Report for JICA's KCCP

Project: Development of the Duckweed Holobiont Resource Values towards Thailand BCG Economy (Be-HoBiD)

"Wastewater treatment using duckweed holobiont and isolation and characterization of PGPB from wastewater acclimatized duckweed including re-introduction of PGPB to the duckweed"

By

MR. CHAKRIT BUNYOO

18th February – 3rd March 2024

At University of Yamanashi, Kofu Campus

Supported by

The Knowledge Co-Creation Program under JICA and Science and Technology Research Partnership for Sustainable Development (SATREPS)

Course title

Wastewater treatment using duckweed holobiont and isolation and characterization of PGPB from wastewater acclimatized duckweed including re-introduction of PGPB to the duckweed

Under supervision of Prof. Dr. Tadashi Toyama

Trainee name: Mr. Chakrit Bunyoo, G5 / G3 Be-HoBiD, PhD Student, Faculty of Science, Kasetsart University

Objectives of the course:

- To learn a series of basic techniques and knowledge necessary for studying about the duckweed culture.
- To learn a series of basic techniques and knowledge necessary for studying about the wastewater treatment using duckweed holobiont.
- To learn a series of basic techniques and knowledge necessary for studying about the isolation, characterization, and evaluation of benefit bacteria for wastewater purification and enhancement of duckweed holobiont-based wastewater treatment.

Schedule:

Date	Key activities
Week 1 19 – 23 Feb 2024	• Chemical and duckweed culture media preparation • Duckweed cultivation in culture media and environmental water • Isolation of duckweed-associated bacteria
Week 2 24 Feb – 1 Mar 2024	• Co-culture of PGPB* and duckweed in synthetic wastewater • Chemical component of wastewater analysis

*PGPB = Plant growth promoting bacteria

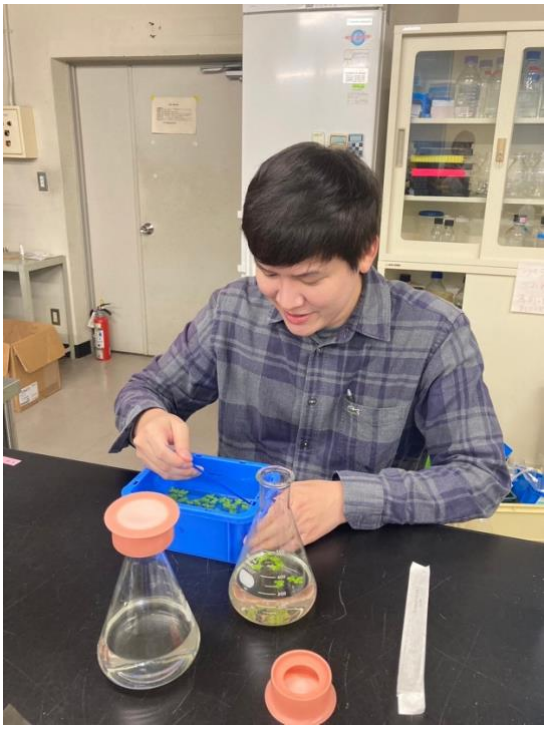
1. Duckweed cultivation in culture media and environment water

Surface-sterile duckweed, *Spirodela polyphiza* was cultivated in either Hoagland or Hutner medium in growth chamber for 10 days. To investigate wastewater remediation by duckweed, *Spirodela* were transferred to flasks containing environmental water (sewage water) collected from Kofu city and then cultivate in growth chamber. The growth of *Spirodela* were observed by counting the number of fonds every day.

Key messages learned

- Due to culture media (Hoagland or Hutner) contains tiny amounts of elements, each composition should be prepared as a concentration of 100X or 1,000X stock solution.
- The chemical composition of environment water may vary depending on its type (eg. sewage from houses, factory wastewater). *Spirodela* may not be tolerant to high concentration of sewage water. Environment water should be diluted to many dilutions before studying.
- Microorganisms can be isolated from *Spirodela* which growing in environmental water.

