–2851.
Qiao, J., Yu, X., Liang, X., Liu, Y., Borriss, R., Liu, Y., 2017. Addition of plant-growth-promoting *Bacillus subtilis* PTS-394 on tomato rhizosphere has no durable impact on composition of root microbiome. BMC Microbiol. 17 (1), 1–12.
Sree, K.S., Sudakaran, S., Appenroth, K.J., 2015. How fast can angiosperms grow? Species and clonal diversity of growth rates in the genus *Wolffia* (Lemnaceae). Acta Physiol. Plant. 37, 204.
Standard Methods for the Examination of Water and Wastewater, nineteenth ed., 1995. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.
Sugawara, M., Hosoyama, A., Yamazoe, A., 2015. Draft genome sequence of *Acinetobacter calcoaceticus* strain P23, a plant growth-promoting bacterium of duckweed. Genome Announc. 3 (1) e00026-15.
Suzuki, W., Sugawara, M., Miwa, K., Morikawa, M., 2014. Plant growth-promoting bacterium *Acinetobacter calcoaceticus* P23 increases the chlorophyll content of the monocot *Lemna minor* (duckweed) and the dicot *Lactuca sativa* (lettuce). J. Biosci. Bioeng. 118 (1), 41–44.
Tang, J., Zhang, Y., Cui, Y., Ma, J., 2015. Effects of a rhizobacterium on the growth of and chromium remediation by *Lemna minor*. Environ. Sci. Pollut. Res. 22 (13), 9686–9693.
Toyama, T., Yu, N., Kumada, H., Sei, K., Ike, M., Fujita, M., 2006. Accelerated aromatic compounds degradation in aquatic environment by use of interaction between *Spirodela polyrrhiza* and bactreia in its rhizosphere. J. Biosci. Bioeng. 101 (4), 346–353.
Toyama, T., Kuroda, M., Ogata, Y., Hachiya, Y., Quach, A., Tokura, K., Tanaka, Y., Mori, K., Morikawa, M., Ike, M., 2017. Enhanced biomass production of duckweeds by inoculating a plant growth-promoting bacterium, *Acinetobacter calcoaceticus* P23, in sterile medium and non-sterile environmental waters. Water Sci. Technol. 76 (6), 1418–1428.
Toyama, T., Hanaoka, T., Tanaka, Y., Morikawa, M., Mori, K., 2018. Comprehensive evaluation of nitrogen removal rate and biomass, ethanol, and methane production yields by combination of four major duckweeds and three types of wastewater effluent. Bioresour. Technol. 250, 464–473.

Verma, R., Suthar, S., 2015. Utility of duckweeds as source of biomass energy: a review. Bioenergy Res 8 (4), 1589–1597.
Xie, W., Su, J., Zhu, Y., 2015. Phyllosphere bacterial community of floating macrophytes in paddy soil environments as revealed by Illumina high-throughput sequencing. Appl. Environ. Microbiol. 81 (2), 522–532.
Xu, J., Cui, W., Cheng, J.J., Stomp, A.M., 2011. Production of high-starch duckweed and its conversion to bioethanol. Biosystem. Eng. 110, 67–72.
Yamaga, F., Washio, K., Morikawa, M., 2010. Sustainable biodegradation of phenol by *Acinetobacter calcoaceticus* P23 isolated from the rhizosphere of duckweed *Lemna aoukikusa*. Environ. Sci. Technol. 44 (16), 6470–6474.
Yamakawa, Y., Jog, R., Morikawa, M., 2018. Effects of co-inoculation of two different plant growth-promoting bacteria on duckweed. Plant Growth Regul. 86 (2), 287–296.
Yu, C., Sun, C., Yu, L., Zhu, M., Xu, H., Zhao, J., Ma, Y., Zhou, G., 2014. Comparative analysis of duckweed cultivation with sewage water and SH media for production of fuel ethanol. PLoS One 9 (12), e115023.