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Novel Sustainable Bio-fertilizer Formulated with Mangrove-associated Bacteria Enhances Duckweed Growth and Protein Content

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ABSTRACT

Duckweed is a future food and a source of affordable protein that has the potential to replace animal protein. This study aims to formulate a bio-fertilizer consisting of mangrove-associated bacteria to boost the growth and protein of duckweeds as a sustainable approach to increase plant-based protein yields. The culture-depending technique was performed by using Aleksandrow agar, Pikovskaya's agar, and Jensen agar to screen potassium-solubilizing bacteria, phosphate-solubilizing bacteria and nitrogen-fixing bacteria, respectively, from mangrove soil sediments. Mangrove-associated bacteria that are close to *Acinetobacter radioresistens*, *Brachybacterium paraconglomeratum*, and *Enterobacter cloacae*, which are known as nitrogen-fixing bacteria, *Klebsiella quasipneumoniae*, *Bacillus tropicus*, and *Paenibacillus pasadenensis* known as potassium-solubilizing bacteria, and *Bacillus cereus* and *Bacillus thuringiensis* known as phosphate-solubilizing bacteria were identified through 16S rRNA gene sequencing. After that, three sets of bio-fertilizers were randomly formulated. Each set consisted of nitrogen-fixing bacteria, potassium- and phosphate-solubilizing bacteria, as well as commercial compost as a carrier. These formulated bio-fertilizers were evaluated for plant growth promotion and protein production on duckweed plants under temperatures between 26 and 30°C. The results showed that each set of our formulated bio-fertilizer can increase the

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nitrogen (N), phosphorus (P), and potassium (K), duckweed growth, and protein content when compared to the control group. It indicates that bio-fertilizers formulated with mangrove-associated bacteria and high NPK contents could enhance the growth of duckweed as well as its protein content, which could supply our future plant-based protein sustainably.

Keywords: Bio-fertilizer, duckweed, Lukut river Malaysia, mangrove-associated bacteria, nitrogen-fixing bacteria, phosphate-solubilizing bacteria, potassium-solubilizing bacteria

INTRODUCTION

Duckweed, the world's tiniest flowering plant, is gaining attention for its rich nutrient content and versatile applications in various industries, including animal feed, aquaculture, health supplements, bio-fertilizers, biofuels, and emerging human food products (de Beukelaar et al., 2019; Naseem et al., 2020). Its protein content, ranging from 20 to 30%, surpasses that of cereals, making it a sustainable and cost-effective source of protein (Appenroth et al., 2018; Yahaya et al., 2022). Duckweed's primary protein, ribulose-1, 5-bisphosphate carboxylase (RuBisCO), is an excellent source of essential amino acids and possesses favorable properties as a functional food (Chakrabarti et al., 2018). This aquatic green plant thrives in specific conditions, including an optimal temperature range of 17.5 to 30°C and ample sunlight, while also requiring supplemental nutrients like nitrogen (N), phosphorus (P), and potassium (K) for growth (Hasan & Chakrabarti, 2009). Additionally, duckweed is a phytoremediation plant capable of extracting pollutants, including metals and radionuclides, from wastewater and accumulating them in its tissues (Kamyab et al., 2017; Radulovic et al., 2018).

Therefore, to ensure the safety of duckweed as a food source for humans, it must be cultivated in a controlled environment, such as axenic culture, which can produce a substantial quantity of duckweed with minimal bacterial presence and fewer contaminants. However, setting up a laboratory for large-scale duckweed production is expensive. Hence, an open-air system that harnesses direct sunlight and minimizes nutrient usage becomes necessary to make duckweed a commercially viable future food option for low-income communities. In our previous study (Yahaya et al., 2022), we found that adding N:P:K fertilizer to the water containing duckweed served as the most effective growth medium, resulting in a substantial yield of duckweed. However, in the long-term condition, the commercial compost medium exhibited an even greater proliferation of duckweed. Furthermore, combining the commercial compost with the N:P:K fertilizer in water showed the potential to enhance the growth of duckweed and its protein content (Yahaya et al., 2022). However, excessive and long-term chemical application could cause negative effects on the environment and human health.

Bio-fertilizers are fertilizer preparations containing living cells or dormant cells of

effective strains of microorganisms that aid crop plant nutrient uptake through rhizosphere interactions (M. Kumar et al., 2022; Sarbani & Yahaya, 2022). The development of bio-fertilizers has become an important part of agriculture, which can help improve soil fertility and produce disease-resistant, stress-resistant plants with better nutrient uptake (Liu et al., 2023; Sahoo et al., 2018). Bio-fertilizers help maintain a nutrient-rich soil environment by facilitating processes such as N fixation, P and K solubilization, mineralization, synthesis of plant growth regulators, antibiotic production, and organic matter degradation in the soil (Mali & Attar, 2021; Shahwar et al., 2023). Bio-fertilizers containing *Azobacter* sp. and *Azospirillum* sp. bacteria exhibit growth-enhancing and essential oil production-increasing effects on basil (*Ocimum basilicum* L.) plants (Tahami et al., 2017), while bio-fertilizers containing *Rahnella aquatilis* and *Variovorax paradoxus* prove to be effective in promoting the growth of *Crocus sativus* (Saffron) plants and enhancing the production of secondary metabolites (Chamkhi et al., 2023) by facilitating plant-beneficial activities such as P solubilization, siderophore production, and auxin production.

Mangroves, found in the transitional zone between land and sea, are valuable sources of biotechnological resources like microbial cellulase, endophytes, and salinity-tolerant glucanase enzymes (Behera et al., 2017; Castro et al., 2018; dos Santos Goncalves et al., 2020). These ecosystems host 27 true mangrove species across 10 selected systems (Sreelekshmi et al., 2020), recognized for their carbon-rich nature (Adame et al., 2022; Gu et al., 2022; Morrissette et al., 2023). They harbor diverse microbial life, including rhizosphere bacteria with the potential to stimulate plant growth by producing phytohormones (Pham et al., 2022; Smaill et al., 2010; Talaat, 2019), mitigating environmental stressors (Chandra et al., 2021; David & Rostkowski, 2020; Muñoz-García et al., 2022; Ramakrishna et al., 2020), and preventing pathogen-induced diseases (Cheng et al., 2021; Gomez-Aparicio et al., 2022; Zhou et al., 2023).