Preparation of duckweed culture media and other chemical required for the experiments



Transferring *Spirodela* to environmental water collected from Kofu city

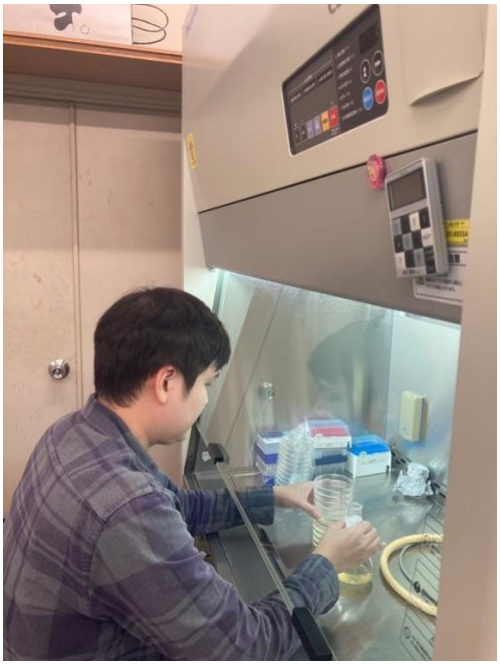
Day 0	Day 7
	
Cultivation of *Spirodala* in environmental water (sewage water)	

2. Isolation of duckweed-associated bacteria

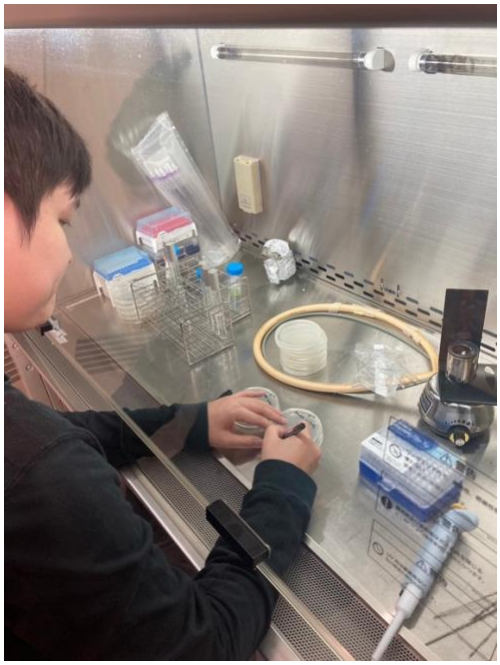
To isolate duckweed-associated bacteria that may facilitate wastewater remediation, 10-day-cultured and surface sterile *Spirodela* was transfer to flasks containing environmental water and then cultivated in a growth chamber for 1 day. Subsequently, 10 -15 fonds of *Spirodela* were rinsed in sterile medium. To capture associated bacteria, duckweed fonds was washed in sterile medium by vortexing and shaking. The mixture was diluted and spread on R2A or 1/10 diluted agar and incubated for 14 days.

