STUDIES ON RECONSTRUCTION OF A FUNCTIONAL DUCKWEED HOLOBIONT TO REDUCE NUTRIENT COMPETITION WITH MICROALGAE FOR HIGH-YIELD BIOMASS PRODUCTION

（高収率バイオマス生産に向けたウキクサホロビオントの再構築
に関する研究
～微細藻類との栄養競合の低減によるウキクサの成長促進）

DOCTORAL DISSERTATION

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ABSTRACT

Duckweed (Lemnaceae) is a small floating aquatic plant. Due to the significant high-rate growth and potential nutrient value, duckweed has become an important and suitable material for basic studies and applications for low-carbon industries. However, the outbreak of microalgae is a big problem in decreasing duckweed production yield, especially under strengthened nutrient conditions. Thus, reduction of microalgal growth for enhancing duckweed productively is a critical challenge.

Duckweed naturally forms a holobiont with its associated microorganisms. The goal of this research is to design and reconstruct a bifunctional duckweed holobiont that enables duckweed growth promotion and microalgal growth inhibition. In this study, I targeted a factory wastewater treated pond where a duckweed *Lemna aequinoctialis* was naturally growing. A microalga, *Coelasterella* sp. KC10 was isolated from the pond water.

After screening of sixty-nine bacterial strains isolated from *L. aequinoctialis* I found that the growth of *L. aequinoctialis* colonized with four plant growth-promoting bacteria (PGPB) was significantly enhanced in both suspension and attachment conditions, while the growth of microalga, *Coelastrella* sp. KC10, was inhibited in co-culture with two microalgal inhibiting bacteria (MGIB). Moreover, in triple co-culture of either single PGPB or MGIB with *L. aequinoctialis* and *Coelastrella* sp. KC10 in MA medium, the growth inhibition of *L. aequinoctialis* by *Coelastrella* sp. KC10 was successfully relieved. PGPB *Kinneretia* sp. KLaR20, *Runella* sp. KLaDT11 and MGIB *Rhizobium* sp. KLaR16 recovered the growth inhibition of *L. aequinoctialis* by 65%, 36%, and 33% respectively compared to the co-culture without bacteria. Additionally, the triple co-culture of those two selected PGPB with *L. aequinoctialis and Coelastrella* sp. KC10 or the quadruple co-culture of a PGPB and a MGIB with *L. aequinoctialis and Coelastrella* sp. KC10 recovered the growth of duckweed to the similar level as the culture without microalga. This observation indicated that these bacteria could maximize the use of beneficial plant-microbe interaction. This study has demonstrated for the first time that duckweed holobiont can be re-constructed to tolerate against growth inhibition by microalga.

In the last chapter of the study, I focus on studying the algicidal mechanism of MGIB *Rhizobium* sp. KLaR16 by extracting, purifying, and identifying algicidal substances. Understanding these mechanisms helps elucidate the role of MGIB KlaR16 in the holobiont of duckweed and leads to the development of natural and environmentally friendly methods for controlling algae blooms.

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INTRODUCTION

1. Duckweed

Duckweeds, belonging to the family Lemnaceae, are the smallest flowering plants and comprise five genera and 36 species of aquatic monocotyledonous plants (Acosta, 2021; Landolt, 1986). Duckweed can be distinguished morphologically from other flowering plants and also closely aquatic plants due to its reduced, simple structure body. The plant body consists of a "frond", a leaflike structure, and "root," except for two genera of root-less duckweed, *Wolffia* and *Wolffiella*. Their propagation is mainly due to asexual vegetative growth, in which a daughter frond begins to develop and emerges from a pouch in the basal sections of the mother frond. At the time of emergence, each daughter frond already contains developed primordia for the next generation of fronds (Landolt, 1986; White and Wise, 1998). Therefore, with this simple propagation mechanism, the growth rate of duckweed is almost exponential, enabling rapid biomass production. The number of duckweed fronds can double in 1 to 2 days. Duckweed biomass, in dried form, contains up to 40–45% crude protein, making it valuable as an animal feed (Anderson et al., 2011; Leng et al., 1995) and allergen-free human nutrition with no anti-proliferative or cytotoxic activity (Sree et al., 2019). Moreover, duckweed can take up nitrogen and phosphorus as nutrients from polluted water, including sewage and wastewater (Culley Jr and Epps, 1973; Scheffer et al., 2003), resulting in water purification. Duckweed biomass has demonstrated its high potential as a human food with high nutritious values, and it can also be used for the efficient production of biofuels including ethanol and methane (Appenroth et al., 2017; Sela et al., 2020; Toyama et al., 2018). Recently, attempts have also begun to use it as a material for bioplastic production (Yoksan and Boontanimitr, 2023)

2. Microalgae

2.1. General introduction

Microalgae are a diverse group of photosynthetic organisms including cyanobacteria (blue-green algae), green algae (chlorophyta), diatoms (Bacillariophyta) and dinoflagellates (Dinophyta) whose systematics is based on the kinds and combinations of photosynthetic pigments present in different species. The mechanism of photosynthesis in microalgae is similar to that of higher plants, they have the ability to capture solar energy with higher efficient than that of terrestrial plants.

Moreover, because the cells grow in aqueous suspension, they have more efficient access to water, CO_2 and other nutrients (Mandal and Mallick, 2014).

Cyanobacteria, for example genera of *Anabaena, Nostoc, Mirocystis*, are prokaryotes, while other species are eukaryotes. The size of microalgae cells can be 1-2 micrometers or even reaching several hundred of micrometers. Microalgal cells can be influenced by environmental conditions and growth stages.

Microalgae can grow in diverse environmental conditions, including freshwater and marine ecosystems and are able to produce a wide range of chemical products including the harmfull substances. Some microalgae, especially cyanobacteria, can produce toxins (cyanotoxins) that are harmful to other organisms. (Borowitzka, 2013; Smith et al., 1999)

2.2. The impact of the microalgae on duckweed

Microalgae grow rapidly in environmental water and cause algal blooms or eutrophication, which is a phenomenon of water pollution caused by the overgrowth of microalgae. During algal blooms, algal cells blanket the water surface, diminish water transparency, and hinder sunlight penetration. This adversely affects aquatic ecosystems. Microalgae exhibit a rapid growth rate akin to that of duckweed, fostering robust competition for nutrient minerals. Nitrogen and phosphorus are removed by phytoplankton and other mobile algae as they reach the sediment, resulting in rotten sludge. When duckweed and algae grow together, the adverse effects of algae on duckweed can be very strong because of nutrient limitations. In a previous study, the growth rate of duckweed was reduced to 62%, and the reduction in chlorophyll content of the duckweed fronds was even more serious, up to 80% (Roijackers et al., 2004). Microalga strongly inhibited the growth of *Lemna gibba* by reducing nutrients and increasing pH, of the five factors that significantly inhibited duckweed growth by microalga, depletion of N was strongest, increase pH beyond 10 was second and followed by depletion of P, and the elements Fe and Mn (Szabó et al., 2005)
Thus, suppressing microalgal growth without the use of herbicides or pesticides is a critical challenge for increasing duckweed production in an environmentally safe manner.

3. Holobiont

A “holobiont” is defined as an ecological unit composed of a host and associated microorganisms.

The holobiont concept emphasizes the symbiotic relationships and interdependence between the host plant and its associated microorganisms, where the associated microbiome enhances nutrient acquisition, protects against pathogens, modulates growth hormones, and improves resilience to abiotic stresses like drought or salinity which collectively influence the plant’s health, growth, development and adaptation to the environmental stresses (Vandenkoornhuyse et al., 2015). Moreover, these interactions are bidirectional, while receiving the benefits from the microbiome, the plant can also influence the composition and function of its microbiome through the secretion of root exudates and other biochemical signals (Hassani et al., 2018)

Research on plant holobiont and understanding these complex interactions can lead to the development of innovative strategies for improving crop productivity and reducing the reliance on chemical fertilizers and pesticides. Therefore, it is significant for the agriculture and environmental sustainability. Additionally, studies on holobionts hold promise for unravelling the fundamental nature and potential of living organisms (Agler et al., 2016; Haney et al., 2015; Richardson, 2017). In recent years, the idea that unknown but sophisticated symbiotic mechanisms drive the environmental adaptation and coevolution of holobionts has been proposed (Koskella and Bergelson, 2020; Vandenkoornhuyse et al., 2015). Therefore, strengthening holobiont robustness represents nature-oriented biotechnology for the future.

4. Plant growth-promoting bacteria

Plant growth-promoting bacteria (PGPB) play a key role in strengthening holobionts for high-yield biomass production. PGPB promote the growth of host plants through direct and indirect mechanisms. Direct plant-growth promotion involves phytohormone production, nutrient solubilization, and enhanced metabolism (Lugtenberg and Kamilova, 2009). The indirect mechanisms are mainly attributed to the suppression of pathogenic fungi and insect larvae *via* exclusion and antagonism (Bravo et al., 2011; Bulgarelli et al., 2013; Khan et al., 2018). Several PGPB have been reported to be beneficial for duckweed production. Phenol-degrading *Acinetobacter calcoaceticus* P23, which was isolated from *Lemna aoukikusa*, has been reported to increase duckweed frond number and enhance chlorophyll production in not only duckweed but

also lettuce (Suzuki et al., 2014; Yamaga et al., 2010; Yamakawa et al., 2018). *Ensifer* sp. SP4 promotes the growth rate and biomass productivity of the giant duckweed *Spirodela polyrhiza* by enhancing nitrogen metabolism and photosynthesis (Toyama et al., 2018). The relationship between PGPB and their host plants varies depending on the species and environmental conditions. Thus, it is crucial to consider both environmental factors and bacteria associated with duckweed species to create suitable duckweed holobiont for high-yield duckweed biomass production. *Chryseobacterium* sp. isolated from low-nitrogen factory wastewater was capable of promoting the growth of *Lemna gibba* without nitrogen competition with the plant (Khairina et al., 2021). Moreover, the diazotrophic *Azotobacter vinelandii* stably colonized *Lemna minor* and allowed it to grow even under nitrogen-free water conditions because of its nitrogen fixation activity (Shuvro et al., 2023).

5. Microalgal growth-inhibiting bacteria

Microalgal growth-inhibiting bacteria (MGIB) or algicidal bacteria are a group of bacteria that negatively affect the growth, and development or kill microalgae *via* two main mechanisms. In the direct mode, bacteria directly contact microalgal cells to exert their inhibiting or killing effects (Wang et al., 2021; Zeng et al., 2021). Basically, MGIB can attach to microalgal cells or invade microalgal cells, directly feeding on microalgae cellular contents or causing structural damage. Conversely, in the indirect mode, MGIB release functional substances extracellularly, that are toxic to microalgae. Reported bacterial algicidal substances primarily consist of peptides, proteins, alkaloids, amino acids, antibiotics, pigments, and fatty acid compounds, which function either individually or synergistically (Sun et al., 2018). These algicidal compounds can disrupt cellular processes, inhibit photosynthesis or lead to cell lysis. Some bacteria can generate reactive oxygen species (ROS) which cause oxidative stress in microalgal cells and therefore damage cellular components. The indirect mode can include the competition for the nutrients and alteration of environmental conditions, in which, MGIB outcome microalga for essential nutrients or MGIB can modify the pH or oxygen level in ways that are unfavorable for microalgal growth and thereby limiting algal growth. (Grossart and Simon, 2007; Ramanan et al., 2016)

The use of bacteria to control microalgal blooms is an environmentally friendly approach. I hypothesized that introducing MGIB into duckweed holobionts could also facilitate the recovery of duckweed growth from reduction by co-existing microalgae.

OBJECTIVES

The main objective of this study is to design and reconstruct a bifunctional duckweed holobiont that enables duckweed growth promotion and microalgal growth inhibition. The specific objectives are:

1. To obtain duckweed-associated bacteria from a duckweed *L. aequinoctialis*, which is indigenous to a factory wastewater treatment tank
2. To design and reconstruct a bifunctional duckweed holobiont that enables duckweed growth promotion and microalgal growth inhibition.
3. To extract, purify, and identify algicidal substances produced by MGIB

STATISTICAL ANALYSIS

Statistical analysis was analyzed using SPSS software ver.28.0.1.1(14) (IBM, Armonk, NY, USA). The results are shown as mean ± standard deviation (SD) with the values of three sample replicates per experiment. Significance ($P < 0.05$) was calculated using one-way ANOVA (followed by post-hoc test Tukey HSD if among groups value is significant) for experiments with more than two conditions or using Student's t-test for experiments within two conditions.

CHAPTER 1

PLANT GROWTH CONDITIONS AND DUCKWEED-ASSOCIATED BACTERIA (DAB) ISOLATION

1. Objective

Duckweed, *Lemna aequinoctialis,* is indigenous to factory wastewater, naturally derived from a holobiont with its associated microorganisms. This chapter aims to obtain the duckweed-associated bacteria, DAB, by isolating and purifying from the natural *L. aequinoctialis*. Moreover, this chapter also aims to obtain aseptic *L. aequinoctialis,* which will be used for further experiments in this study and the stock collection.

2. Plant growth condition

2.1. Duckweed

Lemna aequinoctialis was collected from a sedimentation tank of treated wastewater at KEWPIE Corporation (KP) Goka plant in Tokyo, Japan (Fig. 1). This was the only species of duckweed naturally growing in the final sedimentation pond after wastewater treatment.

Figure 1. Sedimentation tank of treated wastewater where duckweed was collected (Photo was provided by Kewpie Corporation, Tokyo, Japan)

2.1.1. Sterilization of duckweed

Duckweed was sterilized using 5% sodium hypochlorite as described previously by Suzuki et al. (2014), ten fronds of duckweed *Lemna aequinoctialis* were surface sterilized by washing in 5% sodium hypochlorite for 4 minutes and then washed five times with sterilized water and the duckweed was transferred into 6 wells plates containing NF medium (2.7 mM

Figure 2. Sterile *L.aequinoctialis* on R2A agar. Verification of no bacteria

$CaCl_2$, 1.2 mM $MgSO_4$, 1 mM KH_2PO_4, 5 mM KNO_3, 18 µm $MnCl_2$, 46 µm H_3BO_3, 0.77 µm $ZnSO_4$, 0.32 µm $CuSO_4$, 0.49 µm MoO_3, 20 µm $FeSO_4$, 50 µm Na_2EDTA; pH was adjusted to 5 with KOH) (Muranaka et al., 2015) supplemented with 1% sucrose. Sucrose helps to recover the growth of duckweed and supplies a carbon source for bacteria growth. The plates were placed in the plant growth chamber (BiOTRON NK system; NIPPON MEDICAL & CHEMICAL INSTRUMENTS CO., LTD, Tokyo, 28^0C, 5,000 lux,16h-photoperiod, RH 60%). After one week, green duckweed in the well containing no bacteria was transferred into the flask containing fresh NF medium and cultured in the plant culture chamber. The sterility of *L. aequinoctialis* was confirmed by the absence of bacteria colony formation on R2A plates incubated at 30ºC for 7-14 days (Fig. 2).

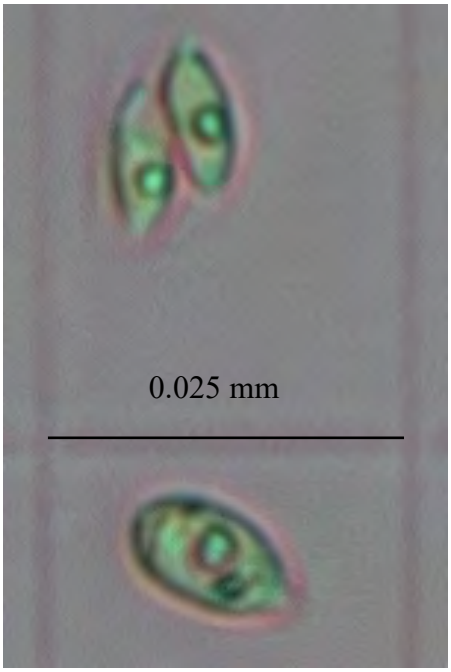


Figure 3. *Coelastrella* sp. KC10 cells under light microscope

2.1.2. Duckweed growth condition

The aseptic duckweed was cultivated in a plant growth chamber (BiOTRON NK system; NIPPON MEDICAL & CHEMICAL INSTRUMENTS CO., LTD, Tokyo) at 28°C, 50% relative humidity, 5,000 lx (75 µmol $m^{-2}s^{-1}$) illumination, and 16 h-photoperiod in NF medium. All plant cultivations in this study were conducted under the same light and thermal conditions as described above.