There are limited studies regarding the effect of beneficial microbes on the growth and performance of duckweed cultivated in an open-air system. Hence, in this study, the integration of mangrove-associated microbes into our formulated bio-fertilizer was demonstrated, significantly boosting both duckweed growth and protein yield, underscoring the potential of duckweed as a viable future food option. In the past decades, many researchers have formulated various bio-fertilizers with extensive and advanced effects on crop growth to preserve the environment and ecosystem (Sarbani & Yahaya, 2022). Therefore, the bio-fertilizer formulation in this study aims to benefit plant and crop productivity.

MATERIALS AND METHODS

Sample Collection

Soil samples with a depth of 5 cm were collected at the freshwater riverine mangrove located at Lukut River, Negeri Sembilan,

Malaysia (coordinate 2° 35' 25.2342" N, 101° 48' 9.831" E) on 20th February 2020, during the low tide at three sampling points, with temperature ranging from 26.1 to 30°C. Soils 1 and 2 were collected in the 5 m x 5 m area populated by *Rhizophora mucronata* and *Avicennia officinalis* trees. Meanwhile, Soil 3 was collected from the riverbank near *the Nypa fruticans* tree and 50 m distance from Soil 1 and Soil 2. The samples were placed in a 50 ml tube and stored at -80°C until ready for analysis.

Screening of Nitrogen-fixing Bacteria, Phosphate-solubilizing Bacteria, and Potassium-solubilizing Bacteria

Soil samples were individually suspended in 0.85% of sodium chloride (NaCl, Merck, USA) (Yahaghi et al., 2018) for inoculation into Jensen agar (HiMedia Laboratories, India), Pikovskaya`s agar (HiMedia Laboratories, India), and Aleksandrow agar (HiMedia Laboratories, India). Jensen agar, Pikovskaya's agar, and Aleksandrow agar were used to screen and culture nitrogen-fixing bacteria, phosphate-solubilising bacteria, and potassium-solubilising bacteria, respectively. The Jensen medium was aerobically incubated at 27°C for 48 hr, while Aleksandrow agar and Pikovskaya agar were incubated at 35°C for 7 days after spreading the soil suspension.

DNA Extraction and 16S rRNA Gene Sequence Analysis

Bacterial cultures were prepared by proliferating a single colony from Jensen agar, Pikovskaya's agar, and Aleksandrov agar in a nutrient broth at 28°C with 200 rpm shaking and incubated for 48 hr to obtain the optical density at 600 nm. DNA from the bacterial cultures was extracted using the PROMEGA Wizard® Genomic DNA Purification Kit (USA). The purity, integrity, and quantity of extracted DNA were determined using agarose gel electrophoresis and a nanodrop (BioDrop, Thailand).

The PCR amplification of the 16S rRNA gene was performed by using a universal primer set of 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (GGT TAC CTT GTT ACG ACT T-3') (Zhou et al, 2009). The components for the PCR reaction were 12.5 µl of Go Taq® Green Master Mix 2X (Promega, USA), 0.5 µl of each forward and reverse primer (final concentration of each primer is 0.5 µM), 5 µl of DNA template (approximately 250 ng), and 6.5 µl of nuclease-free water. Amplification was performed on a Mastercycler nexus PCR cycler (Bio-Rad T100™ Thermal Cycler, USA), a program to perform an initial denaturation at 94°C for 5 min; 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C; and the final extension at 72°C for 10 min, followed by cooling to 4°C until the sample is recovered (Fatima et al., 2011). Amplicons were then visualized with a UV transilluminator after resolving 5 µl of the products in the 1% agarose gel and staining with GelRed (Biotium, USA) Lastly, purified PCR products were subjected to DNA sequencing at Next Gene Scientific Sdn. Bhd. (Malaysia).

The raw sequences of the 16S rRNA gene were analyzed and compared with sequences available in the National Centre for Biotechnology Information (NCBI) sequence database (MT367790, MN833494, MT214134, MT124566, KY908508, KY908503, MZ317924, ON222566, ON000559, KM100367, NR113987, OM510015, MW363212, KX216390, and OM351571). Then, MEGA 11 software performed the CLUSTALW alignment on the sequence. The maximum likelihood technique based on the Tamura-Nei model was applied to infer evolutionary history.

Development of Bio-fertilizers

At the beginning of the study, commercial compost (Growmate Eazy Mix, MR. DIY, Malaysia) was used as a carrier material for developing bio-fertilizer. The commercial compost was packed in autoclave polythene covers and sealed using an electric sealer. It was then sterilized at 121°C for 20 min to destroy contaminated microbes.

Nine bacterial isolates were cultured in nutrient broth (Oxoid, United Kingdom) at 28°C with shaking at 200 rpm for 48 hr until the optical density at 600 nm (OD) reached the value of 0.3 (10^7 CFU). The bacterial cultures were centrifuged at 11,057 × *g* at 4°C for 15 min and resuspended in sterilized nutrient broth. The process was repeated twice. Then, the pre-sterilized commercial compost was inoculated with bacteria at a ratio of bacteria pellet to pre-sterilized commercial compost 1:50, as recommended by Stella et al. (2019). Mangrove-associated microbes were randomly divided into three sets, each consisting of nitrogen-fixing, phosphate-solubilizing, and potassium-solubilizing bacteria. Set A bio-fertilizer consists of *A. radioresistens*, *K. quasipneumonia*, and *B. cereus*; Set B bio-fertilizer consists of *B. paraconglomeratum*, *B. cereus*, and *B. tropicus*; and Set C bio-fertilizer consists of *E. cloacae*, *P. pasadenensis*, and *B. thuringiensis*. The mixture was manually shaken by hand until the microbial inoculum was uniformly distributed in the commercial compost. Microbial inoculum and autoclaved commercial compost were packed into the polythene bag and immediately sealed. The package containing pre-sterilized commercial compost without bacterial inoculation was used as a control. All the packages were then incubated at 30°C for 7 days. After the 7th-day interval, formulated bio-fertilizers were tested for bacteria survivability and NPK content.