Key messages learned

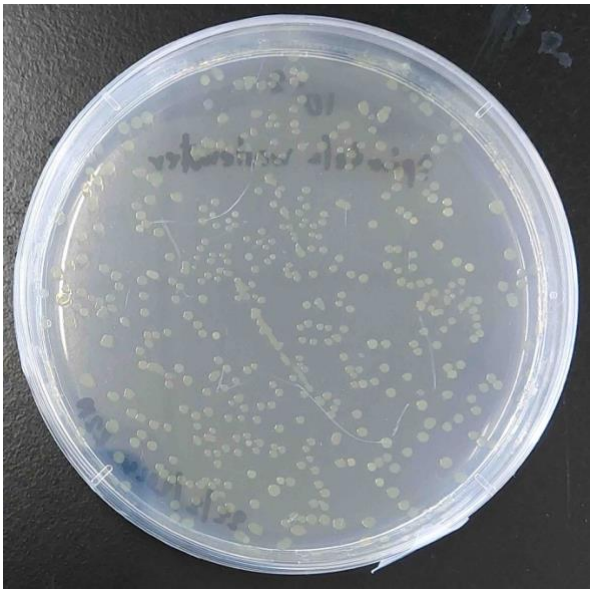
- *Spirodela* may cultivated in environmental water more than a day. Isolation of microorganisms from well-grown *Spirodela* in environmental water will provide potential microbial strains that are useful for remediation.
- Low-nutrient media (eg R2A agar or diluted TS agar) allow slow-growing bacteria to grow which are inhibited by fast-growing microbes in rich nutrient media.



Preparation of R2A medium for bacteria isolation experiment



Isolation of bacteria from *Spirodela* cultivated in environmental water



Isolation of bacteria associated with *Spirodela* on R2A agar incubated at 28° C for 3 days (1/100 dilution)

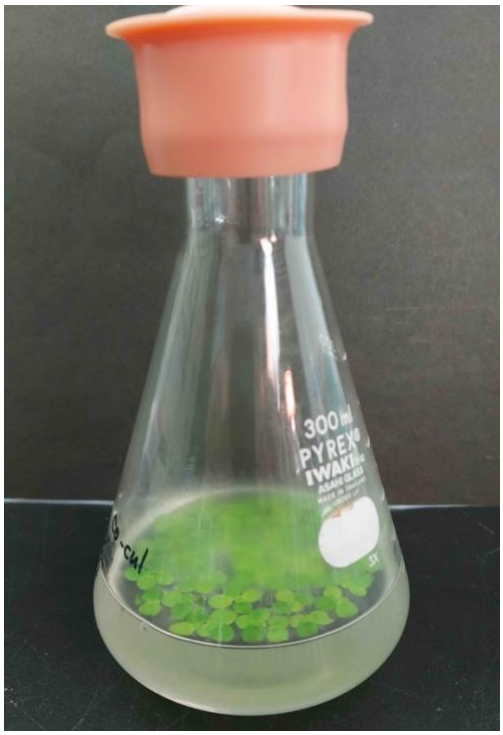
Isolation of bacteria associated with *Spirodela* on R2A agar incubated at 28° C for 3 days (1/1,000 dilution)

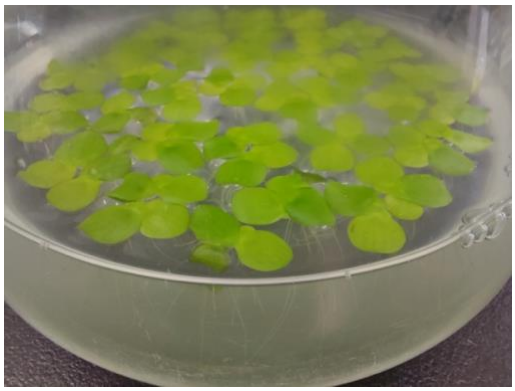
3. Co-culture of PGPB and duckweed in synthetic wastewater

Surfaced sterile *Spirodela* was co-cultivated in PGPB suspension (*Acinetobacter calcoaceticus* P23; OD=0.1 in Hutner medium) for 1 day. Subsequently, five-fond *Spirodela* were transfer to synthetic wastewater and then cultivated in growth chamber for 10 days. Bacterial-free *Spirodela* (without co-culture) was used as a control. *Spirodela* growth was evaluated by monitoring number of fond and investigating wastewater remediation of P23 co-culture with *Spirodela* by analyzing wastewater properties (TOC, total nitrogen and phosphorus concentration) everyday.