2.2. Microalga

The microalga used in this study, KC10, was obtained from the same wastewater (WW) where duckweed was growing. Several microalgae of different shapes were observed in the tank, and KC10 was selected as the dominant microalga. KC10 was first recovered using a 3 µm pore size membrane filter (T300A/PP-25; ADVANTEC, Tokyo), followed by treatment with an antibiotic mixture (500 mg/L each of ampicillin, tetracycline, chloramphenicol, and streptomycin) for sterilization. The purity of the microalgal culture was confirmed by the absence of bacterial colony

formation on R2A agar plates. Finally, a single colony of pure KC10 was obtained on an MA agar plate ($NaNO_3$ 50 mg/L, KNO_3 100 mg/L, $Ca(NO_3)_2 \cdot 4H_2O$ 50 mg/L, Na_2β-glycerophosphate$\cdot 5H_2O$ 50 mg/L, Na_2SO_4 40 mg/L, $MgCl_2 \cdot 6H_2O$ 50 mg/L, H_3BO_3 20 mg/L, $Na_2EDTA \cdot 2H_2O$ 5 mg/L, $FeCl_2 \cdot 6H_2O$ 0.5 mg/L, $MnCl_2 \cdot 4H_2O$ 5 mg/L, $CoCl_2 \cdot 6H_2O$ 5 mg/L, $ZnCl_2$ 0.5 mg/L, $Na_2MoO_4 \cdot 2H_2O$ 0.8 mg/L, bicine 0.5 g/L, and agar 15 g/L; pH = 8.6, adjusted with NaOH). The 18S rRNA gene was PCR-amplified using the primers ss3 (5′-GATCCTTCCGCAGGTTCACCTACGGAAACC-3′) and ss5 (5′-GTGATCCTGCCAGTAGTCATATGCTTG-3′) (Khaw et al., 2020). The nucleotide sequence of the 18S rRNA gene was determined and deposited in DDBJ/GenBank/EMBL under the accession number LC801618. It revealed that KC10 (LC801618) was 98.07% (1,640 bp) identical to *Coelastrella saipanensis/tenuitheca* (MF407353.1/MH176106.1). *Coelastrella* sp. KC10 cells (Fig. 3) were cultured in an MA medium in the plant growth chamber under the same conditions as those for duckweed.

3. Isolation of duckweed associated bacteria (DAB)

3.1. Method

Bacteria including actinomycetes were isolated from surface-sterilized and non-surface sterilized *Lemna aequinoctialis* samples, which were collected from wastewater

Ten fronds of duckweed *Lemna aequinoctialis* were surface sterilized by washing in 5% sodium hypochlorite, 70% ethanol for 2-3 min, and distilled water three times for 1 min. The surface-sterilized duckweed and the same number of fronds of non-surface sterilized duckweed were collected and homogenized to release bacteria in 1 mL of sterilized MiliQ water using a Biomasher (Nippi Inc., Tokyo, Japan). The homogenized solution was then diluted by a 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. 40 µl of each serial dilution of 10^{-2}, 10^{-3}, and 10^{-4} was spread onto TSA (Difco^{MT} Tryptic Soy Broth plus 1.5% agar) and R2A. The same volume of each serial dilution of 10^{-1}, 10^{-2}, and 10^{-3} was spread onto the other two solid media 1:100 diluted TSA and SCA (Kuester and Williams, 1964). The plates were incubated at 30°C for up to 3 weeks. All morphologically distinct colony-forming bacteria were isolated and stored at –80°C in 15% glycerol.

3.2. Result and discussion

Sixty-nine morphologically distinct colony-forming bacteria were isolated from *L. aequinoctialis* collected from food factory wastewater. The isolation was performed on four different media in two conditions of duckweed, sterilized with 5% sodium hypochlorite for endophyte, and non-sterilization for surface bacteria. R2A and TSB for general bacteria, 100X diluted TSB (dTSB) for oligotrophic bacteria and SC *Actinobacteria.*

The number of bacterial strains in each condition and medium is given in Table 1. A single colony was purified (Fig. 4) and maintained in 15% (v/v) glycerol at –80º C.

Table 1. Total DAB were isolated from *L. aequinoctialis*

Media	R2A	TSB	dTSB	SC	Total
Sterilized with 5% sodium hypochlorite	13	2	8	9	32
Non-sterilized	17	7	5	8	37
Total					**69**

The highest number of bacteria has appeared on R2A medium, followed by SC, dTSB and the lowest number was grown on TSB. On SC medium, the number of bacteria forming on non-sterilized and sterilized with 5% sodium hypochlorite was not so different. On R2A and TSB sterilized conditions got fewer colonies than the non-sterilizeed one. Interestingly, on dTSB three more morphologically distinct colonies were formed on sterilized conditions. However, since all the colonies were selected solely based on their morphological appearance, there is a possibility that the same colonies were mistakenly picked multiple times. Therefore, further analysis is needed for the identification of the isolated bacteria.

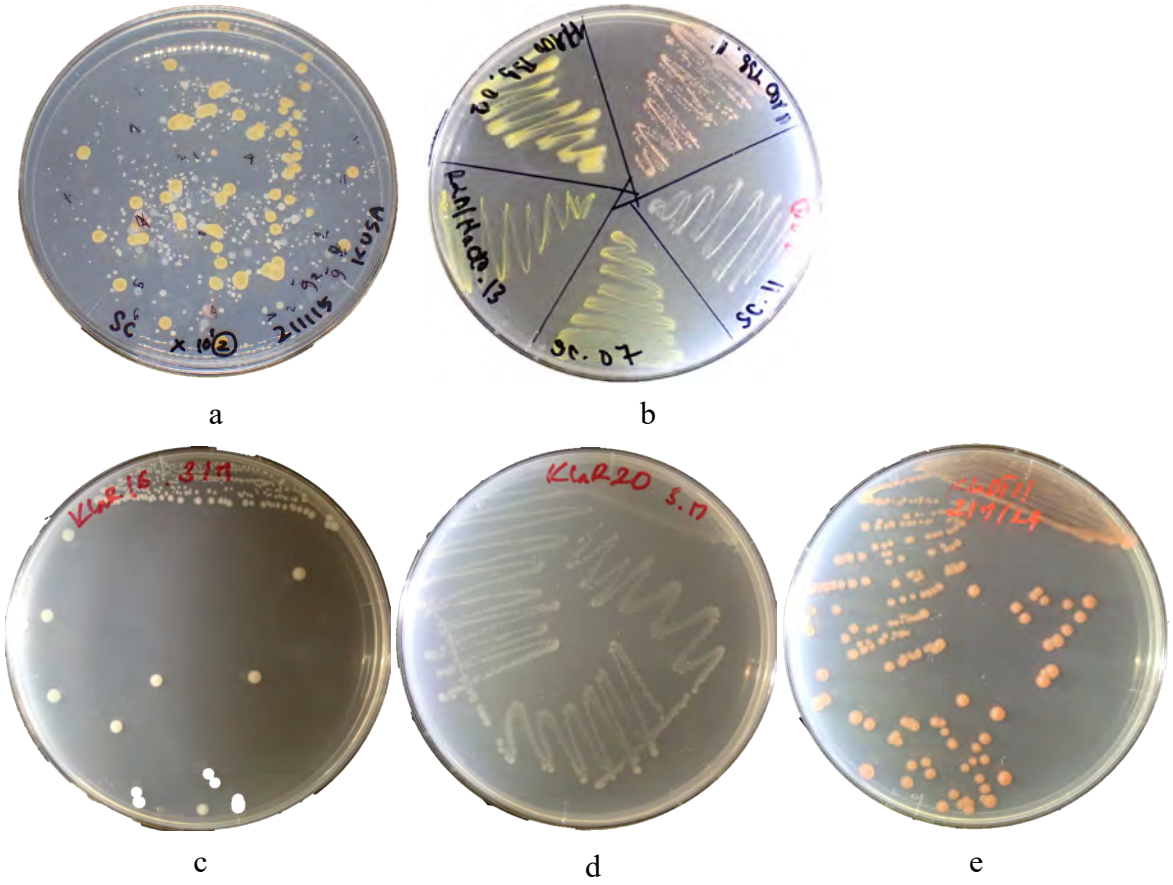


Figure 4. Bacterial colonies on cultural media.

a, Bacterial colonies on SC; b, Isolated bacteria on R2A; c, KLaR16 colonies on R2A; d, KLaR20 colonies on R2A; e, KLaDT11 colonies on R2A

4. Conclusion

This chapter aimed to obtain DAB from *L. aequinoctialis* which is indigenous to a factory wastewater, from the isolation sixty-nine morphologically distinct colony-forming bacteria were successfully obtained from duckweed *L. aequinoctialis,* and all these isolated bacteria were purified and preserved in 15% glycerol in –80ºC. Duckweed was also successfully recovered and grew after the sterilization process and the aseptic duckweed was maintained in NF medium for the stock collection and further experiments. Microalga *Coelastrella* sp. KC10 also passed the purity check and aseptic *Coelastrella sp. KC10* was maintained in MA medium for stock collection and for further experiments.

CHAPTER 2

SCREENING FOR PLANT GROWTH-PROMOTING BACTERIA (PGPB) AND MICROALGAL-INHIBITING BACTERIA (MGIB)

1. Objective

This chapter mainly aims to obtain PGPB and MGIB from duckweed *L. aequinoctialis* which is indigenous to a factory wastewater treatment tank. The specific objectives are to obtain PGPB from the isolated bacteria which promotes the growth of *L. aequinoctialis* in terms of increasing the number of duckweed fronds and dry weight compared to the control sample without bacteria and to obtain MGIB which inhibits the growth of microalga *Coelastrella* sp. KC10 without direct effect on the growth of duckweed

2. Screening for Plant growth promoting bacteria (PGPB)

2.1. Methods

2.1.1. Suspension condition

Two fronds of aseptic *L. aequinoctialis* were transferred into a plant culture dish (Cat. No. 310100, SPL LIFE SCIENCES, Gyeonggi-do, Korea, internal dimension 91.3 x 38.2 [diameter x height, mm]) containing mH medium and bacterium cells with a final OD_{600} of 0.1. The growth of *L. aequinoctialis* was examined by tallying the duckweed frond numbers every two days for 14 days followed by measuring the dry-weight biomass at the end of the cultivation period. The control condition contained no bacteria.

2.1.2. Attachment condition

Aseptic *L. aequinoctialis* was placed into an mH medium containing bacterium cells with a final OD_{600} of 0.3 for 48 hours. After bacterium inoculation, two fronds of duckweed were washed with sterilized water to remove unattached cells. Only strong attached cells were transferred with duckweed into a plant culture dish containing freshly sterilized mH and cultivated in a plant culture chamber. Control was set up with no attached bacteria.

The colony-forming unit (CFU) per frond of duckweed was performed after the unattached cells were removed and after the duckweed cultivation period. Ten fronds of duckweed were smashed by Biomasher (Nippi Inc., Tokyo, Japan) in 1 mL of sterilized water. The homogenized solution was then diluted by a 10^{-1} to 10^{-4}. 10 μL of each solution was spread onto the R2A medium plate. the plate was prepared in three replications. The plates were incubated at 28º C for up to 3 days. The bacterial colonies were counted after forming and CFU/frond was calculated by the following formula:

CFU = average of colony number X dilution factor X (1000 μL / 10μL) / 10 fronds

2.2. results and discussion

2.2.1. Screening of PGPB candidates under suspension conditions in mH

The effect of bacteria on the growth of *L. aequinoctialis* was evaluated on the basis of frond number and dry weight after 14 days of cultivation in a bacterial cell suspension of mH. Of the 69 bacterial strains, 13 bacterial strains were found to enhance the growth of *L. aequinoctialis*. When compared with the aseptic duckweed. EPG (%) values in frond numbers of *L. aequinoctialis* ranged from 25.6% to 82.2% and in dry weight ranged from 20.5% to 91.9% in co-culture with a single PGPB (Fig. 5). With respect to *L. aequinoctialis* in co-culture with KLaS08, KLaDT11, and KLaSR13, the number starting from 2 on day 0 reached 238.3, 249.6, and 222.3, respectively, on day 14. The aseptic *L. aequinoctialis* reached 137 fonds. Their dry weights were 15.8 mg, 14.0 mg, and 13.4 mg with KLaS08, KLaDT11, and KLaSR13, respectively, whereas the dry weight of control aseptic *L. aequinoctialis* was 8 mg. This result confirmed that the bacterial cells co-cultured with duckweed had a considerable positive impact on the growth of the host plant.

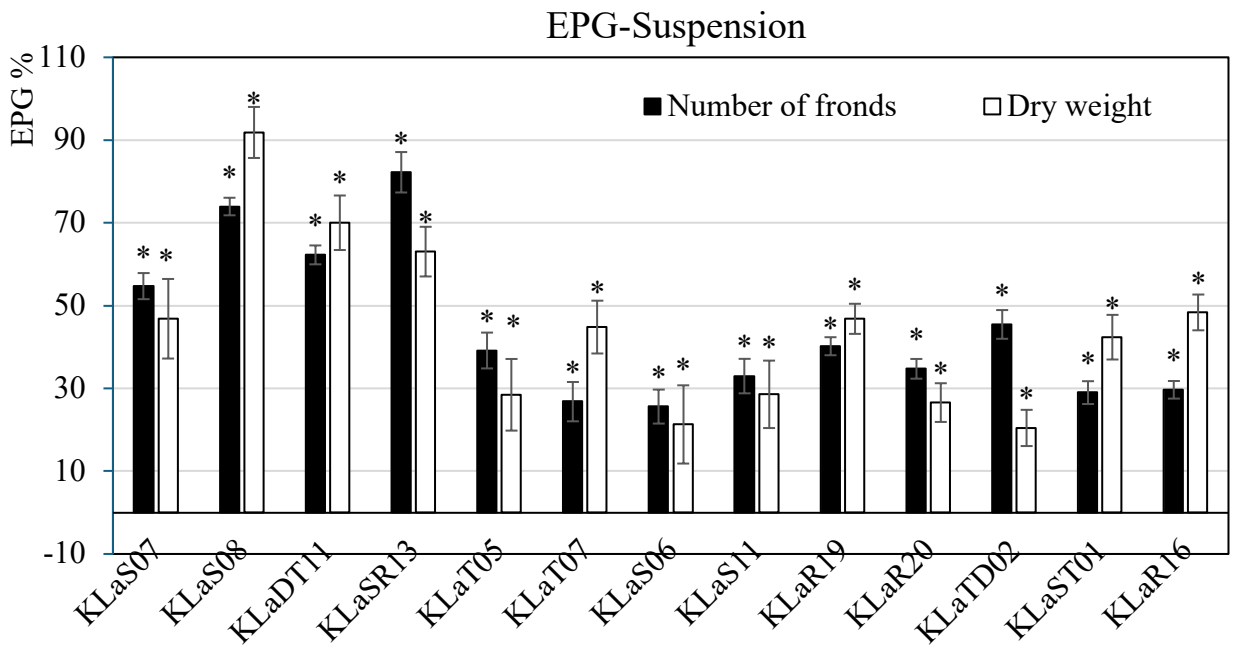


Figure 5. Effect of single PGPB on the growth of duckweed. Percent increase in duckweed frond number and dry weight after 14 days from the corresponding control (aseptic duckweed) are shown. Significant growth promotion (one-way ANOVA, Tukey post hoc test; $p < 0.05$) from the corresponding control experiment is indicated with an asterisk.

2.2.2. Screening of PGPB under attachment conditions in mH

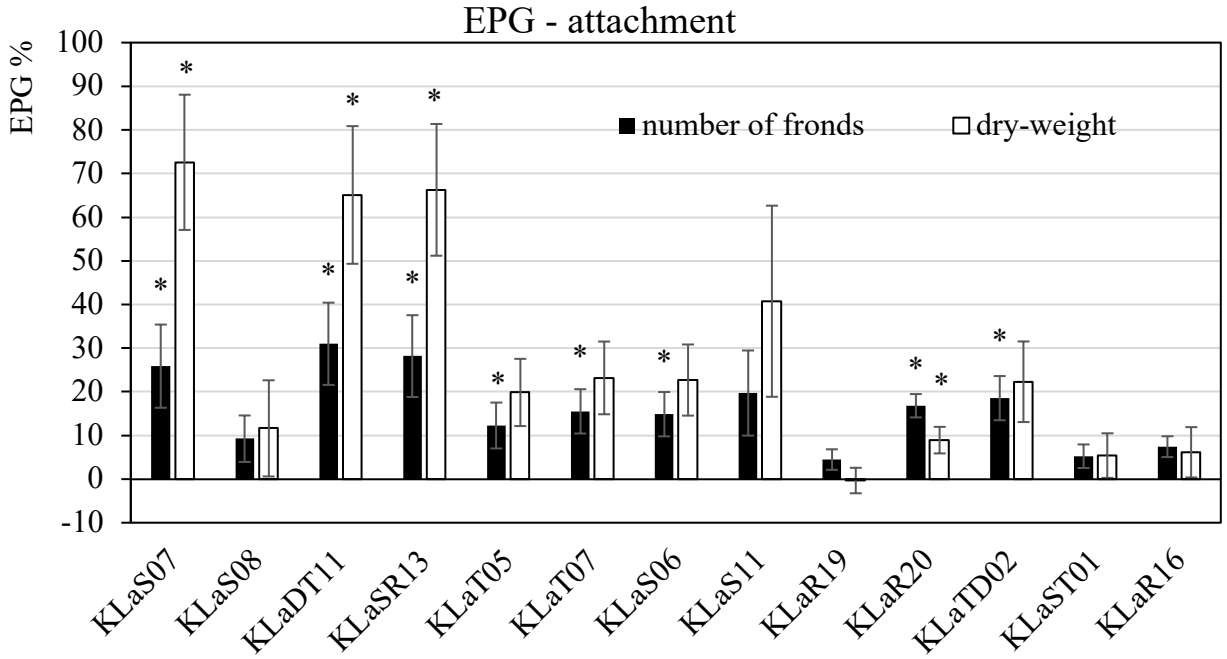


Figure 6. Effect of single PGPB on the growth of duckweed. Percent increase or decrease in duckweed frond number and dry weight after 14 days from the corresponding control (aseptic duckweed) are shown. Significant growth promotion (One-way ANOVA, Tukey post hoc test; $p < 0.05$) from the corresponding control experiment is indicated with an asterisk.