Bacterial Survivability Experiment

One gram of each sample was mixed with 9 ml of sterile distilled water in a ratio of 1:9 and allowed to mix thoroughly in a shaker for 1–2 hr. The suspension was serially diluted before being dispensed into the agar plate and incubated at 35°C for 24 hr. The number of bacterial growths on the plate was calculated using Equation 1.

$$\text{Population density (CFU/ml)} = \frac{\text{Number of colonies X Dilution factor}}{\text{Volume of culture plate}}$$

[Equation 1]

N, P, and K Determination in Mangrove Soils and Formulated Bio-fertilizer

After seven days of incubation, the formulated bio-fertilizers were analyzed for their N, P, and K contents. N content was measured using the Kjeldahl method. Meanwhile, P and K were analyzed using X-ray fluorescence (XRF).

Three samples of mangrove soil (Soils 1, 2, and 3) and three samples of formulated bio-fertilizers (Sets A, B, and C) and control were used for this analysis. The analysis was performed in triplicates. For the Kjeldahl method, a digestion tube mixed 1 g of soil samples or formulated bio-fertilizer with 10 ml of sulphuric acid (H_2SO_4, Merck, USA). A Kjeldahl tablet was then added as a catalyst to the sample solution. Then, the sample solution was digested using Gerhardt KJEDAHLTEM (Germany) for 60 to 120 min until the digestion solution turned clear green. After that, the digestion tubes were allowed to cool and placed in Gerhardt VAPODEST 500 (Germany) for titration and distillation processes. Finally, the N content of the samples was calculated based on a volumetric standard solution (Yahaya et al., 2022).

In XRF determination (S8 TIGER, Bruker, Germany), three soil samples and three sets of formulated bio-fertilizer and the control were dried, homogenized and sieved to get smaller particle sizes. Plastic cups lined with a 3.6 m thick Mylar® polymer were used to hold samples and placed inside the XRF analyzer. The X-ray tube operated at 15 W with a 50 kV generator in operation conditions. The spot size of the sample was typically 10 mm × 14 mm. The detector has a high resolution of 135 eV.

Duckweed (*Lemna minor*) Growth Experiment

A duckweed *L. minor* growth experiment was conducted to evaluate the efficiency of formulated bio-fertilizers. Ten fronds of *L. minor* plants were sterilized using 70% ethanol (R&M, Switzerland), bleach (CLOROX®, USA), and sterilized distilled water before being transferred into a container with a size of 122 mm x 173 mm x 62 mm that contains 200 ml of water and 25 g of control medium and three sets of formulated bio-fertilizer (Sets A, B, and C). In this experiment, duckweed plants were grown in a greenhouse with a temperature range between 26 to 30°C. The number of duckweed fronds was recorded every two days for 15 days (Figure 1).

Quantification of Protein Content in Duckweed Fronds

Fresh *L. minor* was dried in an oven at 65°C for 24 hr and ground into a fine powder. Dried duckweed was then soaked in sterilized distilled water at a ratio of 1:10 (1 g of dried duckweed in 10 ml of distilled water) overnight to allow cell expansion before applying physical enforcement to break the cells. Then, the soaked material and water were microwaved at 100 W for 15 min using a home-based microwave. The microwaved duckweed was filtered to separate the solids and the green juice. Protein content in the green juice was quantified using the Bradford reagent

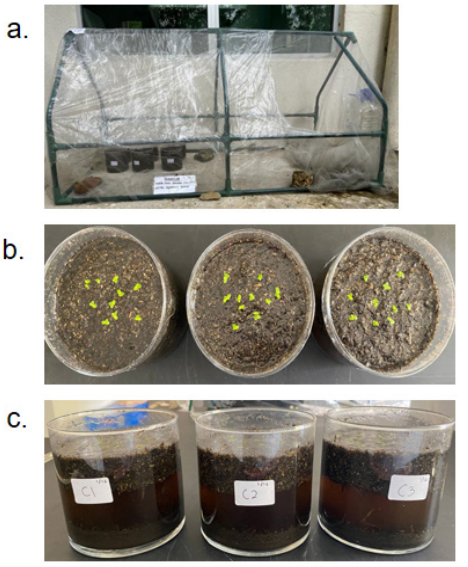

Figure 1. Experimental design for duckweed growth. (a) Greenhouse setup to grow duckweed, (b) top view, and (c) from the view of an experimental glass jar containing formulated bio-fertilizer

(Bio-Rad, USA) and measured using a UV spectrophotometer (Varian Cary 50 UV-Visible Spectrophotometer, Australia). The protein concentration was measured by using Equation 2.

$$y = mx + c \quad \text{[Equation 2]}$$

where, y = absorbance at 595 nm; x = protein concentration.

Statistical Analysis

All collected data on the elemental analysis, duckweed growth, and duckweed protein content were analyzed using MINITAB16 software (Minitab Ltd., Unted Kingdom) and analysis of variance (ANOVA) available in the software. Mean difference analysis was conducted using Tukey's method, with significant differences defined between the sample means ($P < 0.05$).

RESULTS

Morphology of the Bacteria Colonies Isolated from the Mangrove Soil

As the initial step in identifying the species of each bacterial isolate, the morphological characteristics of each were observed and recorded. The colonies of the selected isolates were characterized based on their shape, texture, and appearance. The nitrogen-fixing ability of isolated strains was determined on Jensen agar. Visible colony growth on the agar indicated positive nitrogen fixation, while bacteria unable to fix nitrogen did not grow on this medium. Specifically, J1, J2, and J3 were identified as nitrogen-fixing bacteria, exhibiting light yellow, yellow, and milky white colonies on Jensen agar.

On Pikovskaya's medium and Aleksandrow agar, the abilities of isolated strains to solubilize inorganic phosphorus and potassium were screened, respectively, using A1, A2, and A3 for potassium-solubilizing bacteria, and P1, P2, and P3 for phosphate-solubilizing bacteria. The isolates' potassium- and phosphate-solubilizing activities were qualitatively evaluated by the formation of halos (clear zones) around the colonies growing on Pikovskaya's medium. The bacterial colonies corresponding to Jensen agar, Aleksandrow agar, and Pikovskaya's agar are shown in Figure 2.