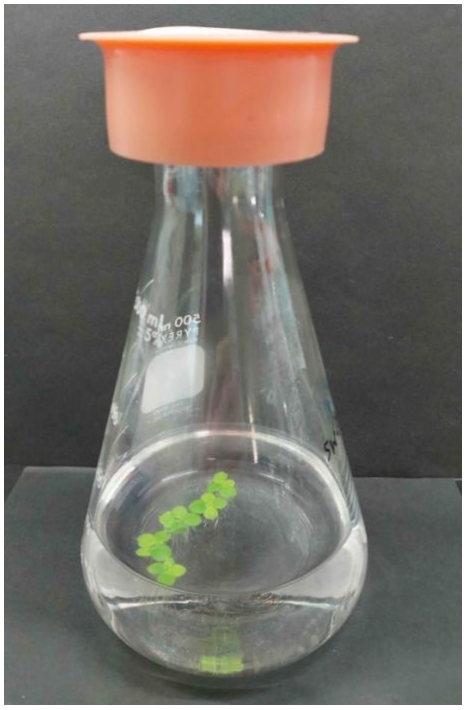
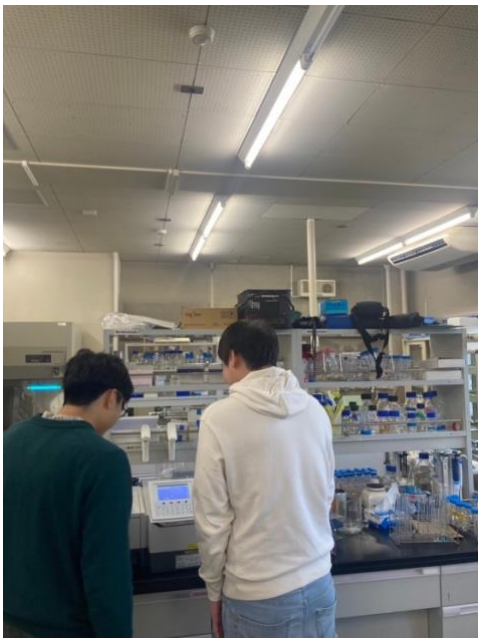
Key messages learned

- Depending on PGPB strain, optimization of preculture time and bacterial cell suspension (OD600) preparation may be needed before co-cultivation with duckweed.
- Synthetic wastewater recipes may vary depending on the objective of the study. Here, the synthetic wastewater contains a source of nitrogen that provided by organic compounds such as meat extract and urea. However, *Spirodela* was not well-tolerant to certain concentrations of synthetic wastewater, thus, further experiment should be involved dilution.
- Apart from wastewater composition analysis, re-isolation of P23 should be done to verify its association to duckweed during remediation process.





Spirodela was co-cultivated with previously reported PGPB (*A. calcoaceticus* P23) in Hutner medium for 1 day before transferred to synthetic wastewater.

Day 0	Day 3
 *Spirodela* associated PGPB (P23) were cultivated in synthetic wastewater.	*Spirodela* was not well-tolerant to certain concentrations of synthetic wastewater, thus, further experiment should be involved dilution.
 Analysis of NH_4-N concentration in wastewater using spectrophotometry	 Analysis of total organic carbon (TOC) using TOC analyzer (Shimadzu)

Culture learning and holiday activities

In addition to academic learning, the training course provided me with the opportunity to learn Japanese culture by exchanging Thai culture to Japanese and other international students in the lab. Also, I had wonderful socialize activities such as having Japanese lunch and dinner with sensei and friends. During the weekend, I enjoyed the fascinating nature of Yamanashi Prefecture especially Mt. Fuji and lake Kawaguchiko.

Acknowledgements

This training course was supported by KCCP under JICA and Science and Technology Research Partnership for Sustainable Development (SATREPS). I would like to extend my appreciation to Professor Dr. Tadashi Toyama, Prof Dr. Kazuhiro Mori and his laboratory members for sharing valuable knowledge with me and for their hospitality during my stay in University of Yamanashi.