Attachment conditions, in which only the bacterial cells colonizing the duckweed affect the growth of the duckweed plant, are useful for the practical production of duckweed biomass because of lower costs and bacterial contamination than those observed under suspension conditions. The 13 PGPB selected from the above-mentioned experiment under suspension conditions were tested under attachment conditions. Four bacterial strains were shown to increase *L. aequinoctialis* frond number and dry weight when compared with aseptic duckweed. These four PGPB strains showed EPG (%) values in *L. aequinoctialis* frond numbers from 4.5% to 31.0% and in dry weights from -0.29% to 72.5% (Fig. 6). Although KLaS08 showed one of the best PGPB activities under suspension conditions (Fig. 5), it showed one of the worst PGPB activities under attachment conditions. This is probably because KLaS08 has less cell adhesion and colonization capacity for duckweed than other bacteria. Finally, four bacterial strains, KLaS07, KLaDT11, KLaSR13, and

KLaR20, exhibited significant PGPB activity in terms of both frond number and dry weight. These strains were further examined for growth recovery in the presence of antagonistic microalgae

3. Screening for microalgal growth-inhibiting bacteria (MGIB)

3.1. Method

The bacterial cell was collected by centrifugation and washed several times with MA medium. Bacterial cells with final OD_{600} of 0.1 were inoculated into a *Coelastrela* sp. KC10 culture with 0.01 OD_{680}. The co-culture was cultivated for 14 days, the cells of *Coelastrela* sp. KC10 were counted for every 2 days, and chlorophyll content was measured after the cultivation periods. Microalga cell was counted under a light microscope using a hemocytometer. Chlorophyll content was obtained in methanolic extracts using the freeze-thaw method, followed by storage at –80ºC for 12–18 hours. Subsequently, the sample was incubated in a water bath at 55ºC for one hour with intermittent vortexing to facilitate efficient extraction (Pushpalatha et al., 2021). Contents of Chlorophyll *a* (Chl_a), and Chlorophyll b (Chl_b) were calculated following the formula (Dere et al., 1998): $Chl_a = 15.65\ A_{666} - 7.340\ A_{653}$, $Chl_b = 27.05\ A_{653} - 11.21\ A_{666}$

3.2. Result and discussion

The screening began with the PGPB to obtain the bacteria with a dual function of PGPB and MGIB. However, four selected PGPB did not significantly inhibit the growth of microalga (Fig. 7). The strain KLaR20 also did not inhibit the growth of microalga KC10 (data not shown). The screening was continued with the other DAB. Two bacteria were found to inhibit the growth of microalga *Colatrella* sp. KC10 which are KLaST01 and KLaR16 (Fig. 8). Compared to the positive control where only KC10 grows in MA medium, the cell numbers of microalgae in the co-culture of KC10 with KLaR16 and KLaST01 were significantly lower after 6 days (Fig. 8a). Additionally, chlorophyll contents also were proportionally lower at the end of the culture period (Fig. 8b). KLaST01 and KLaR16 promoted the growth of duckweed in suspension condition, however, in attachment condition they were neutral, neither promotion nor inhibition the growth of duckweed. (Fig. 5 and Fig. 6).

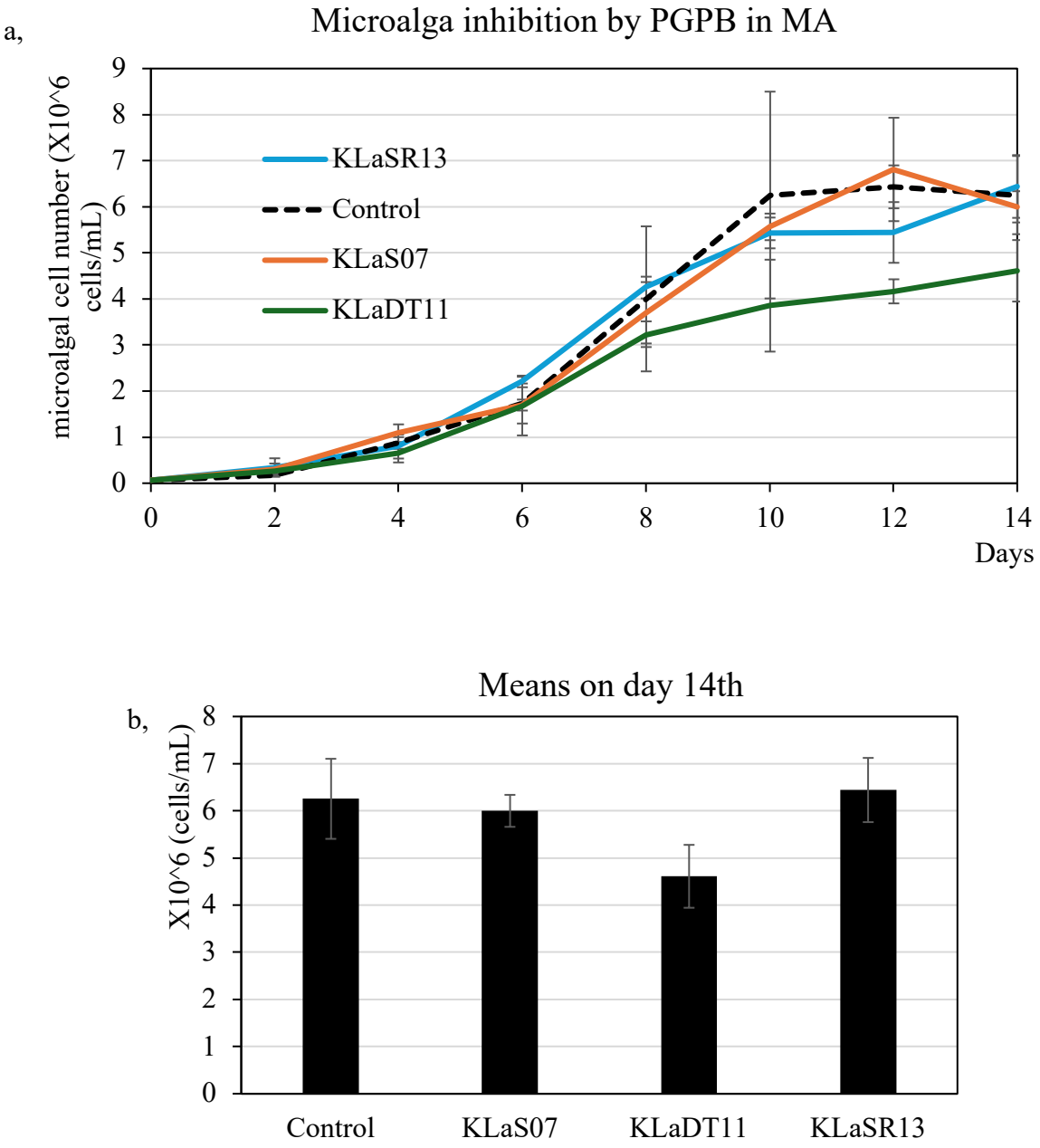


Figure 7. Algicidal activity of PGPB. a, microalgal inhibitory by three PGPB over 14 days, b, number of cells of microalga on day 14th. Values are mean ± SD (n = 3). Significant inhibition (one-way ANOVA Tukey post hoc test; $p<0.05$) indicate by different alphabet between treatments.

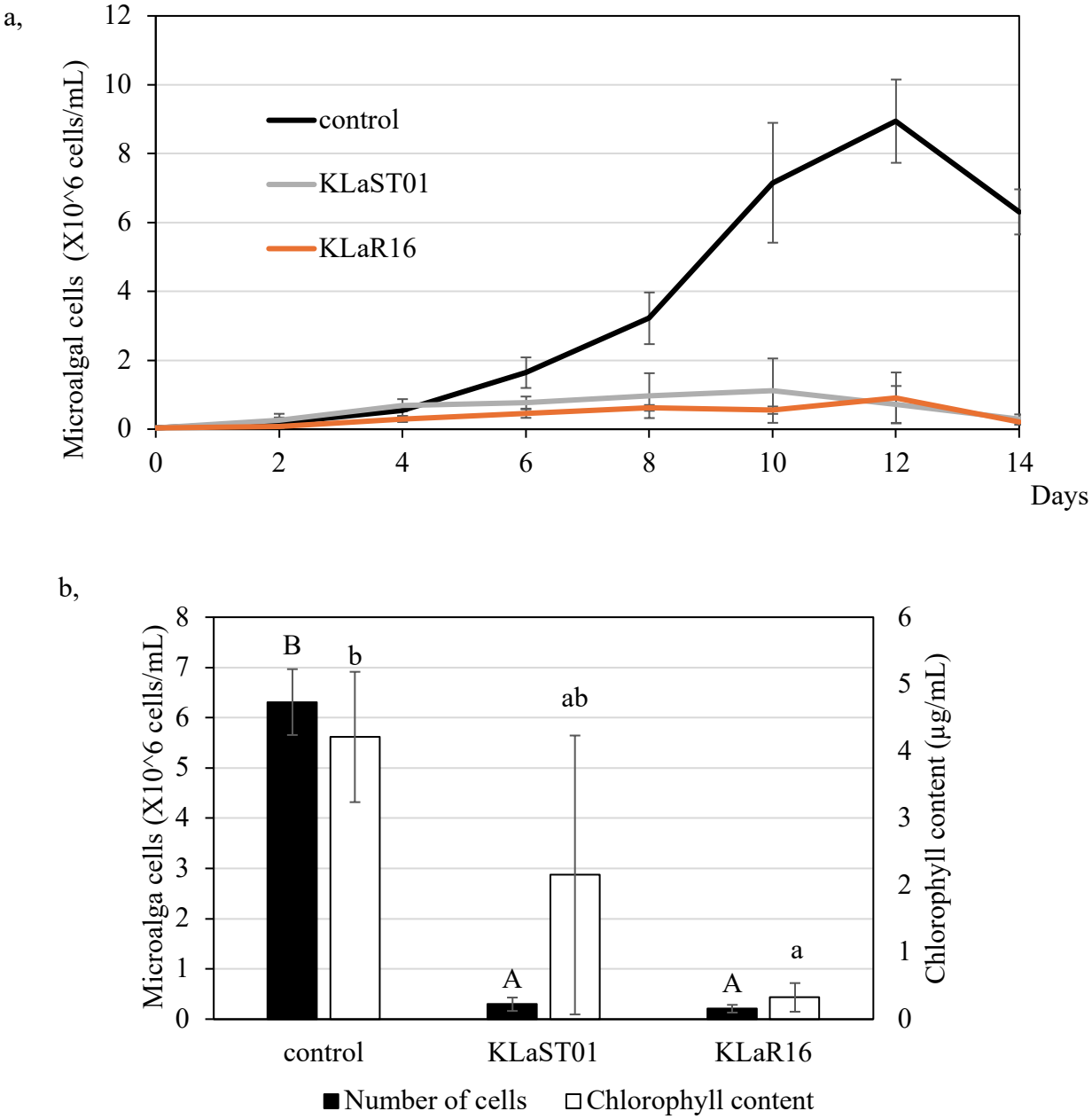


Figure 8. Microalga growth inhibition by single MGIB. a, Microalga growth inhibition by single MGIB over 14 days; b, Number of cells and chlorophyll content of microalga on day 14th. Values are mean ± SD (n = 3). Significant inhibition (one-way ANOVA Tukey post hoc test; $p<0.05$) indicate by different alphabet between treatments.

4. Conclusion

Screening from sixty-nine DAB, thirteen PGPB were selected as beneficial for duckweed *L. aequinoctialis* which promoted duckweed growth in terms of frond number and dry weight, in suspension conditions. The second screening was performed on these thirteen PGPB and four PGPB were chosen as excellent candidates for enhancing duckweed growth in attachment condition. However, none of the four selected PGPB was significantly inhibited the growth of microalga *Coelastrella* KC10. Therefore, MGIB were screened from the other DAB, out of sixty-nine DAB, two MGIB were selected. Which also promoted the growth of duckweed in suspension condition. Finally, six bacterial strains including four PGPB and two MGIB were chosen for further studies.

CHAPTER 3:

IDENTIFICATION OF SELECTED BACTERIA BY 16S rRNA GENE SEQUENCING AND CHARACTERIZATION OF PLANT GROWTH-PROMOTING TRAITS

1. Objective

This chapter has two main goals, The first aim is to analyze some general PGP traits of selected bacteria, and the second aim is to identify the genus/species of selected PGPB and MGIB by 16S rRNA gene sequence

2. Characterization of Plant Growth-Promoting Traits

2.1. Method

Auxin (indole-3-acetic acid, IAA) production activity was determined by the colourimetric method (Gordon and Weber, 1951). Each bacterial strain was cultured in R2A broth supplemented with 200 µg/mL tryptophan at 30°C for 24 hours. The culture was then centrifuged, and 2 mL of supernatant was mixed with 1 mL of Salkowski's reagent (29.16 mL of 60% $HClO_4$, 1 mL of 0.5 M $FeCl_2$, and 19.84 mL of MilliQ water). The mixture was incubated in the dark for about 25 minutes and the positive reaction by developing pink-red in colour and was quantified by measuring absorbance at 530 nm. IAA productivity of these (Pushpalatha et al., 2021) isolated strains in the present and the absence of tryptophan was calculated using the standard curve which was constructed using different concentrations of pure IAA (5 – 100 µg/mL). Phosphate solubilization and Siderophore production were detected by the developing of the clear and yellow halo around the bacterial plug on Pikovskaya's agar (Pikovskaya, 1948), supplemented with 2% (w/v) tricalcium phosphate and the chrome azurol S (CAS) agar (Schwyn and Neilands, 1987), respectively.

2.2. Results and discussion

IAA production, phosphate solubilization and siderophore production were examined in six selected bacteria, four PGPB and two MGIB.

Six tested bacteria produced IAA at different concentrations from 25 to 900 µg /mL (Table 2). IAA is a plant hormone which is essential for growth and development. It is the most common naturally occurring auxin and plays a critical role in various physiological processes such as cell elongation and division, root development or stress response, etc... IAA generally has a positive effect on soil plants, however, the effect on aquatic plants is still uncertain, previously reported by Utami et al. (2018), IAA at 0 – 50 µM has no growth promotion on *Lemna minor.* In Ishizawa et al. (2017b), IAA was shown to correlate with neither growth promotion nor growth inhibition and only phosphate solubilization was correlated with growth promotion.

All tested bacteria produced siderophores by showing a yellow halo around the bacterial plugs on blue agar CAS assay (Fig. 10a) and Phosphate solubilization was only observed in MGIB, KLaR16. It produced a clear dissolution halo on Pikovskayas'medium supplemented with 2% (w/v) tricalcium phosphate (Fig. 10b). Phosphate solubilization refers to the process by which insoluble forms of phosphorus are converted into soluble forms that plants can absorb. This is primarily carried out by certain microorganisms, including bacteria.

Iron is often in an insoluble form, making it difficult for plants to access. Siderophores are small, high-affinity iron-chelating compounds secreted by microorganisms to sequester iron from the environment. Siderophores bind to iron and make it soluble, facilitating its uptake by both the microorganisms and plants.