Microbial Identification Using 16S rRNA Gene Sequencing Analysis

The phylogenetic tree was constructed using Mega 11 software with the maximum-

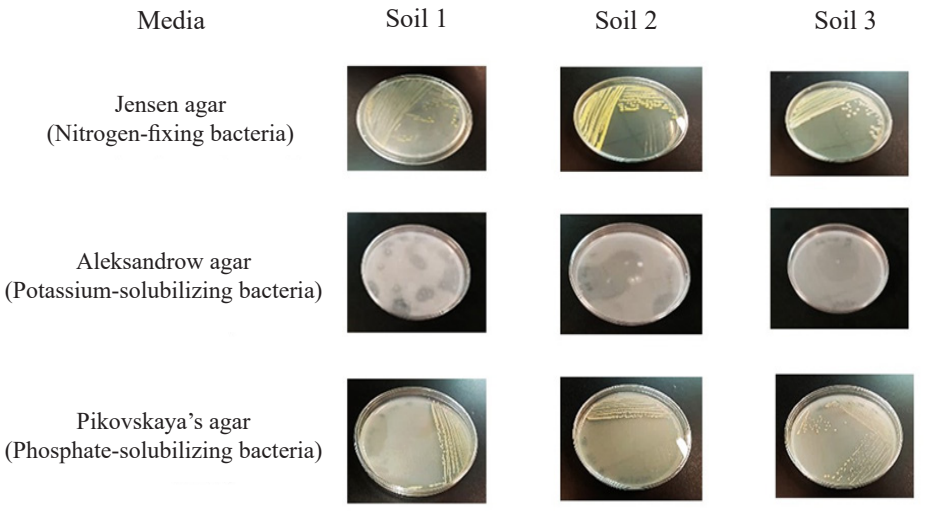

Figure 2. Morphologies of bacteria colonies isolated from soil 1, 2 and 3 on Jensen agar, Aleksandrow agar, and Pikovskaya's agar. Jensen agar, Pikovskaya`s agar, and Aleksandrow agar were used to screen and culture nitrogen-fixing bacteria, phosphate-solubilizing bacteria, and potassium-solubilizing bacteria, respectively

likelihood (Tamura-Nei model) analysis. The bootstrap values are presented as percentages of 1,000 replications at branch points. Subsequent Basic Local Alignment Search Tool (BLAST) analysis unveiled that all the sequences originated from bacteria commonly found in Earth's diverse habitats, including soils.

Taxonomic identification was carried out by analyzing the 16S rRNA gene sequence amplified from nine bacterial isolates. This analysis revealed nine distinct bacterial phylotypes exhibiting sequencing similarities ranging from 98 to 100% (Table 1). Interestingly, these phylotypes belonged to six genera: *Bacillus*, *Acinetobacter*, *Brachybacterium*, *Enterobacter*, *Klebsiella*, and *Paenibacillus*.

Of particular interest were three bacterial strains isolated from Jensen agar: (1) *Acinetobacter radioresistens* (J1), (2) *Brachybacterium paraconglomeratum* (J2), and (3) *Enterobacter cloacae* (J3). Notably, *A. radioresistens* strain OsEp Plm 15B15 (MT367790.1) demonstrated a remarkable 99.25% similarity with J1 isolate. Similarly, *B. paraconglomeratum* strain AS53 (MT214268.1) exhibited a high similarity value of 99.65%, closely matching J2. Likewise, *E. cloacae* strain SUK83 (KY908479.1) shared a significant similarity of 99.63% with J3 (Figure 3).

Meanwhile, the phylogenetic analysis of the 16S rRNA genes from three different strains isolated from Aleksandrow agar revealed their predicted identities as *Klebsiella quasipneumoniae* (A1), *Bacillus tropicus* (A2), and *Paenibacillus pasadenensis* (A3) with a sequence identity of 98–99%. The first bacterium isolated from Aleksandrow agar, *K. quasipneumoniae* strain cjy02 (MN177200.1), exhibited an impressive 99.70% similarity with A1. Additionally, *B. tropicus* strain WSB89

Table 1
A list of bacterial species obtained from soil samples 1, 2, and 3, and is provided through 16S rRNA sequencing

Samples	Identity	Query cover	Percent identity	Accession number
J1	*Acinetobacter radioresistens* strain OsEp Plm 15B15	100%	99.25%	MT_367790.1
J2	*Brachybacterium paraconglomeratum* strain AS53	99%	99.65%	MT_214268.1
J3	*Enterobacter cloacae* strain SUK83	99%	99.63%	KY_908479.1
A1	*Klebsiella quasipneumoniae* strain cjy02	100%	99.70%	MN_177200.1
A2	*Bacillus tropicus* strain WSB89	98%	98.67%	OP_630954.1
A3	*Paenibacillus pasadenensis* strain zp09	100%	99.33%	KM_100367.1
P1	*Bacillus cereus* strain R1	99%	99.34%	MN_213372.1
P2	*Bacillus cereus* strain E1	100%	100%	OP_597695.1
P3	*Bacillus thuringiensis* strain PDKV Bt I-3	100 %	99.74%	OP_209990.1