Bacteria that solubilize phosphate and produce siderophores are essential for plant health and productivity. These bacteria enhance nutrient availability, support plant growth, and improve plant resilience against stress and diseases. In this study, selected PGPB and MGIB commonly produced IAA and siderophores. IAA productivity of bacteria seemed to correlate with PGP activity, except for *Rhizobium* sp. KLaR16 (Table 2, Fig. 6, Fig.10). In the case of KLaR16, the microalgal growth-inhibiting compounds it produced together with IAA may counteract the PGP effect of IAA. On the other hand, the duckweed growth-promoting bacterium *Acinetobacter calcosceticus* P23 (Yamaga et al., 2010), isolated from a pond where duckweed grows naturally, produces exopolysaccharides with a unique structure and attaches to duckweed such as *L. minor*. When the polysaccharides were purified and added to duckweed, the growth rate of duckweed increased in a dose-dependent manner (Morikawa et al., 2017). This suggests that the exopolysaccharides may

also act as one of the PGP factors in bacteria such as *Novosphingobium* sp. KLaS07, *Runella* sp. KLaDT11, and *Brevundimonas* sp. KLaSR13, which significantly promotes duckweed growth in the colonized state (Fig. 6). This assumption needs to be verified in the future. It is also interesting to uncover the molecular mechanism of duckweeds response to the PGP factors provided by each PGPB.

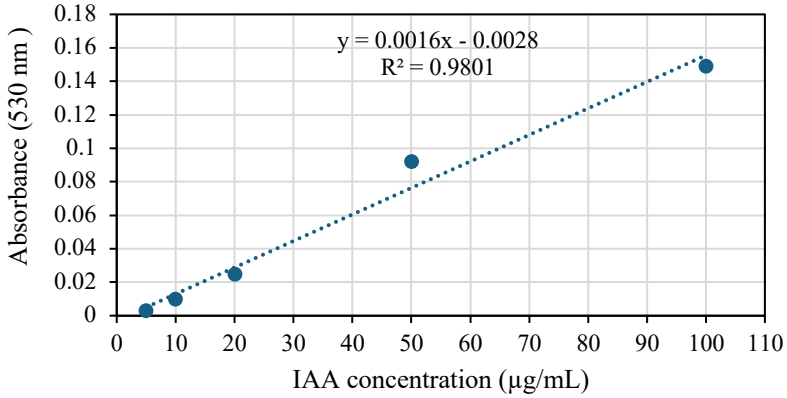
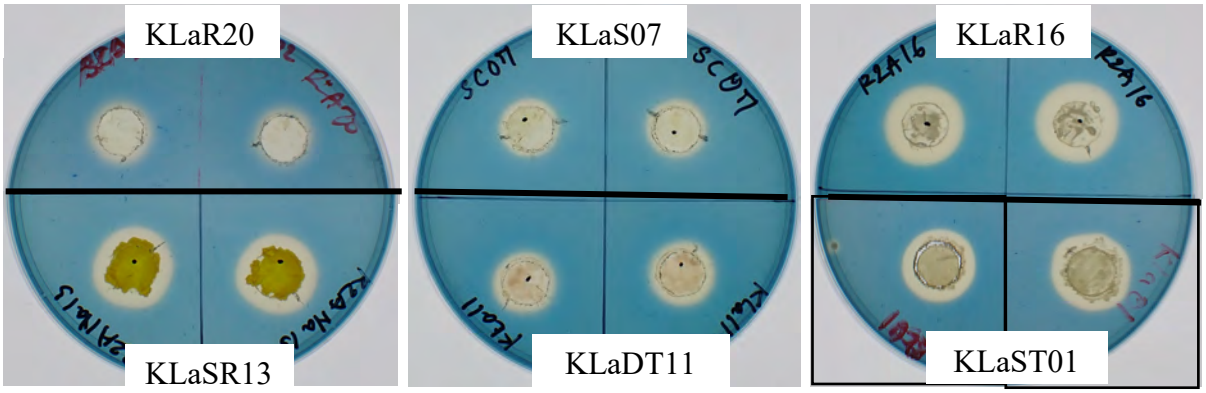


Figure 9. IAA standard curve

Table 2. Production of general PGP factors of PGPB and MGIB. +, positive; –, negative

Bacterial strain	**IAA**	**Phosphate solubilization**	**Siderophore**
KLaS07	+ (118.62 µg /mL)	–	+
KLaDT11	+ (201.75 µg /mL)	–	+
KLaSR13	+ (614.25 µg /mL)	–	+
KLaR20	+ (47.75 µg /mL)	–	+
KLaST01	+ (25.75 (µg /mL)	–	+
KLaR16	+919.25 (µg /mL)	+	+

a



b

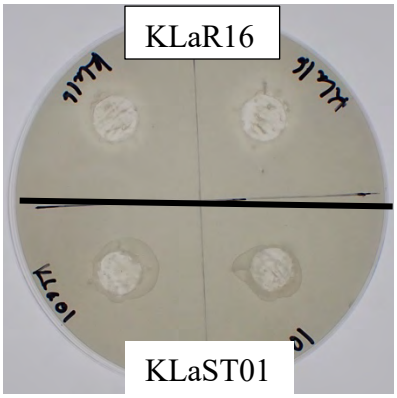


Figure 10. PGPB traits of PGPB and MGIB. a. siderophore production, b. phosphate solubilization

3. Identification of selected bacteria by 16S rRNA sequence analysis

3.1. Method

Genomic DNA was extracted from pure bacterial colonies by using Isoplant II (Nippon Gene Co., Ltd), according to the manufacturer's protocol. The 16S rRNA gene fragment was PCR-amplified using the KOD-Plus-Neo DNA polymerase (Toyobo, Kyoto) with a standard protocol recommended by the manufacturer and a set of universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACGACTT-3′). Then, the PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The resulting sequences were analyzed and compared with the 16S rRNA gene sequences of the reference type strains in the EZbioclound server database (Yoon et al., 2017) for taxonomic identification. The 16S rRNA gene sequences of the six selected bacterial strains were deposited in DDBJ/GenBank/EMBL under accession

numbers LC801612 (KLaS07), LC801613 (KLaTD11), LC801614 (KLaSR13), LC801615 (KLaR20), LC801616 (KLaST01), and LC801617 (KLaR16).

3.2. Result and discussion

Based on 16S rRNA gene sequences nine PGPB which are KLaS07, KLaDT11, KLaSR13, KLaDT02, KLaT05, KLaS06, KLaR19 and KLaR20 belong to the genus *Novosphingobium, Runella, Brevundimonas, Sphingomonas, Asticcacaulis, Acidovorax, Vogesella and Kinneretia* respectively. Two MGIB which are KLaST01 and KLaR16 belong to the genus *Pannonibacter and Rhizobium* respectively (Fig. 11)

Results of partial 16S rRNA gene sequence analysis indicated that strain KLaS07 (accession number LC801612) was 98.58% similar to *Novosphingobium hassiacum* W-51^T (Kämpfer et al., 2002) which was isolated from wastewater of a sewage pond. There is a report that *Novosphingobium* sp. FID3 stably degraded bisphenol A upon colonization on duckweed, *S. polyrhiza* (Li et al., 2014). Recently, significant PGP activity of *N. subterraneum* W5-13 has been also reported for *S. polyrhiza* (Boonmak et al., 2024)

Strain KLaDT11 (1333bp, accession number LC801613) was closely related to *Runella zeae* (99.8% sequence similarity) which was isolated from *Zea mays* (Chelius et al., 2002) but no PGP activity has been reported for bacteria in the genus *Runella*.

Strain KLaSR13 (1297bp, accession number LC801614) was most closely related to *Brevundimonas balnearis* FDRGB2b^T (99.8% sequence similarity) (Tóth et al., 2017). *B. diminuta* MYS6 has been reported to enhance plant growth of a sunflower *Helianthus annuus* and its Cu uptake (Rathi and Yogalakshmi, 2021). Strain KLaDT02 (1337 bp) was most closely related to *Sphingomonas azotifigens* NBRC 15497 (Xie and Yokota, 2006) with 99.1% sequence similarlity. *Sphingomonas azotifigens* is a nitrogen-fixing bacterium isolated from the roots of *Oryza sativa*. Up to date, there is no other report linking *Sphingomonas azotifigens* to duckweed. However, its general PGPB activities, such as nitrogen fixation, might suggest potential benefits when it interacts with duckweed, as in this study.

Strain KLaT05 (1314 bp) was most closely related to *Asticcacaulis excentricus* CB48 with 99.24% sequence similarity. *Asticcacaulis excentricus* was initially isolated from freshwater environments belonging to the Alphaproteobacteria class. This genus of bacterium is known for its unique morphological feature of producing a prostheca, a stalk-like appendage (Poindexter, 1964). A study by Ishizawa et al. (2017b) reported this bacteria strain inhibited the growth of *L. minor*. The inhibitory effect of this bacterial strain was due to its enhancement of reactive oxygen species accumulation and stimulation of antioxidant enzymes in plant cells (Ishizawa et al., 2017a)

Strain KLaS06 (1,351 bp) was closely related to *Acidovorax kalamii* KNDSW-TSA6(T) (Pal et al., 2018) with 99.26% sequence similarlity. *Acidovorax kalamii* KNDSW-TSA6 was isolated from a water sample of the river Ganges. It is also having ability to fix atmospheric nitrogen, converting it into a form that plants can utilize. However, its interaction with duckweed is limited. Study by Ishizawa et al. (2017b) mentioned the species of this genus affected the growth of *L. minor* from –5% to +5%. A study by Boonmak et al. (2024) reported that *Acidovorax kalamii* increased *S. polyrhiza* growth by 156.96 EPG%

Strain KLaR19 (1,401 bp) *Vogesella urethralis* YM-1(T) was first isolated from human urine (Lan et al., 2020) 99.86 %. The research on this bacterium interaction with duckweed are limited. No reports have indicated the effect of this bacteria on duckweed so far.

Strain KLaR20 (1425 bp, accession number LC801615) was most closely related to *Roseateles*/*Kinneretia asaccharophila* KIN192^{T} (Gomila et al., 2010) (99.57 % sequence similarity), which was isolated from freshwater of Lake Kinneret, Israel. No report is available about PGP activity of the genus *Kinneretia*.

An MGIB bacterium strain KLaST01 1348bp, accession number LC801616) was most closely related to *Pannonibacter indicus* DSM23407^{T} (99.3% sequence similarity). *P. indicus* W11-28 isolated from a wastewater effluent from a poultry farm has been reported to enhance the growth of duckweed, *S. polyrhiza*, while *P. phragmitetus* W9-8 from the same wastewater effluent strongly inhibited the duckweed growth (Boonmak et al., 2024). *P. phragmitetus* has been also reported for its growth-inhibiting activity against microalga *Synechocystis* (Zhu et al., 2017)
Strain KLaR16 (1355bp, accession number LC801617) was most closely related to *Rhizobium wuzhouense* W44^{T} (Wang et al., 2021) (98.81 % sequence similarity), *Rhizobium* strain has been

reported for algicidal activity against cyanobacterium *Microcystis aeruginosa* (Pal et al., 2021) but no report available for microalgal growth inhibition.

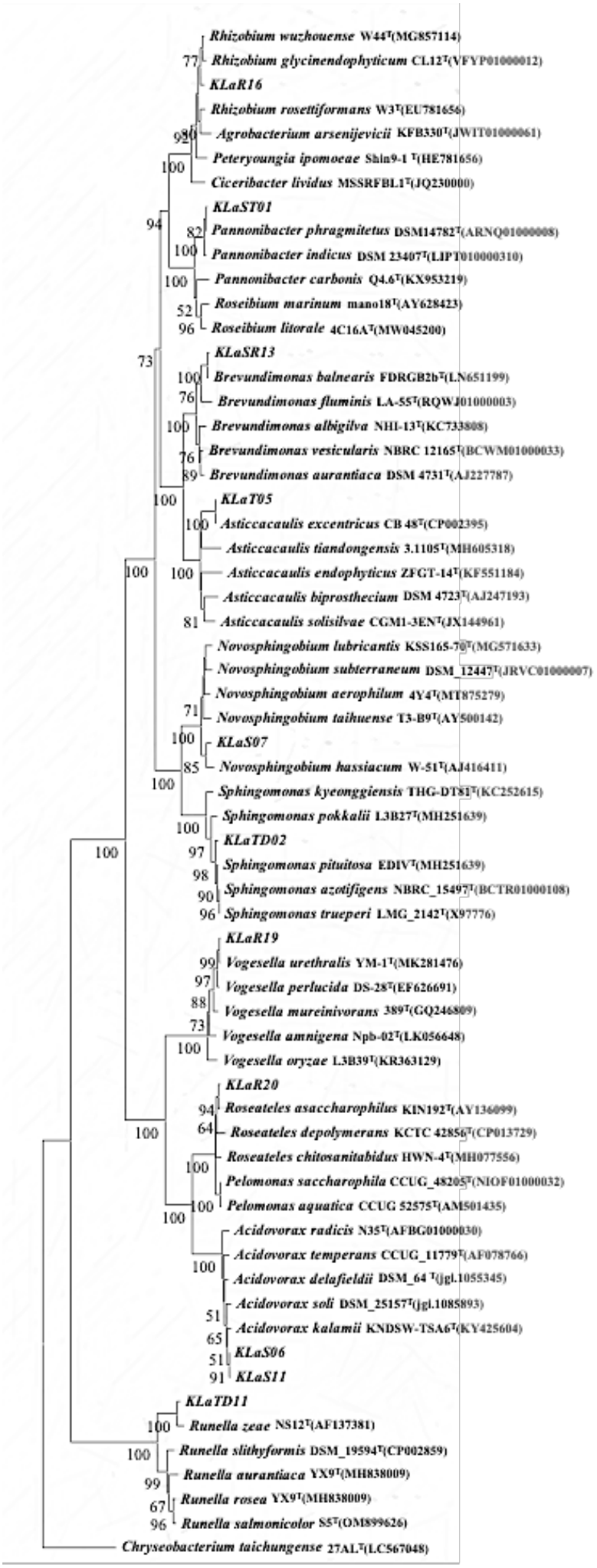


Figure 11. Neighbor-joining phylogenetic tree on the basic of 16S rRNA gene sequences of isolated strains and related species. *Chryseobacteium taichungense* 27AL was used as an outgroup. Numbers at nodes refer to bootstrap values (based on 1000 replicates, only values >50% are shown)

4. Conclusion

Several PGP traits of six selected bacteria were analyzed, however, the promotion factors and traits required for PGPB differ between soil and aquatic environments, further research on the duckweed PGPB and MGIB identified in this study could reveal unforeseen mechanisms underlying aquatic plant-microbe interactions

Eight PGPB and two MGIB were identified by 16S rRNA gene sequencing, it provides a foundation for advancing knowledge, and a comprehensive understanding of the bacteria, ensuring effective application, regulatory compliance and contributing to the broader scientific and commercial utility.

CHAPTER 4:

THE GROWTH RECOVERY OF DUCKWEED IN THE PRESENCE OF THE MICROALGA

1. Objective:

This chapter aims to obtain the best PGPB, MGIB, and the combination of PGPB and MGIB from above-selected strains, which recovers the growth of duckweed *L. aequinoctialis* in the presence of microalga *Coelastrella* sp. KC10 in MA medium and in sterilized wastewater.

2. The growth recovery of re-constructed duckweed holobiont in the presence of microalga in MA

2.1. Method

Duckweed *L. aequinoctialis* was inoculated with bacteria as an attachment condition. After 48 hours of co-culture, 2 fronds of duckweed with strong attached bacteria were transferred into 50 mL of MA medium containing 0.01 OD_{680} of *Coelastrella* sp. KC10. The co-culture was placed in a plant culture chamber for 14 days, *L. aequinoctialis* frond was counted every 2 days. Dry-weight was measured at the end of co-culture periods. The control was prepared as same as the sample conditions except it contained no attached bacteria.

Suspension condition: Bacteria cells were prepared at a final OD_{600} of 0.1, then 2 fronds of Duckweed *L. aequinoctialis* were inoculated with bacteria cell suspension in MA in each plant culture dish containing 0.01 OD_{680} of *Coelastrella* sp. KC10. The co-culture was placed in a plant culture chamber for 14 days, *L. aequinoctialis* fronds were counted every 2 days. Dry-weight was measured at the end of co-culture periods. The control was prepared as same as the sample conditions except it contained no attached bacteria.

The effect on plant growth (EPG) and the effect on plant growth recovery (EPGR) of each bacterial strain was calculated following the previous study (Ishizawa et al., 2017b) EPG(%) or EPGR(%)= $(G(T) - G(C)) \div G(C) \times 100$, where *G(T)* is the mean of *L. aequinoctialis* growth in the

presence of bacteria, which was evaluated by the frond number or dry weight of *L. aequinoctialis* after 14 days of cultivation, and *G(C)* is that in the aseptic *L. aequinoctialis*. The standard error (SE) of the EPG and EPGR was calculated from the following formula.

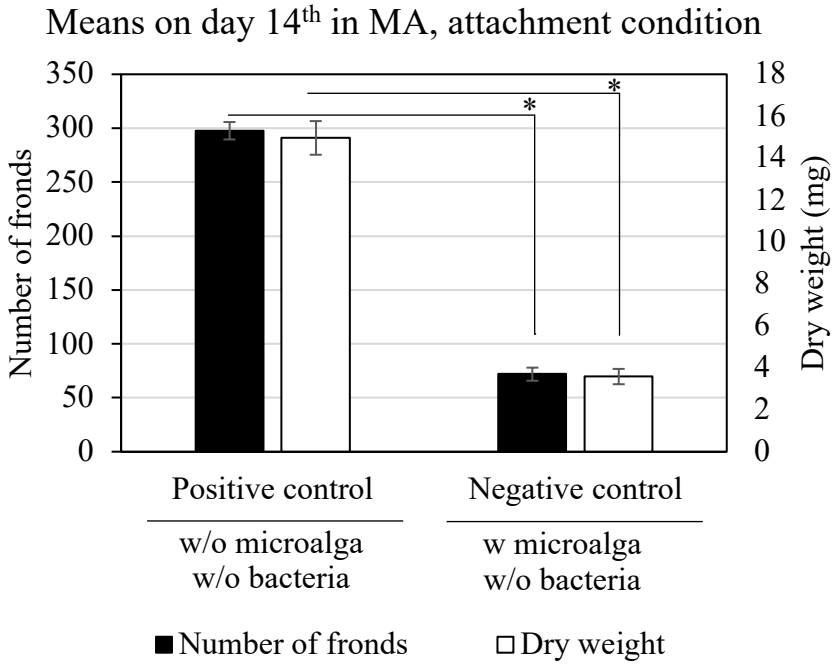
$$\text{SE } (EPG \text{ or } EPGR) = \sqrt{SE\big(G(T)\big)^2 + SE\big(G(C)\big)^2} \div G(C) \times 100$$

2.2. Results and discussion

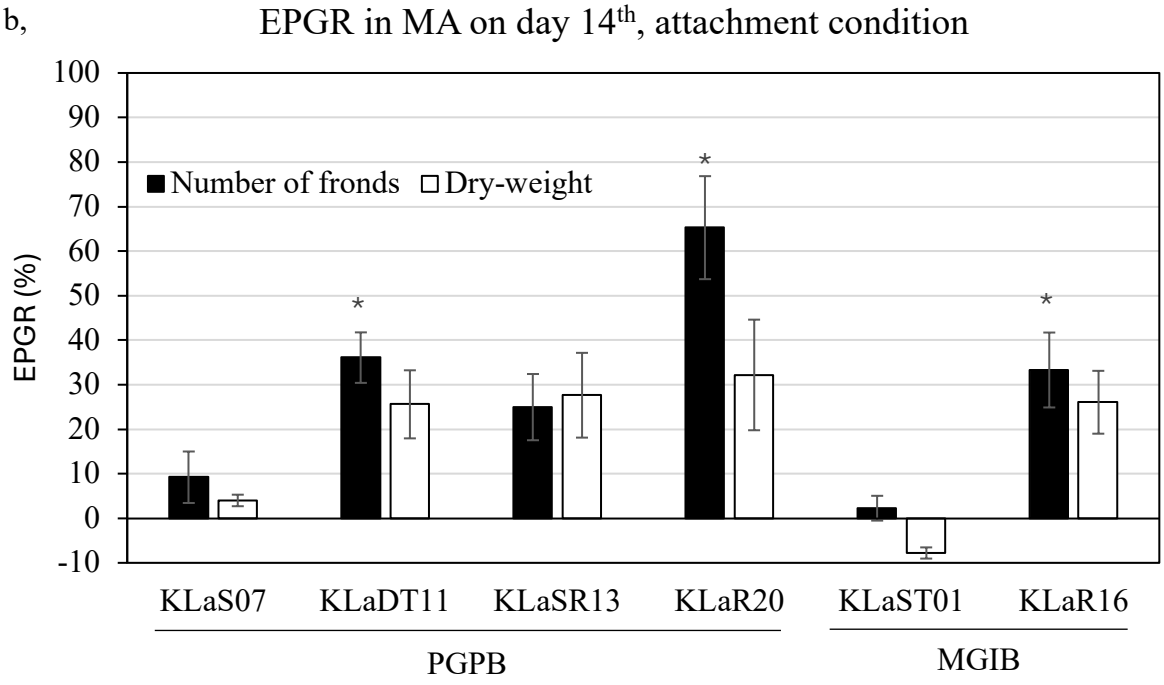
2.2.1. The growth recovery of re-constructed duckweed holobiont in the presence of microalga in MA, attachment condition

The triple co-culture of duckweed colonized with selected single PGPB or MGIB, and microalga KC10 was performed in MA medium, for 14 days, in attachment condition. Aseptic *L. aequinoctialis* was co-cultured with bacterial cell suspension for 48 hours to allow bacteria to colonize. Then, the bacterial colonized duckweed were transferred into MA medium containing microalga *Coelastrella* sp. KC10. Frond numbers and dry weight of the duckweed were measured as described elsewhere. It was shown that growth of *L. aequinoctialis* was severely inhibited in co-culture with microalga. *L. aequinoctialis* increased the frond number and dry weight up to 298 fronds and 15 mg in the single culture, respectively. However, these values markedly decreased to 72 fronds and 3.6 mg when co-cultured with *Coelastrella* sp. KC10 (Fig. 12 a, c). Assisted by PGPB, and MGIB, the number of duckweed fronds was partly but restored. Among the selected four PGPB and two MGIB, two of PGPB, *Kinnneretia* sp. KLaR20 and *Runella* sp. KLaDT11, and one of MGIB *Rhizobium* sp. KlaR16 functioned to the growth recovery of *L. aequinoctialis* from inhibition by microalga. The best recovery rate was observed in co-culture with *Kinnneretia* sp. KLaR20, 65%, followed by *Runella* sp. KLaDT11, 36% and *Rhizobium* sp. KLaR16 recovered 33% in frond numbers. (Fig. 12 b, c).

a,


Means on day 14th in MA, attachment condition
Number of fronds
Dry weight (mg)
350
300
250
200
150
100
50
0
18
16
14
12
10
8
6
4
2
0
*
*
Positive control
Negative control
w/o microalga
w/o bacteria
w microalga
w/o bacteria
Number of fronds
Dry weight

b,


EPGR in MA on day 14th, attachment condition
EPGR (%)
100
90
80
70
60
50
40
30
20
10
0
-10
Number of fronds
Dry-weight
*
*
*
KLaS07
KLaDT11
KLaSR13
KLaR20
KLaST01
KLaR16
PGPB
MGIB

c,

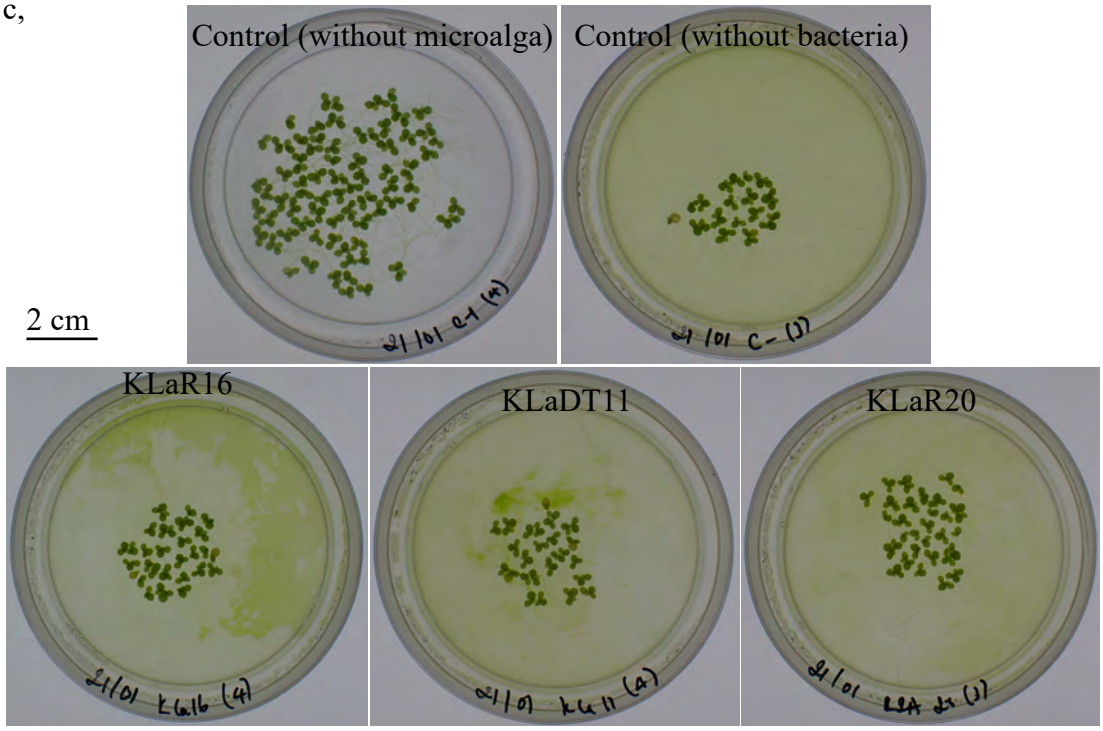


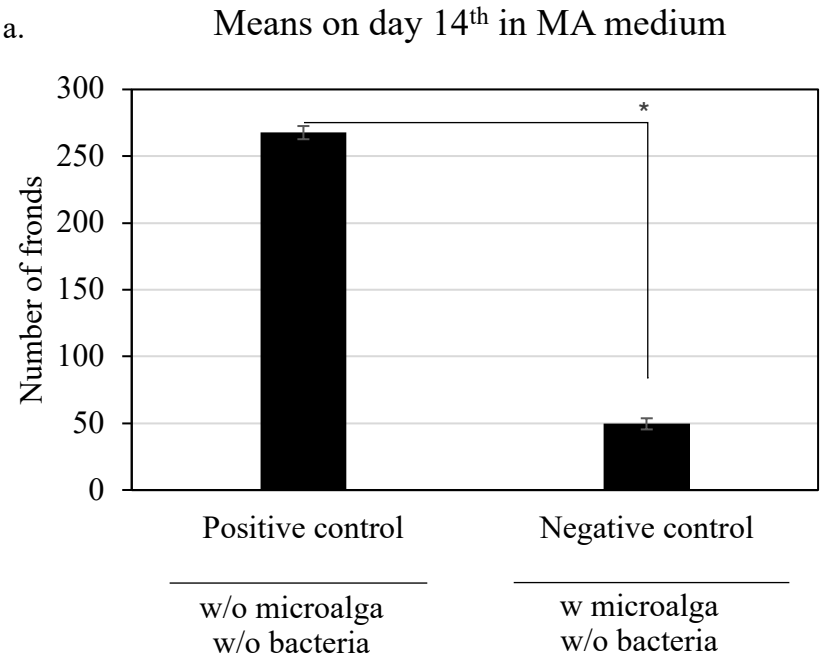
Figure 12. a, The growth of duckweed in MA, with (w) and without (w/o) microalga, on day 14th. Values are mean ± SD (n = 3), significant growth inhibition, (T-test, $p < 0.05$) from the control experiment is indicated with an asterisk from the control experiment; b, The effect of a single bacterium on duckweed growth recovery in the presence of microalga in MA. Percent increase or decrease in duckweed frond number and dry weight after 14 days from the corresponding control (aseptic duckweed) are shown. Significant growth promotion (One-way ANOVA, Tukey post hoc test; $p < 0.05$) from the corresponding control experiment is indicated with an asterisk; c, Photo image of *L. aequinoctialis* in the presence of microalga after 14 days of cultivation in MA, attached with PGPB, MGIB, and control (without bacteria).

2.2.2. The growth recovery of duckweed in the presence of microalga in MA medium in suspension condition

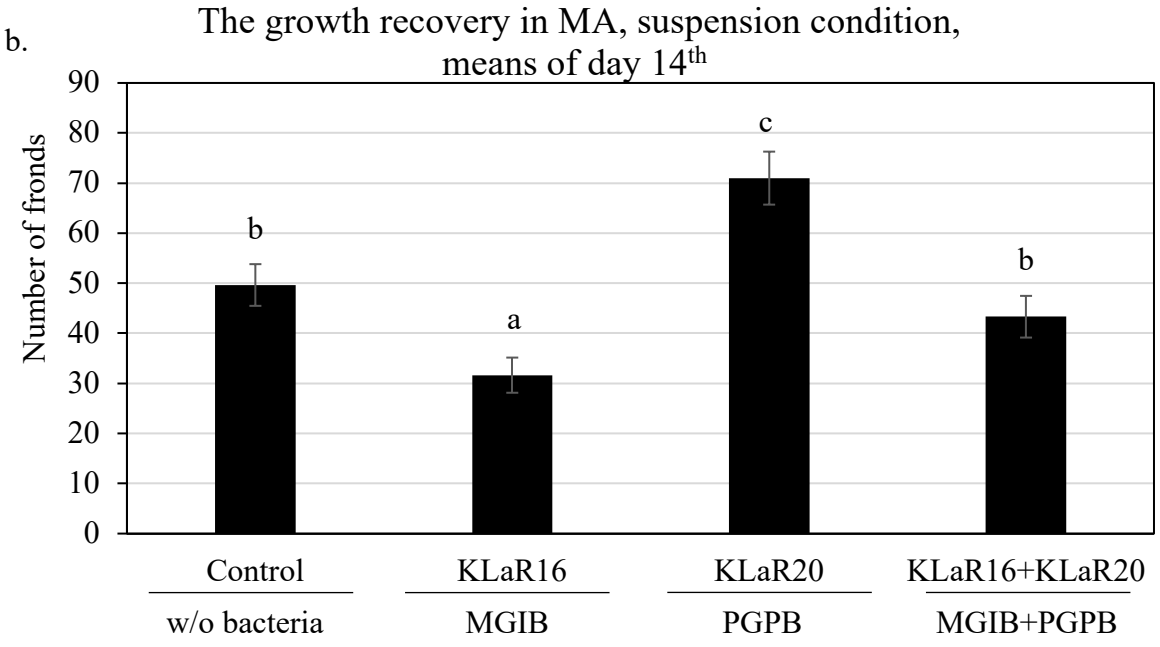
In the presence of *Coelastrella* sp. KC10 , neither a single MGIB *Rhizobium* sp. KLaR16 nor a combination of MGIB and PGPB has any positive effect on the growth recovery of duckweed *L. aequinoctialis* in the suspension condition (Fig. 13). KLaR16 has no inhibition effect on the growth

of duckweed. However, in the recovery experiment, the MGIB was co-culture with microalga, so it is possible that MGIB *Rhizobium* sp. KLaR16 produced the algicidal substance which inhibited the growth of microalga *Coelastrella* sp. KC10 and negatively affected the growth of duckweed *L. aequinoctialis*. Even in combination with PGPB *Kinnneretia* sp. KLaR20, the growth recovery was not better than the control (without bacteria), indicating the strong effect of algicidal substances.

a.



b.



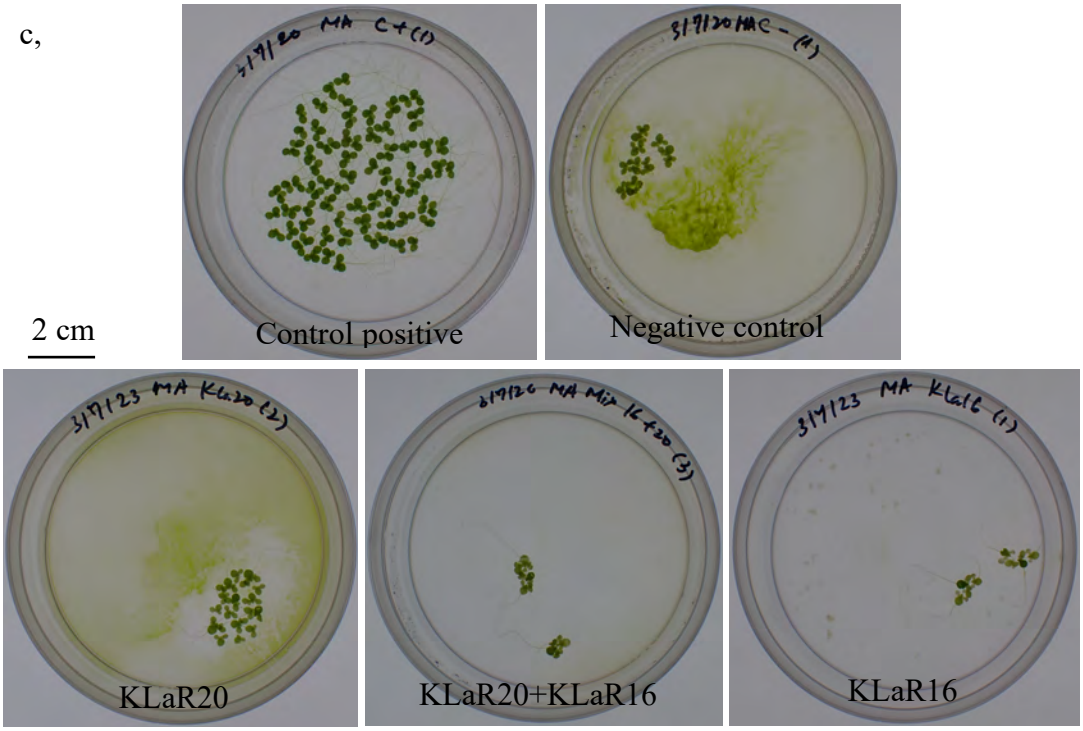


Figure 13. a, The growth inhibition of duckweed in MA with (w) and without (w/o) microalga. Values are mean ± SD (n = 3), and significant growth inhibition (T-test, $p < 0.05$) from the control experiment is indicated with an asterisk; b, The growth recovery of duckweed in the presence of microalga in MA, suspension condition. Values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey as a post-hoc test);

c, Photo image of *L. aequinoctialis* in the presence of microalga after 14 days of cultivation in MA suspension with PGPB, MGIB, BGBP+MGIB, and positive control (without microalga and bacteria), negative control (without bacteria).