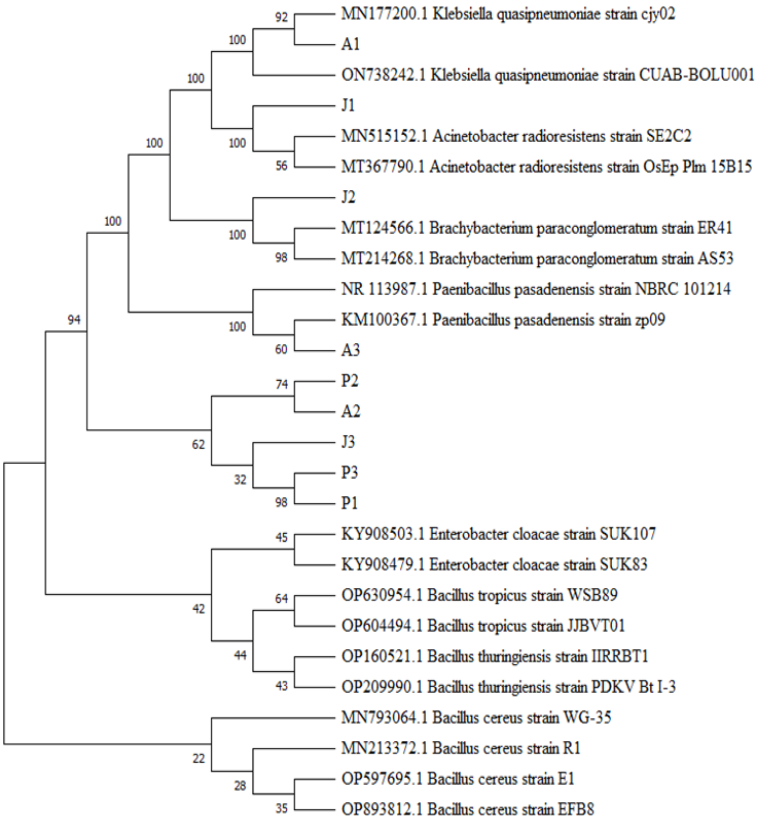

Figure 3. A phylogenetic bacterial species tree was isolated from three agar (Aleksandrow agar, Jensen agar, and Pikovskaya agar). The DNA of the bacteria was extracted, which then being sequenced with 16S rRNA sequencing and the sequences with National Center for Biotechnology Information

(OP630954.1) displayed a sequence similarity of 98.67% with A2, while *P. pasadenensis* strain zp09 (KM100367.1) showed a significant 99.33% similarity with A3.

Furthermore, the 16S rRNA gene sequence analysis identified two strains from Pikovskaya agar, similar to *B. cereus* (P2) and *B. thuringiensis* (P3). Intriguingly, one of the strains isolated from Pikovskaya agar demonstrated similarity with A2, which was earlier predicted to be *B. tropicus* (Figure 3). These findings contribute valuable insights into the phylogenetic relationships and taxonomic diversity of the identified bacterial strains, paving the way for further investigation into their ecological roles and potential applications in various scientific fields.

Bacterial Survivability in Formulated Bio-fertilizer

Total plate counting can be used to test the bio-inoculants in biofertilizers for their survivability. The microbial survivability results indicate that the bio-fertilizer sets A, B, and C had higher counts of viable microbes compared to the control group (Table 2).

Table 2
Total plate count in control and biofertilizer Sets A, B, and C

Samples	Mean $\log_{10}$ (cfu/g)
Control	6.20×10^6a
Set A	8.80×10^6b
Set B	8.00×10^6b
Set C	10^6b

Note. Means that do not share a letter between samples are significantly different ($P < 0.05$) based on Tukey's 95% simultaneous confidence intervals

N, P, and K Elements in Mangrove Soils and Formulated Bio-fertilizer

Next, the isolated bacteria were grouped into three sets of bio-inoculants, each exhibiting N fixation, P and K solubilization activities in three sets of formulated bio-fertilizers. Therefore, this study analyzed N, P, and K content in mangrove soils, as well as three sets of formulated bio- and compared them with the control (Figure 4). The analysis of chemical element content in mangrove soils revealed that the smallest trace element was P, followed by N. K element was the most abundant element in mangrove soil and showed a slight increase ($P = 0.003$) in Soil 2 compared to Soils 1 and 3.

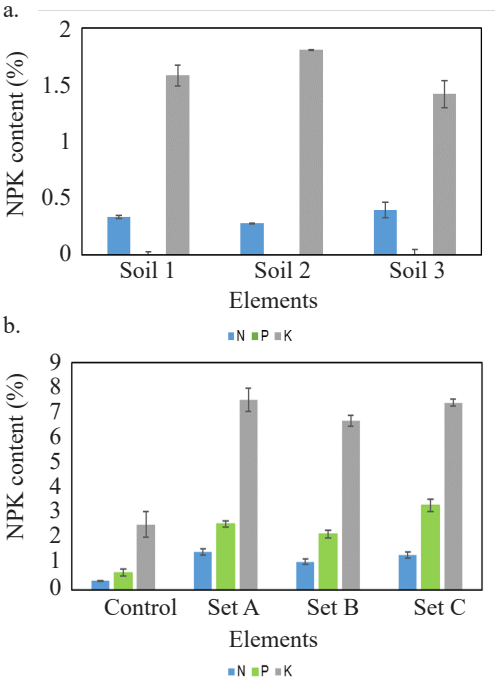

Figure 4. Nitrogen (N), phosphate (P), and potassium elements in (a) mangrove soils and (b) control and formulated bio-fertilizer Sets A, B, and C

Note. All data are mean±standard deviations (n = 3). Means that do not share a letter between samples are significantly different ($P < 0.05$) based on Tukey's 95% simultaneous confidence intervals

Meanwhile, from the analysis of chemical element content in our formulated bio-fertilizers, it was found that the K element was also the most abundant in all three sets of formulated bio-fertilizers and showed a significant increase (P = 0.000) compared to the control, which is similar to N and P elements. However, P content was slightly increased in formulated bio-fertilizer Set C compared to Sets A and B. At the same time, the percentage of N element was similar in Sets A and C. The presence of mangrove-associated bacteria in formulated bio-fertilizer is known to reflect the amount of N, P, and K content.

Effect of Formulated Bio-fertilizer on the Growth of Duckweed Plants

The effectiveness of formulated bio-fertilizer sets A, B, and C compared to the control on the growth of duckweed plants is presented in Figure 5. The duckweed growth was evaluated in terms of the number of duckweed fronds. Relative to the control, formulated bio-fertilizer sets A, B, and C exhibited a significant increase (P = 0.00) in duckweed growth from Day 3 until Day 15. The result of this analysis also showed that formulated bio-fertilizer Set C is the most effective medium to boost duckweed growth compared to Sets A and B. Figure 6 shows the impact of formulated bio-fertilizers sets A, B, and C on duckweed fronds on Day 15, contrasting the results with Day 0 and the control group. The results obtained from this experiment indicate that duckweed growth is correlated with the chemical element content in formulated bio-fertilizer Set C (*E. cloacae*, *P. pasadenensis*, and *B. thuringiensis*), which showed an increase of P content compared to Sets A and B.