3. The growth recovery of re-constructed duckweed holobiont in the presence of microalga in sterilized wastewater (SWW).

3.1. Method

Similar to the method of the growth recovery of re-constructed duckweed holobiont in the presence of microalga in MA in attachment condition. However, SWW was used instead of MA in this experiment. SWW is treated WW sterilized using a membrane filter with a pore size of 0.22 μM (Greniner, BIO-ONE) to remove indigenous microorganisms and microalgae.

3.2. Results and discussion

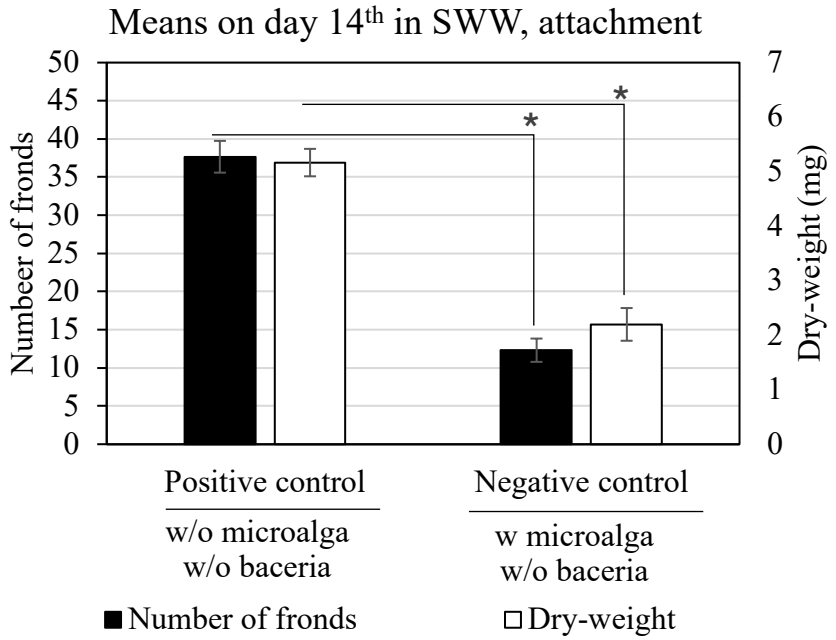
The three competent bacteria chosen from the above-mentioned experiment were tested under SWW co-culture conditions. Experiments were performed using single, double, triple, and quadruple cocultures. Single culture was aseptic duckweed only, double co-culture contained duckweed and microalga, triple co-culture contained duckweed previously colonized by PGPB or MGIB and microalga, and quadruple co-culture contained duckweed colonized by both PGPB and MGIB and microalga. The PGPB tested in this study were *Runella* sp. KLaDT11 and *Kinneretia* sp. KLaR20, and MGIB was *Rhizobium* sp. KLaR16. Under SWW conditions, the frond number of *L. aequinoctialis* increased from 2 to 37.7 after 14 days, but in the co-culture with *Coelastrella* sp. KC10, the final frond number was decreased to 12.3. A dry weight of 5.2 mg was obtained on day 14 in the single culture of *L. aequinoctialis*. However, when co-cultured with *Coelastrella* sp. KC10, the final dry weight of *L. aequinoctialis* decreased to 2.2 mg (Fig. 14). This observation demonstrates that, regardless of the different nutrient conditions of the MA medium and SWW, microalgae have a detrimental impact on duckweed yields (Fig. 12a and 14a). The growth of duckweed in SWW was significantly lower than that in MA, owing to the limitation of nutrient minerals such as nitrogen, phosphorus, and potassium. In contrast, when assisted by PGPB, *L. aequinoctialis* largely recovered its growth rate in the triple co-culture containing microalgae (Fig. 14b). KLaDT11 recovered the final frond number of *L. aequinoctialis* to 27.3 and dry weight to 3.5 mg, whereas KLaR20 recovered the frond number to 28.7 and dry weight to 3.9 mg. The MGIB *Rhizobium* sp. KLaR16 did not prevent duckweed growth inhibition by microalgae in the triple co-culture. The best recovery rate was achieved with the combination of PGPB, KLaR20, and MGIB—KLaR16 in quadruple co-culture yielded 44.3 fronds (117.5% of single culture) and 4.4 mg dry weight (84.5% of single culture), followed by the combination of PGPB, KLaDT11 and MGIB—KLaR16 recovered 31.3 fronds (83.0%) and 3.8 mg dry weight (73.0%). Notably, EPGR (%) values were much higher in nutrient-poor SWW than in nutrient-rich MA medium (table 3). The maximum EPGR (%) of duckweed in quadruple co-culture was 259.5% in frond number and

98.5% in dry weight (Fig. 14c). These results confirm that the holobiont technology proposed here is key to practical high-yield duckweed biomass production.

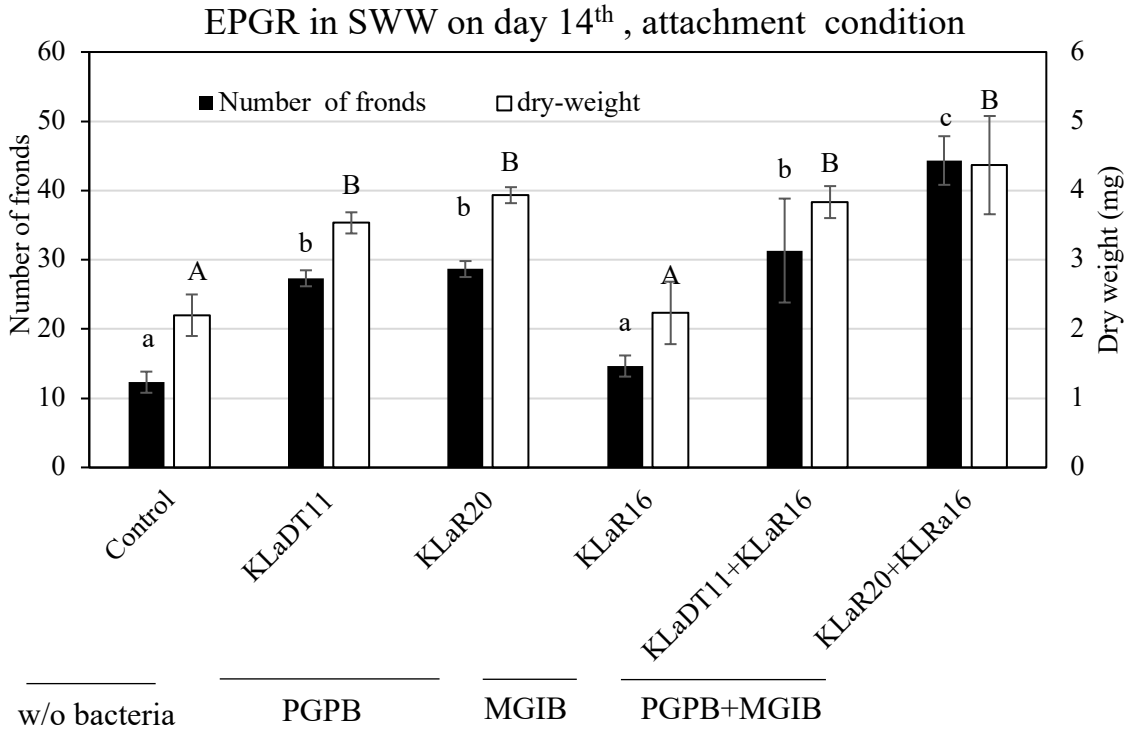
Table 3. Ion contents of MA medium and sterilized wastewater, SWW. nd, not detected. Anions and cations were measured by IC2010 using TSKgel super IC-AR (C-No. 088FA00130F) and TSKgel super IC-CR (C-No. 081FA00052F) colums (4.6 mm I.D. x 15 cm, Tosoh, Osaka, Japan), respectively.

	Ions	**concentration (mg/L)**	
		MA	**SWW**
Anions	F	0.47	nd
	Cl	19.4	428.6
	NO_2	nd	nd
	NO_3	129.5	0.4
	PO_4	0.5	nd
	SO_4	25.8	6.7
Cations	Li	nd	nd
	Na	98.4	318.2
	NH_4	nd	18.3
	Mg	0.3	8.9
	Ca	8.0	41.4
	K	45.5	nd

a,


Means on day 14th in SWW, attachment
Numbeer of fronds
Dry-weight (mg)
50
45
40
35
30
25
20
15
10
5
0
7
6
5
4
3
2
1
0
*
*
Positive control
w/o microalga
w/o baceria
Negative control
w microalga
w/o baceria
Number of fronds
Dry-weight

b,


EPGR in SWW on day 14th , attachment condition
Number of fronds
dry-weight
Number of fronds
Dry weight (mg)
60
50
40
30
20
10
0
6
5
4
3
2
1
0
a
A
b
B
b
B
a
A
b
B
c
B
Control
KLaDT11
KLaR20
KLaR16
KLaDT11+KLaR16
KLaR20+KLRa16
w/o bacteria
PGPB
MGIB
PGPB+MGIB

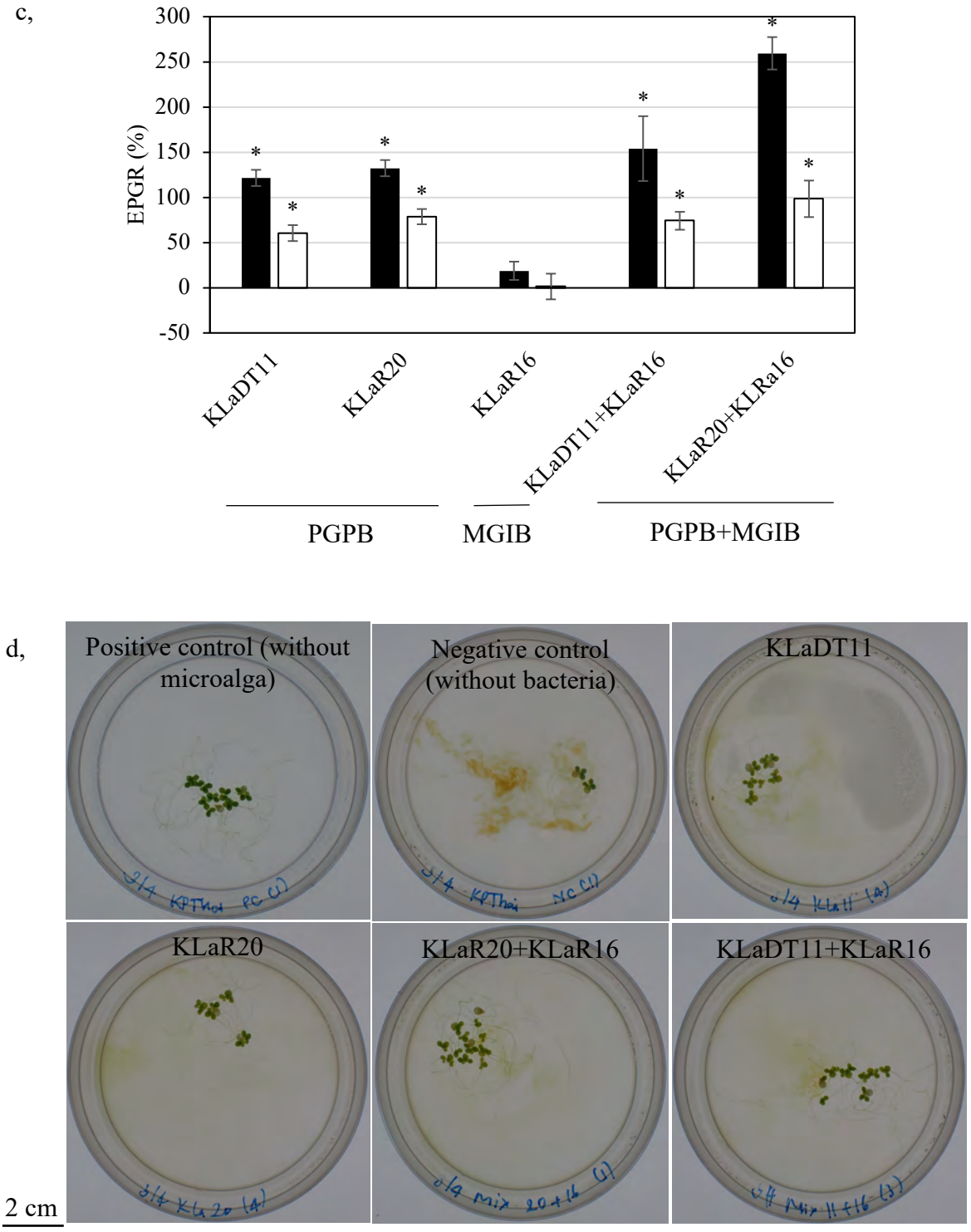


Figure 14. Effect of PGPB and MGIB on the growth recovery of duckweed in SWW. **a**, Growth of duckweed, with (w/) and without (w/o) microalga, on day 14th. Symbols are closed bar,

duckweed frond number, and open bar, dry weight. Values are mean ± SD (n = 3), and significant growth inhibition (T-test, $p < 0.05$) from the positive control experiment is indicated by an asterisk. **b**, **c**, Effect of PGPB and MGIB on the growth recovery of duckweed in the presence of microalga. Symbols are closed bar, duckweed frond number, and open bar, dry weight. Values are mean ± SD (n = 3), and different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey as a post-hoc test). Percent increase or decrease in duckweed frond number and dry weight on day 14th from experiment w/o bacteria (negative control) are shown in **c**. Significant growth promotion (one-way ANOVA, Tukey post hoc test; $p < 0.05$) from the corresponding control experiment is indicated with an asterisk. **d**, Photo image of *L. aequinoctialis* on day 14th with single, double, and triple co-culture in SWW.

4. Conclusion

The microalga *Coelastrella* sp. KC10 was shown to be a growth competitor of the duckweed, which severely reduced duckweed frond number and dry weight in both conditions of culture media and sterilized wastewater. However, in co-culture with two PGPB *Runella* sp. KLaDT11, *Kinneretia* sp. KLaR20 and one MGIB *Rhizobium* sp. KLaR16 (MGIB) in MA medium, the growth of duckweed *L. aequinoctialis* was recovered.

In SWW, two selected PGPB *Runella* sp. KLaDT11, *Kinneretia* sp. KLaR20 and the combination of each PGPB and MGIB *Rhizobium* sp. KLaR16 successfully restored duckweed growth in the presence of microalga *Coelastrella* sp. KC10. The best recovery rate was observed in the combination of *Kinneretia* sp. KLaR20 and *Rhizobium* sp. KLaR16, recovered the growth of duckweed at a similar level to those observed in the absence of microalga.

CHAPTER 5

EXTRACTION AND PURIFICATION OF ALGICIDAL SUBSTANCES PRODUCED BY MGIB, KLAR16

1. Objective

This chapter aims to understand the algicidal mechanism of MGIB *Rhizobium* sp. KLaR16 against microalga *Coelastrella* sp. KC10 by extracting, purifying, and identifying algicidal substances which are produced by bacterial strain KLaR16.

2. Algicidal activity of culture supernatant

2.1. Materials

KC10 is a unicellular green microalga which belongs to the genus *Coelastrella. Coelastrella* mainly occur in freshwater environments as unicellular or several-celled aggregation with spherical or oval-shaped cells. Cells of KC10 are typically 5-20 micrometers in diameter.

KLaR16 is a rod-shaped and motile bacterium which belongs to the genus *Rhizobium. Rhizobium* are known for their ability to form symbiotic relationships with leguminous plants. *Rhizobium* can fix nitrogen in root nodules thus enhance plant growth. *Rhizobium* strain AQ_MP has been reported for algicidal activity against the cyanobacterium *Microcystis aeruginosa* (Pal et al., 2021). To my best knowledge, there is no report available for the algicidal activity of *Rhizobium* against eukaryotic microalgae including *Coelastrella*.

2.2. Methods

2.2.1. Preparation of culture supernatant

Monoculture supernatant of KLaR16 in R2A medium (namely Mo.KLaR16): Bacterium strain *Rhizobium* sp. KLaR16 was grown in R2A medium for 24h and the cell-free supernatant was collected by centrifugation and passing through 0.22 µM membrane.

Co-culture supernatant of bacterial strain KLaR16 and microalga *Coelastrella* sp. KC10 in MA medium (Co.KLaR16-KC10): KlaR16 was grown in R2A overnight, then the culture was centrifuged, and the bacterial cell pellet was washed twice in MA medium. OD_{600} 0.1 of KLaR16 cell suspension was prepared in MA medium and OD_{680} 0.01 of KC10 was added into the suspension. The co-culture of KLaR16 and KC10 was placed in the plant culture chamber for 4 days. The co-culture supernatant was collected by centrifuge and passed through 0.22 µM membrane.

Monoculture supernatant of *Coelastrella* KC10 (namely Mo.KC10): OD_{680} 0.01 of KC10 was solely grown in MA of the same volume as above co-culture experiment. After 4 days, monoculture supernatant was collected by centrifuge and passed through 0.22 µM membrane

2.2.2. Algicidal activity of culture supernatant

Either Mo.KLaR16, Mo.KC10 or Co.Kla16-CK10 was tested activity to grow *Coelastrella* sp. KC10 at final OD_{680} 0.01. The growth of microalga KC10 was evaluated by cell number after 8-10 days. The microalgal cells were counted under a light microscope using a hemocytometer. R2A and MA medium only were also used as control experiments.

2.3. Results

It was found that Mo.KlaR16 clearly inhibited the growth of microalga *Coelastrella* KC10. Compared to the control MA and R2A media, the number of KC10 cells after 8 days culture was apparently less than these control samples, confirming the active algicidal substances in the culture supernatant (Fig. 15). Moreover, the growth of KC10 was better in MA added by organic medium R2A than MA only (data not shown). Therefore, Mo.KlaR16 that contains R2A will not be used for further experiments because separation of algicidal compounds from organic compounds in R2A may not be easy.

Co.KLaR16-KC10 of 4 days was collected and its algicidal activity against microalga KC10 was checked. It was shown that the higher concentration of Co.KLaR16 used, the higher algicidal activity (Fig. 16 a). When compared to Mo.KC10 and MA medium only control, the cells number

of KC10 remaining after 8 days in the 100% Co.KLaR16-KC10 was significantly less (Fig. 16 b). It was indicated that algicidal substances were produced in the co-culture supernatant. These results suggest the indirect mode of algicidal activity of KLaR16 against KC10. Additionally, Mo.KC10 did not inhibit the growth of KC10 (Fig. 16 b) indicating that the algicidal substances were not produced by KC10 but by KLaR16. The pH of Mo.KC10 and Co.KLaR16-KC10 were not much different. Therefore, pH is not an algicidal factor of the Co.KLaR16-KC10.

Ion chromatography analysis was performed on Co.KLaR16-KC10. Anions and cations were measured by IC2010 using TSKgel super IC-AR (C-No. 088FA00130F) and TSKgel super IC-CR (C-No. 081FA00052F) colums (4.6 mm I.D. x 15 cm, Tosoh, Osaka, Japan), respectively. The result is shown in Table 4. There was no significant difference in the ion contents of Co.KlaR16-KC10 and Mo.KC10, suggesting that there is no nutrient deficiency in Co.KlaR16-KC10.

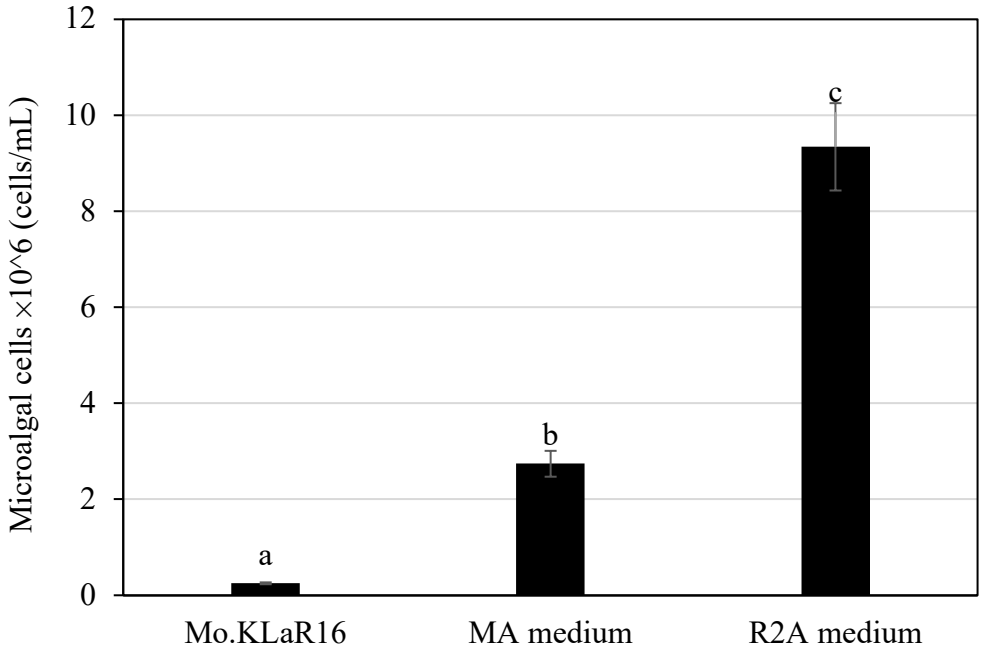


Figure 15. Algicidal activity of Mo.KLaR16 on day 8th.

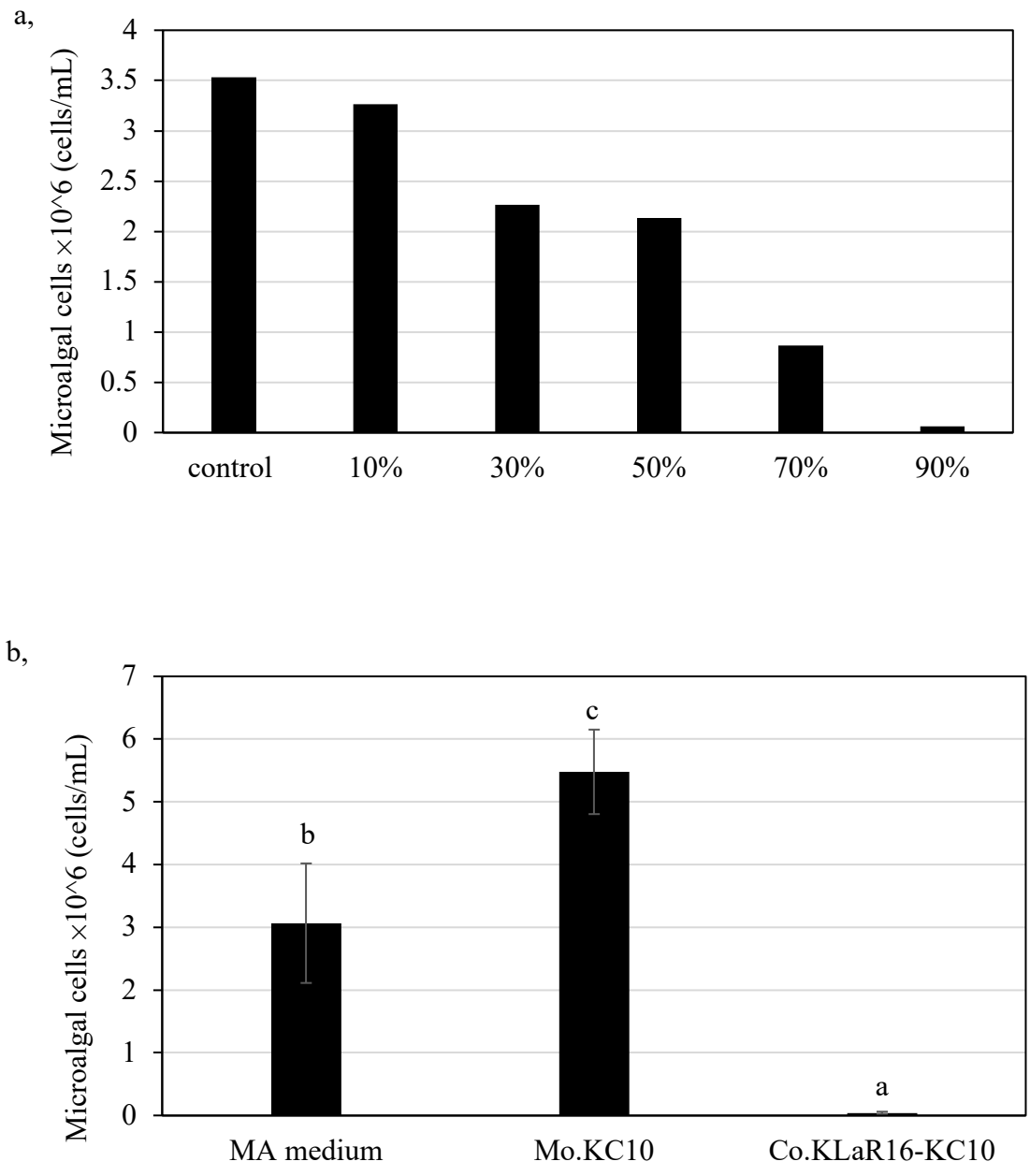


Figure 16. Algicidal activity of co-culture supernatant of KLaR16 and KC10 (Co.KLaR16-KC10) on day 8th. a, at different concentrations of Co.KLaR16-KC10 added to MA medium; b, at 100% concentration without supplementing MA.

Table 4. Ion contents of MA medium and Co.KLaR16-KC10 and Mo.KC10, nd: not detected

Ion chromatography	Ions	Concentration (mg/L)		
		MA	Co.KLaR16-KC10	Mo.KC10
Anion	**F**	0.5	0.9	1.0
	Cl	19.4	21.0	19.6
	NO_2	nd	1.3	3.3
	NO_3	124.9	125.6	95.7
	PO_4	1.0	nd	nd
	SO_4	25.6	27.2	25.2
Cation	**Li**	nd	nd	nd
	Na	94.6	105.4	101.0
	NH_4	nd	0.2	1.1
	Mg	0.2	0.2	0.3
	Ca	9.0	10.1	10.1
	K	49.7	53.5	49.9

3. Extraction of algicidal substances

3.1. Algicidal activity of heat-treated co-culture supernatant of KLaR16 and KC10 (Co.KLaR16-KC10)

Co.KLaR16-KC10 was heat-treated at 100^0C for 20 min and examined the algicidal activity. The results are shown in Fig. 17. Algicidal activity of Co.KLaR16-KC10 was fully retained after the heat treatment,. This result suggested that the algicidal compounds were thermostable.

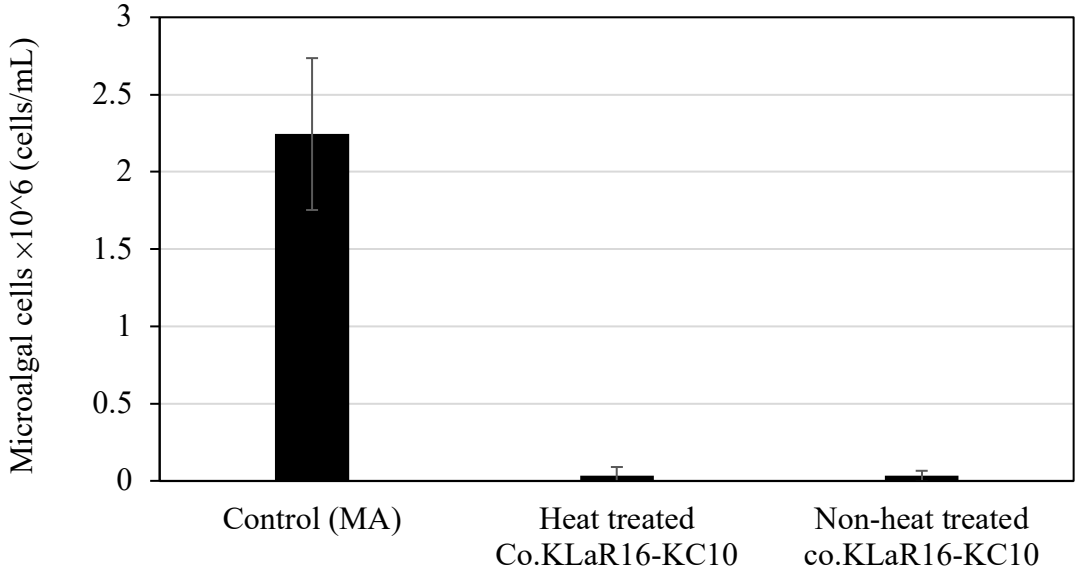


Figure 17. Algicidal activity of heat-treated and non-heat treated Co.KlaR16-KC10 on day 8th.

3.2. Separation of algicidal compounds by ultrafiltration

Co.KLaR16-KC10 was centrifuged using an ultrafiltration tube (MILIPORE Amicon Ultra-15, regenerated cellulose 10,000 MWCO) at 4,000g for 30 min. The volume of samples recovered were 11.6 % in the concentrates (High MW) and 88.3 % in the filtrates (Low MW). MA medium was added to High MW and low MW to adjust the concentration of these samples to the original Co.KLaR16-KC10 and inoculated KC10 to test each algicidal activity. After 8 days of culture, the Low MW sample clearly inhibited the growth of KC10, whereas KC10 almost normally grew in high MW sample (Fig.18 a, b). The algicidal activity of the samples was reconfirmed using 100% Low MW and 100% High MW without supplementing new MA medium (Fig. 18 c). Low MW sample again showed algicidal activity.

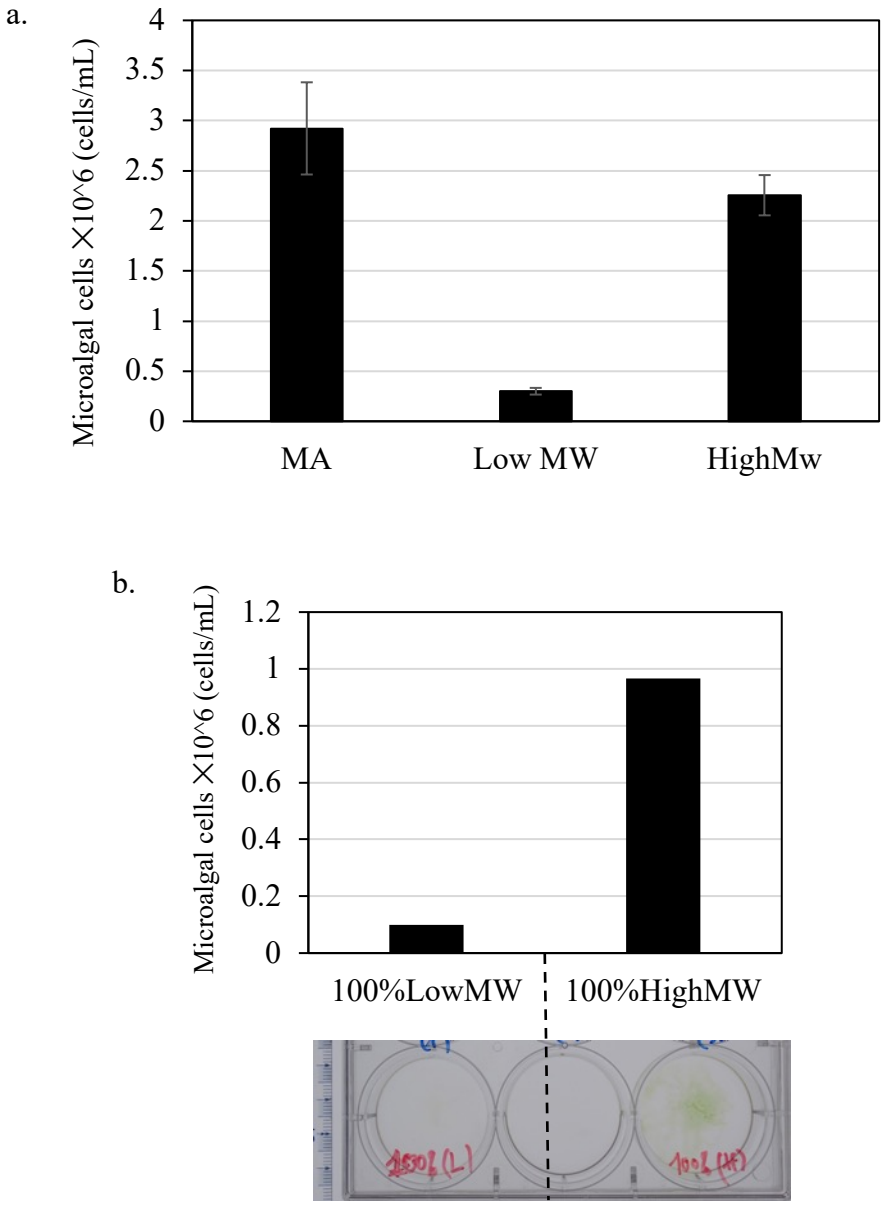


Figure 18. a, Algicidal activity of ultrafiltration samples, Low MW and High MW, at original culture concentrations. b, Algicidal activity of 100% Low MW and 100% High MW samples.