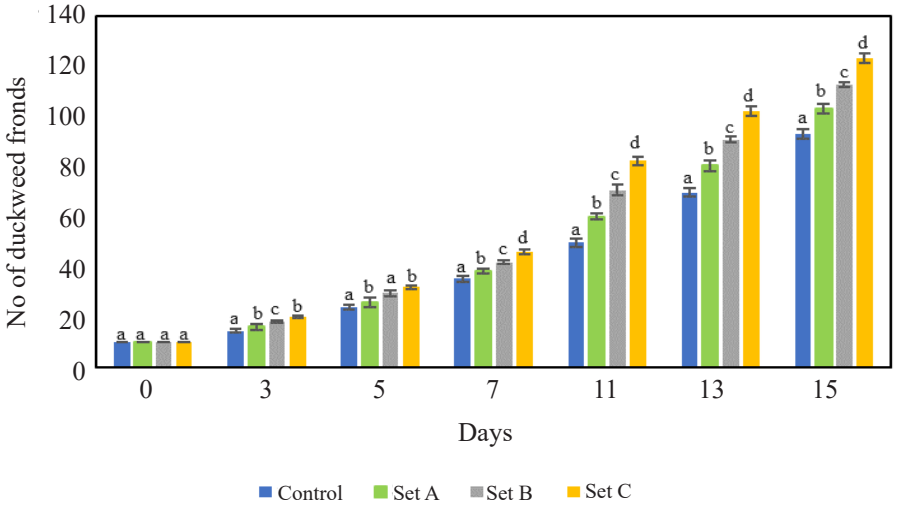

Figure 5. The growth of duckweed in control and formulated bio-fertilizer Sets A, B and C in 15 days

Note. All data are mean±standard deviations (n = 3). Means that those that share a letter between samples are significantly different (P < 0.05) based on Tukey's 95% simultaneous confidence intervals

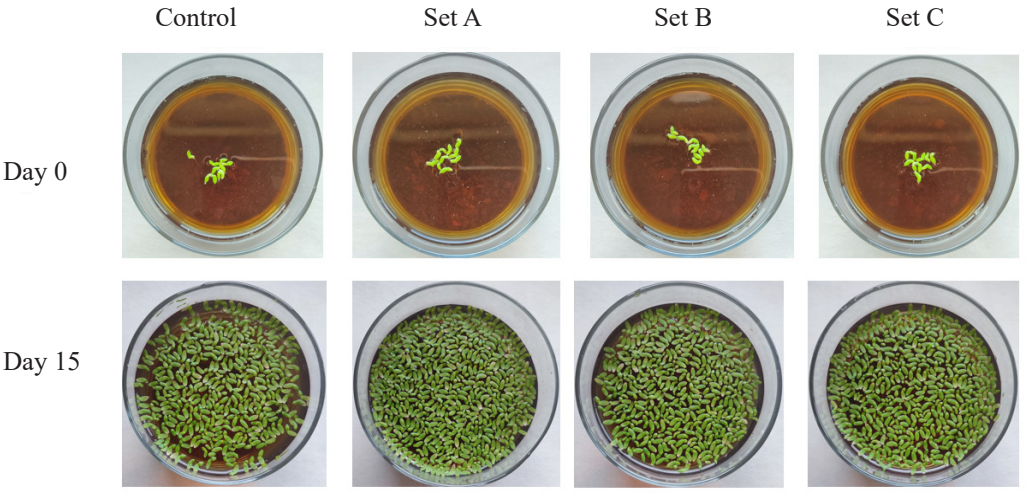

Figure 6. The growth of duckweed on 0 and 15 days in control and formulated bio-fertilizer Sets A, B, and C, respectively

Effect of Formulated Bio-fertilizer on the Duckweed Protein Content

The effectiveness of formulated bio-fertilizer sets A, B, and C compared to the control on the duckweed protein amount is presented in Figure 7. Overall, formulated bio-fertilizer sets A, B, and C displayed an increasing trend ($P = 0.00$) in the amount of protein in duckweed plants compared to the control. It indicates that the presence of mangrove-associated microbes in the formulated bio-fertilizer used as a duckweed growth medium is known to influence the amount of protein in this plant.

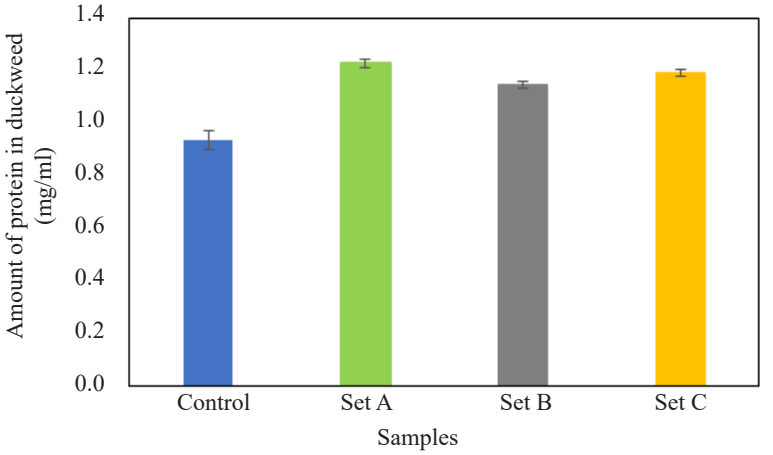

Figure 7. Amount of protein harvested from duckweed after 15 days grown in control and formulated bio-fertilizer Sets A, B, and C in 15 days

Note. All data are mean±standard deviations (n = 3). Means that those that share a letter between samples are significantly different (P <0.05) based on Tukey's 95% simultaneous confidence intervals

DISCUSSION

N, P, and K Content Analysis

Bio-fertilizer has been recommended as a more environmentally friendly substitute for conventional chemical fertilizers and pesticides. It has been established that rhizosphere soil has a rich source of plant growth promoting bacteria (PGPB) (Iniesta-Pallarés et al., 2023; Pii et al., 2015). In this study, bacteria were isolated from the mangrove soil. Nine bacterial strains were identified according to the 16S rRNA gene sequencing and grouped into three bio-fertilizer sets (Set A containing *A. radioresistens*, *K. quasipneumonia*, and *B. cereus*; Set B contains *B. paraconglomeratum*, *B. cereus*, and *B. tropicus*; and Set C containing *E. cloacae*, *P. pasadenensis*, and *B. thuringiensis*) with each having the ability to fix nitrogen, solubilize potassium and phosphorus, grown on the duckweed.