3.3. **Solvent extraction of algicidal compounds**

3.3.1. Methods

Organic solvents: Either ethyl acetate or 1-butanol was used for extraction of the KLaR16 cell-free supernatant (Mo.KLaR16) at a ratio of 1/1 (v/v). Solvent extraction was carried out in a separatory funnel for 10 min by strong shaking, followed by standing for 10 min or until the two layers were allowed to separate. The water layer and organic solvent layer were sequentially drained from the funnel and collected. The water layer was extracted again with new solvent and the organic fractions were evaporated to dry and redissolved in aliquot of water. The solvent extracts were passed through a 0.22 μM membrane for sterilization and added into MA containing *Coelastrella* sp. KC10 at final OD_{680} 0.01 to check algicidal activity.

3.3.2. Results

About 260 mL of Co.KLaR16-KC10 was extracted with ethyl acetate and redissolved in 1.3mL of H_2O (concentrated by 200×) followed by adding 10% of that extractes to the culture of KC10. It was shown that no algicidal activity was recovered in the solvent extracts after 10 days of cultivation (Fig. 19).

Similar to ethyl acetate extraction as above described. About 275mL of Co.KLaR16-KC10 or Mo.KC10 was extracted with 1-butanol and redissolved in 2mL of H_2O (concentrated 137.5×). Each extracts was added to the culture of KC10 at 12%. It was shown that the cells of KC10 in

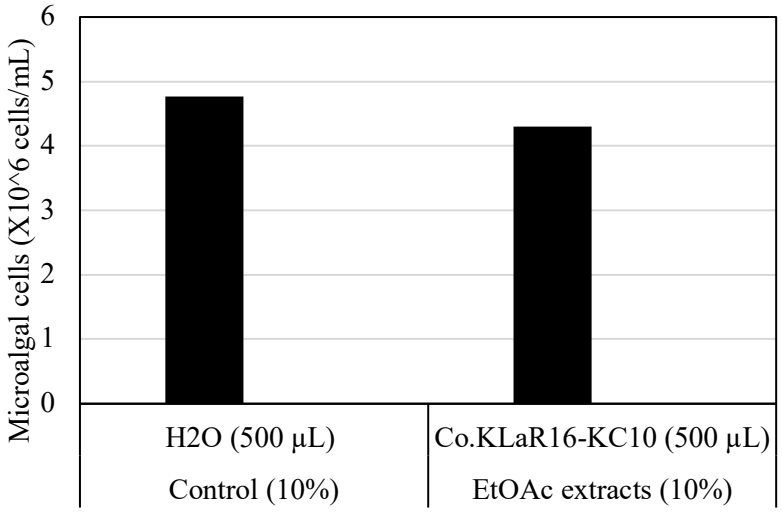


Figure 19. Algicidal activity of ethyl acetate extraction of Co.KLaR16-KC10.

Mo.KC10 and Co.KlaR16-KC10 were not so different after 10 days, suggesting that no algicidal activity was extractable by 1-butanol (Fig.20).

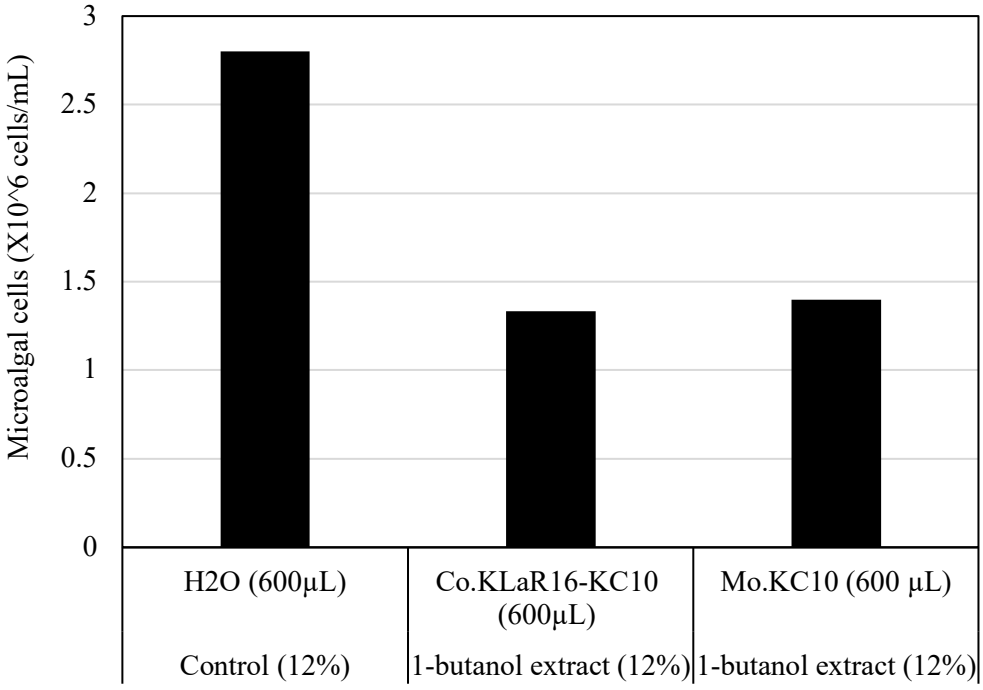


Figure 20. Algicidal activity of 1-btanol extracts of Co.KLaR16-KC10 and Mo.KC10.

4. Conclusion

The functional compounds were produced in the spent medium of the co-culture of KLaR16 with KC10 and the single culture of KLaR16, suggesting a non-direct mode of algicidal activity. Moreover, it was shown that the algicidal substances are constantly produced by KLaR16 regardless of the presence of microalga KC10.

The compounds were suggested to be thermostable compounds with the molecular weight less than 10 kDa. However, they were not extractable by either ethyl acetate or 1-butanol. It was suggested to be compounds different from ever-reported.

GENERAL CONCLUSION

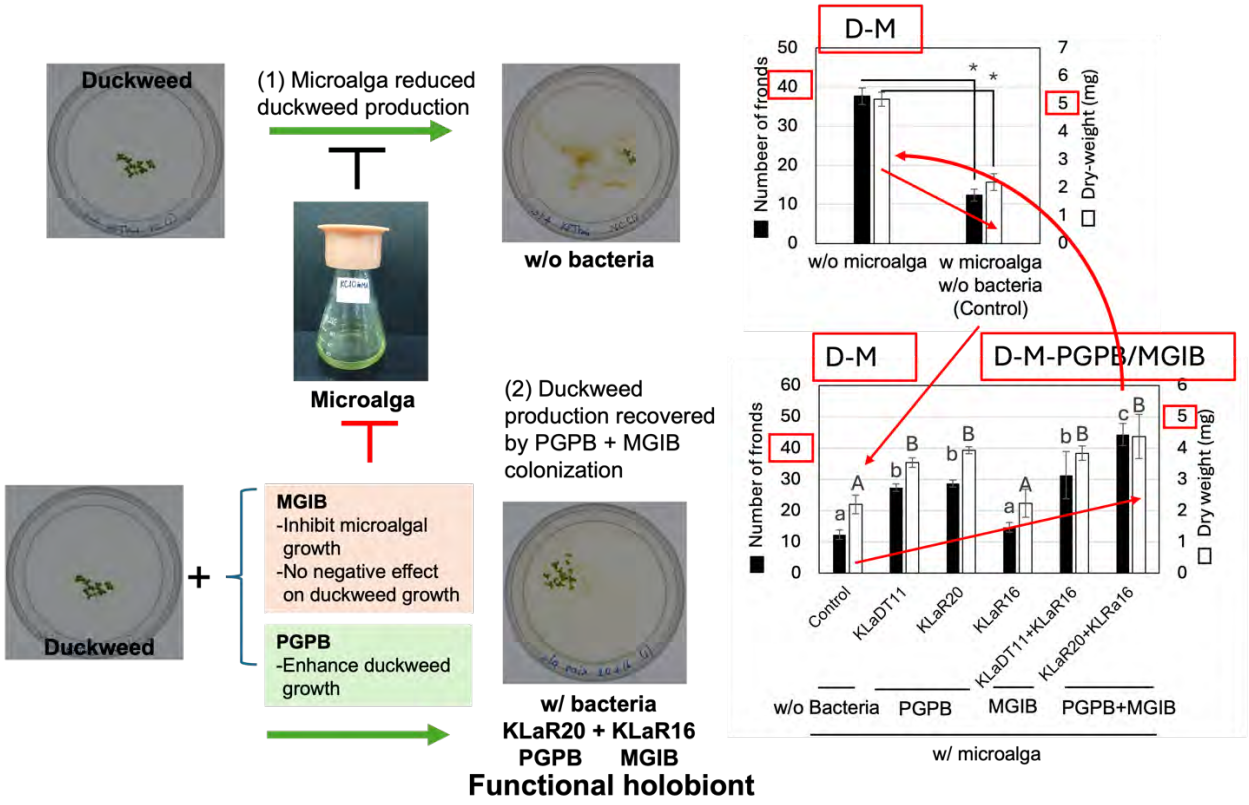


Figure 21. Highlighted achievement of study

In this study, four PGPB strains and two MGIB strains were obtained from 69 duckweed associated bacteria which are indigenous to a food factory wastewater treatment tank. The capacity of these selected bacteria were examined in detail from the aspect of improving duckweed biomass production by both growth promotion and reducing the competition with microalgae. Finally, a highly functional duckweed holobiont was successfully created by mixed association of PGPB and MGIB.

Firstly, the growth of domestic duckweed *Lemma aequinoctialis,* naturally growing in the food factory WWT tank, was shown to be significantly reduced when co-cultured with domestic microalga *Coelestrella* sp. KC10 (D-M in Fig. 21). Secondly, this growth reduction was mitigated in the functional holobiont, assisted by KLaDT11 and KLaR20 (PGPB) or KLaR16 (PGIB) in the triple co-culture. Thirdly, association of both KLaR20 and KLaR16 (PGPB+MGIB) in the quadruple co-culture, the growth of duckweed was perfectly recovered (D-M-PGPB/MGIB in Fig.

21). The best recovery was obtained from the quadruple co-culture of duckweed *L. aequinoctialis*, microalga *Coelastrella* sp., PGPB *Kinneretia* sp. KlaR20 and MGIB *Rhizobium* sp. KlaR16. The recovery of duckweed growth in this quadruple co-culture reached to similar level of the growth of duckweed in the culture without microalga.

The genus *Rhizobium,* which KLaR16 belongs to, generally benefits the soil plants through nitrogen fixing activity. It plays a crucial role in enhancing soil fertility and promoting plant growth. *Rhizobium* sp. KlaR16 was found to have significant growth inhibitory effect on a microalga *Coelastrella* sp. KC10, while no duckweed growth inhibitory effect was observed. Interestingly, the algicidal compounds produced by KLaR16 appeared to be distinct from previously identified substances. This discovery opens new avenues for research into the specific nature and mechanisms of these novel algicidal compounds. Understanding these interactions further could have important implications for agricultural practices, biofertilizer development, and the management of algal blooms, offering potential benefits for both crop productivity and environmental health.

APPENDICES

Appendix A: Media

NF medium

The medium composition per liter includes 396.93 mg of ($CaCl_2 \cdot 2H_2O$), 295.78 mg of $MgSO_4 \cdot 7H_2O$, 136.09 mg of KH_2PO_4, 505.52 mg of KNO_3, 5.56 mg of $FeSO_4 \cdot 7H_2O$, and 18.61 mg of Na_2EDTA. Additionally, the stock solution B contributes micronutrients, including 3.62 mg of $MnCl_2 \cdot 4H_2O$, 2.86 mg of H_3BO_3, 0.22 mg of $ZnSO_4 \cdot 7H_2O$, 0.08 mg of $CuSO_4 \cdot 5H_2O$, and 0.07 mg of MoO_3. The pH of the medium is adjusted to 5

Modified Hoagland medium

The medium composition per liter includes 36.1 mg of KNO_3, 3.87 mg of NaH_2PO_4, 298 mg of K_2SO_4, 103 mg of $MgSO_4 \cdot 7H_2O$, 147 mg of $CaCl_2 \cdot 2H_2O$, 3.33 mg of $FeSO_4 \cdot 7H_2O$, 0.95 mg of H_3BO_3, 0.39 mg of $MnCl_2 \cdot 4H_2O$, 0.03 mg of $CuSO_4 \cdot 5H_2O$, 0.08 mg of $ZnSO_4 \cdot 7H_2O$, and 0.254 mg of $H_2MoO_4 \cdot 4H_2O$. The pH of the medium is adjusted to 7.0 using NaOH to create a balanced environment suitable for plant nutrient absorption and growth.

The MA medium, according to the NIES reference

The composition per liter includes 0.05 g of $Ca(NO_3)_2 \cdot 4H_2O$, 0.1 g of KNO_3, 0.05 g of $NaNO_3$, 0.04 g of Na_2SO_4, 0.05 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of β-Na_2glycerophosphate$\cdot 5H_2O$, 0.005 g of $Na_2EDTA \cdot 2H_2O$, 0.0005 g of $FeCl_3 \cdot 6H_2O$, 0.005 g of $MnCl_2 \cdot 4H_2O$, 0.0005 g of $ZnCl_2$, 0.005 g of $CoCl_2 \cdot 6H_2O$, 0.0008 g of $Na_2MoO_4 \cdot 2H_2O$, 0.02 g of H_3BO_3, and 0.5 g of Bicine. The pH of the medium is adjusted to 8.5 to create an optimal environment for the growth and maintenance of the culture.

R2A medium is composed of 0.5 g/L peptone, 0.5 g/L yeast extract, 0.5 g/L casamino acids, 0.5 g/L glucose,0.5 g/L soluble starch, 0.3 g/L K2HPO4, 0.05 g/L MgSO4·7H2O, and 0.3 g/L sodium pyruvate. The pH of the medium is adjusted to 7.2 using KOH.

The SC medium, as formulated by Kuester and Williams in 1964, includes 1.5 g/L of KNO_3, 0.1 g/L of KH_2PO_4, 0.3 g/L of $MgSO_4 \bullet 7H_2O$, and 0.1 g/L of $CaCl_2 \bullet 2H_2O$. It also contains 0.01 g/L

each of $FeCl_3$ and EDTA, 0.5 g/L of Na_2CO_3, and 0.1 g/L of H_3BO_3. Additionally, the medium incorporates trace elements: 0.002 g/L of $MnCl_2•4H_2O$, 0.0002 g/L of $ZnSO_4•7H_2O$, 0.00005 g/L of $CuSO_4•5H_2O$, and 0.00005 g/L of $Na_2MoO_4•2H_2O$. The pH is usually adjusted to around 7.

Appendix B: Sequences

16S rRNA gene sequences

>KLaS07

GCCTTCGGGCTTAGTGGCGCACGGGTGCGTAACGCGTGGGAATCTGCCCTTGGGTTC
GGAATAACAGTTAGAAATGACTGCTAATACCGGATGATGTCTTCGGACCAAAGATTT
ATCGCCCAAGGATGAGCCCGCGTAGGATTAGGTAGTTGGTGGGGTAAAGGCCTACC
AAGCCGACGATCCTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAG
CCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTCGTAAAGCTCTTTTA
CCCGGGATGATAATGACAGTACCGGGAGAATAAGCTCCGGCTAACTCCGTGCCAGC
AGCCGCGGTAATACGGAGGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGC
GCGTAGGCGGCTACTCAAGTCAGAGGTGAAAGCCCGGGGCTCAACCCCGGAACTGC
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GTGAAATTCGTAGATATTCGGAAGAACACCAGTGGCGAAGGCGACTGACTGGACAA
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CGCAGAACCTTACCAGCGTTTGACATCCCGCGCTATCCAGAGAGATTTGGAGTTCCC
TTCGGGGACGCGGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCTTAGTTGCCATCATTCAGTTGG
GCACTCTAAGGAAACCGCCGGTGATAAGCCGGAGGAAGGTGGGGATGACGTCAAGT
CCTCATGGCCCTTACACGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGAGC
AGCTAGATCGCGAGATCATGCTAATCTCCAAAAGCCGTCTCAGTTCGGATTGTTCTC
TGCAACTCGAGAGCATGAAGGCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG
GTGAATACGTTCCCAGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGGTTC
ACCCGAAGGCGTTG

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18SrRNA gene sequence of *Coelastrella* sp. KC10

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