The results of bacterial screening on Jensen agar and microbial identification by using 16S rRNA analysis demonstrated that *A. radioresistens* (J1), *B. paraconglomeratum* (J2), and *E. cloacae* (J3) exhibit nitrogen-fixing capabilities. Some types of bacteria and cyanobacteria are essential to the nitrogen cycle as they can reduce or fix atmospheric nitrogen gas (N_2), rendering the element accessible to other organisms, including plants and animals (Saha et al., 2017). Soil microbes, influenced by soil nitrogen availability, impact the terrestrial carbon cycle through decomposition and the formation of soil organic matter (SOM) (Cotrufo et al., 2013). High-N substrates lead to rapid breakdown by bacteria, resulting in substantial microbial product accumulation and stable SOM creation. Cycles of extracellular enzyme production, primarily controlled by community composition, serve as markers for microbial nutrient demand, soil nutrient cycling, and soil respiration (Zechmeister-Boltenstern et al., 2015). Meanwhile, *B. cereus* (P2) and *B. thuringiensis* (P3) exhibit phosphate-solubilizing capabilities. Among the essential macronutrients, P plays a crucial role in plants' biological development and growth (Soetan et al., 2009). P solubilizers are crucial in solubilizing soil phosphorus by producing secondary metabolites, including enzymes such as acid phosphatases and phytases. Additionally, they generate phytohormones like indole-3-acetic acid (IAA) and siderophores, which further contribute to increased plant yield (A. Kumar et al., 2014; Kour et al., 2020).

Furthermore, *K. quasipneumoniae* (A1), *B. tropicus* (A2), and *P. pasadenensis* (A3) exhibit potassium-solubilizing capabilities. K is vital for plant development and growth as it involves numerous metabolic processes. It plays a crucial role in the plant's ability to withstand drought and diseases (Billore et al., 2009). Additionally, it contributes to starch production, controls root growth, regulates stomata movement within plant cells, activates enzymes, maintains cell turgor, and transports sugars as well as starches (Meena et al., 2014), ultimately influencing plant quality. Rhizospheric bacteria, known as potassium-solubilizing bacteria, have the ability to convert insoluble potassium into

soluble forms through acidolysis, chelation, exchange reactions, and complexation (Meena et al., 2015). Simultaneously, they decompose organic matter and crop residues to promote plant growth and increase yield (Etesami et al., 2017). Among soil microorganisms, potassium-solubilizing bacteria play the most significant role in plant potassium cycling (Sun et al., 2020). In the soil, K is found at a concentration of 1–2% as a soluble compound, with the other 90%+ present as insoluble rocks and silicate minerals (e.g., mica, muscovite, feldspar, microline, and orthoklas). In this way, the latter type is mostly inaccessible to plants (Parmar & Sindhu, 2013). Bacteria play a vital role in maintaining soil fertility by secreting organic acid during the degradation of silicate minerals, which release K, silicon, and aluminum.

Based on the results obtained from this study, Set C appears to be much better than Sets A and B as a bio-fertilizer option, as Set C showed an increase in the content of N, P, and K when compared to Sets A, B, and Control. Set C contains *E. cloacae*, *P. pasadenensis*, and *B. thuringiensis* in a formulated bio-fertilizer. The main advantage of Set C is its ability to enhance the P content in the soil. Several studies (Ali & Pati, 2023; Ansari et al., 2023; A. Kumar et al., 2014) have reported that *E. cloacae*, one of the bacteria in Set C, is a potent inorganic P solubilizer and can significantly increase P acquisition in plants. Moreover, *E. cloacae* exhibit a variety of growth-promoting actions, including P and K solubilization, as well as N fixation (Chin et al., 2017; Deepa et al., 2010; Ramesh et al., 2014). These actions improve plant health and soil fertility (Ghiglione et al., 2021). *Paenibacillus pasadenensis*, another component of Set C, has also been shown to be involved in the solubilization of soil phosphorus, the production of phytohormones and antimicrobial metabolites (Govindasamy et al., 2011), and is also known to be involved in the fixation of atmospheric nitrogen and the uptake of micronutrients, further benefiting plant growth (Grady et al., 2016). *Bacillus* sp. was also considered an effective nitrogen-fixing bacteria (Awasthi et al., 2011; Zhang et al., 2023). Multiple PGPB favorable characteristics were found in the genus *Bacillus*, including P solubilization and participation in the N cycle (Stegelmeier et al., 2022).

This study also shows that Set A bio-fertilizer, which contains *A. radioresistens*, *K. quasipneumonia*, and *B. cereus*, as well as Set B, which contains *B. paraconglomeratum*, *B. cereus*, and *B. tropicus*, exhibit increased levels of N, P, and K compared to control. Previous research demonstrated that *Acinetobacter* increased the amount of N that duckweed could extract from the pond water (Stegelmeier et al., 2022). *Acinetobacter* sp. is claimed to be a plant growth-promoting bacterium, as it has been found to enhance wheat growth (Egamberdieva et al., 2008). Furthermore, this study revealed that Set A is the second highest in increasing the P content in the soil, similar to Set B. This finding is supported by research conducted by Yamakawa et al. (2018),

in which *Acinetobacter calcoaceticus* P23 exhibited apparent P solubilizing activity. They claim that *Acinetobacter* can increase the amount of P in the cultivation of duckweed (Yamakawa et al., 2018). *Klebsiella quasipneumoniae*, a sister-like *of Klebsiella pneumonia*, is considered a human bacterial pathogen. However, it has been reported that a *Klebsiella* strain was present in the rhizosphere and exhibited PGPB traits (Tangapo et al., 2018). *Klebsiella pneumoniae* possesses various PGPB traits, namely the production of IAA, P solubilization, N–fixing ability, and several other traits (Ashfaq et al., 2022). Similar results have shown that K solubilizing bacteria have been successfully isolated from tobacco rhizosphere, such as *Klebsiella variicola* (Sun et al., 2020). There is extensive evidence that inoculation with *Klebsiella* sp. can increase the available K in the soil (Wang et al., 2020).

Set B is composed of *B. paraconglomeratum*, *B. tropicus*, and *B. cereus*. Results from this study show that Set B has the lowest increase in N, K, and P content compared to Sets A and C. *Brachybacterium paraconglomeratum*, which has demonstrated the ability to promote plant growth. This bacteria species can also produce the plant hormone IAA and siderophores. Furthermore, it can utilize 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole source of N and exhibits ACC deaminase activity in plant growth promotion (Gontia et al., 2011).

The Effect of Bio-fertilizer in Improving Duckweed Growth and Its Protein Content

Microbes naturally found in plants are crucial in promoting plant growth, even in challenging conditions. Extensive research on bio-fertilizers has demonstrated their potential to supply vital nutrients to crops, enriching crop yields without harming the environment (Kour et al., 2020). However, not all microbes can interact with plants, making it essential to analyze the interactions of PGPB with their natural plant hosts (Zamioudis & Pieterse, 2012). Duckweed, a fast-growing aquatic plant, undergoes clonal duplication during its vegetative growth cycle, with a high number of fronds indicating healthy growth and reproduction (Tang et al., 2015). N fixation, P solubilization, and K solubilization have been identified as the mechanisms responsible for the symbiotic connections between bio-fertilizers and duckweed.

Studies have shown that bio-fertilizers from different sets can significantly enhance duckweed growth (Yoneda et al., 2021). Unlike in soil, plant-associated microorganisms in water must adhere to and colonize plant bodies to avoid being washed away by water currents. Aquatic PGPB is hypothesized to possess useful properties such as rapid adhesion and stable colonization. Based on the results obtained from this study, bio-fertilizers from Sets A, B, and C can enhance the growth of the duckweed, with Set C displayed as the best bio-fertilizer, corresponding to the highest number of duckweed fronts after 15 days.

The bio-fertilizer increased the number of duckweed fronds, which is consistent with the findings of Yoneda et al. (2021). Specifically, PGPB strains from the phyla Betaproteobacteria, Gammaproteobacteria, and Alphaproteobacteria have been observed to increase the number of duckweed fronds by more than twofold (Makino et al., 2022). Additionally, certain PGPB strains, such as *Pseudomonas* sp. Ps6 and *Ensifer* sp. strain SP4 have demonstrated the ability to accelerate duckweed growth (Toyama et al., 2017).

The potential for using this PGPB as a bioinoculant was demonstrated by exposing duckweed to all bacterial strains obtained in this study. The PGPB effects of all tested bacterial strains on duckweeds were comparable to those of the well-studied representative PGPB, *Acinetobacter calcoaceticus* strain (Makino et al., 2022; Toyama et al., 2017). The ability of *A. calcoaceticus* P23 to grow in both artificial media and environmental conditions makes it a potential bioinoculant for enhancing duckweed growth (Suzuki et al., 2014; Toyama et al., 2017; Yamaga et al., 2010). Several studies have shown that co-cultivation of duckweed with specific PGPB strains can lead to significant growth benefits. For instance, the rhizobacterium MH3 has been found to boost duckweed development, resulting in a 30% increase in frond number and a 50% increase in dry weight (Tang et al., 2015). Moreover, certain *Bacillus* strains present in different bio-fertilizer sets have successfully functioned as PGPB to stimulate rapid duckweed growth (Idris et al., 2007). The hypothesis of synergistic effects arising from the co-inoculation of these strains further supports establishing and maintaining a mutually beneficial plant-microbe relationship. Thus, the bio-fertilizer set can establish and maintain a mutually beneficial plant-microbe relationship. It is worth noting that the bio-fertilizer bacterial strain can potentially promote growth and rescue plants from growth inhibition synergistically.

In this investigation, bio-fertilizers denoted as Sets A, B, and C exhibited a notable augmentation in duckweed protein content in tandem with escalating concentrations of N, P, and K. These findings align with those reported by Li et al. (2016), observed a similar increase in protein content in *Spirodela polyhiza* duckweed as N and P concentrations were elevated (Li et al., 2016). The correlation between these studies suggests that higher nutrient levels are conducive to enhancing duckweed protein production. Notably, Set A displayed a higher protein content compared to Sets B and C, highlighting the influence of different bacterial species within the bio-fertilizer sets on protein content. Shuvro et al. (2023) also observed increased protein content in *L. minor* when cultured with *Azotobacter vinelandii* for 10 days, relative to the control. However, under stressful conditions, the protein production levels decreased (Shuvro et al., 2023). Furthermore, the growth factor and protein content of duckweed are impacted by light intensities (Petersen et al., 2022).

Our study highlights the potential of mangrove-associated bacteria identified as *A. radioresistens*, *B. paraconglomeratum*, and *E. cloacae*, which are known as nitrogen-fixing bacteria, *K. quasipneumoniae*, *B. tropicus*, and *P. pasadenensis* known as potassium-solubilizing bacteria, and *B. cereus* and *B. thuringiensis* known as phosphate-solubilizing bacteria when integrated into bio-fertilizers. These environmentally friendly alternatives to traditional chemical fertilizers and pesticides represent a novel approach. The microbial composition in each set of our formulated bio-fertilizer includes specific nitrogen-fixing species, potassium-solubilizing, and phosphate-solubilizing bacteria. This specificity distinguished our study distinct from previous research. The synergistic combination of these three types of bacteria in our bio-fertilizer formulation represents a promising strategy for developing bio-fertilizers to enhance plant growth. This study also contributes to the ongoing efforts in bio-fertilizer development by identifying specific microbial compositions that enhance plant growth. This knowledge is crucial for formulating effective bio-fertilizers that can be applied in various agricultural settings. In addition, highlighting the importance of mangrove-associated microbes in bio-fertilizers underscores the potential role of mangrove ecosystems in supporting agricultural practices. This information can contribute to conserving mangrove biodiversity for ecological and agricultural benefits.

CONCLUSION

Our study successfully achieved its aim by showcasing the impactful integration of mangrove-associated microbes into our formulated bio-fertilizer. The discernible outcome was a substantial enhancement in duckweed's growth and the subsequent protein yield. These findings contribute valuable insights into optimizing bio-fertilizer formulations and emphasize the significant potential of duckweed as a viable and promising future food option. As the challenges of sustainable food production are navigated, the demonstrated success of this integration underscores the importance of exploring innovative and environmentally friendly approaches to enhance agricultural productivity and advance the feasibility of alternative protein sources.

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