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Amplicon metabarcoding analysis of bio-extract and its potential on plant growth promotion

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Abstract. *Saengsanga T, Phanurak W. 2023. Amplicon metabarcoding analysis of bio-extract and its potential on plant growth promotion. Biodiversitas 24: 916-922.* Bio-extract is a mixture of existing organic waste and molasses in the presence of a suitable carbon source by aerobic and/or anaerobic bacteria. In this study, the mineral constitution, bacterial diversity, and effects of the bio-extract on rice growth were investigated. The bio-extract contained various mineral elements, especially nitrogen, potassium, calcium, iron, and magnesium, at concentrations of 248.56, 124.48, 2100.75, 732.68, and 112.60 mg L^{-1}, respectively. The amounts of plant growth regulators, including indole-3-acetic acid (IAA) and total cytokinins, were 0.26 and 1.02 mL L^{-1}, respectively. 16S rRNA-based metagenomic libraries yielded 39,892 amplicon sequence reads corresponding to 32 operational taxonomic units (OTUs). The Shannon index, Chao 1 index, Gini-Simpson index, and Good's coverage value were 2.68, 32.0, 0.74, and 1.0, respectively. Firmicutes (49.4%), Proteobacteria (45.5%) and Actinobacteria (5.1%) were the predominant phyla. A various concentration of bio-extract (0.0, 0.1, 0.5, 1.0, and 1.5% (v/v)) was determined for their effects on the growth of rice seedlings (*Oryza sativa* L. var. KDML105). The result revealed that highest root and shoot lengths were 7.64±0.63 and 78±0.33 cm, respectively, when 0.1% (v/v) of the bio-extract was applied. This was most effective in increasing the vigor index of rice seedlings, with the highest value of 1466±66, which corresponded to an increase of 15.2% compared to the controls. However, increasing the concentration of the bio-extract (0.5-1.5%, v/v) drastically decreased the root and shoot elongation and vigor index of rice seedlings. These results indicate the bacterial diversity and quality of the bio-extract, and application should be done carefully at appropriate dilutions.

Keywords: 16S rRNA gene, actinobacteria, plant growth regulators, Shannon index

INTRODUCTION

Bio-extract is liquid organic fertilizer produced by aerobic and/or anaerobic fermentation of plant or animal waste in the presence of an appropriate carbon source. It has a diverse variety of microorganisms living together in harmony or so called liquid microbial consortium (Ali et al. 2021). Bio-extracts contain organic compounds and amino acids, as well as a variety of growth-stimulating substances to varying degrees, depending on the type of initial raw materials used. Several studies have found that the chemical constituents of extracts can successfully stimulate plant growth, increase productivity, and enhance the effectiveness of chemicals and/or biofertilizers (Montoneri et al. 2022). It has been shown to have the potential to act as a direct source of beneficial microorganisms or/and substrates to enhance soil microbial activity in decomposing organic manure and releasing nutrients for plants. Understanding the consortia of beneficial microorganisms in the bio-extract will be important.

Microbiological analysis of fermented bio-extracts from various sources indicated that they frequently contain many species of beneficial microorganisms. Among them, plant growth promoting bacteria (PGPB), have a crucial role in bio-extracts, biofertilizers or liquid organic fertilizers (Olanrewaju et al. 2017). These beneficial bacteria have both direct and indirect strategies for accelerating plant growth. PGPB either directly enhance plant growth by facilitating nutrient acquisition including the secretion of plant hormones, or indirectly by lowering the adverse effects of several pathogens on plant growth and development as a consequence of biocontrol agents. The beneficial microorganisms are functionally classified as lactic acid bacteria (LAB), yeasts, photosynthetic bacteria, actinomycetes, fungi, and other types of microorganisms. According to Hidalgo et al. 2022, Bacillales are the majority group throughout the food waste composting process. However, the number and diversity of microorganisms may vary depending on the raw materials used as fermentation substrates. The consortium microorganisms found in the bio-extracts were identified to assist in the healing of soil ecosystems and conversion of soil components into forms that plants can readily use (Chutichudet and Chutichudet 2022). Microbes play a significant role in the breakdown of substrates. Molasses and rice brans are potential carbon sources for promoting bio-extract fermentation process derived from agroindustry factory. Molasses, a byproduct of sugar production from sugarcane, is a viscous substance with a black color that contains concentrated amounts of vitamins and minerals. It serves as a crucial source of carbon for microbial development (Phibunwatthanawong and Riddech 2019).

Rice bran is a common agricultural byproduct that have been use as culture media for enzymatic solid-state fermentations (Omarini et al. 2019), and use as bio-simulators to stimulate microorganisms in oil reservoirs (Chen et al. 2016).

In the last decade, most studies have employed 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE) techniques to quantify diversity of microorganisms (Janatiningrum et al. 2018), which require time and money and only examine a small number of prominent microbial populations. Next-generation sequencing (NGS) technology, such as metagenomic analysis, has been developed and offers unprecedented opportunities to improve our understanding of the composition and function of microbial communities in food products (Fidien et al. 2021) and the environment, such as rivers (Betiku et al. 2021), the soil microbiome in coffee plantations (Rodríguez et al. 2020), and actinobacteria associated with marine organisms (Retnowati et al. 2021). As mentioned before, metagenomics is now a technique used to study bacterial communities and to determine, examine, and evaluate microbial communities from various habitats by sequencing their genetic material (Rodríguez et al. 2020; Tran 2022). The present study aims to determine the microbiome in the bio-extract using 16S rRNA gene sequencing and investigate effects of the bio-extract on the growth of rice seedlings (*O. sativa* L. var. KDML105).

MATERIALS AND METHODS

Collection of bio-extract samples

Bio-extracts were collected from a local organic farm located in the Huai Thalaeng District, Nakhon Ratchasima Province, Thailand. The major components were 2% (v/v) microbial inoculant, 10 kg of rice bran, 10 L of molasses, and 180 L of freshwater. The mixture was placed in a 200 L plastic drum for 6 months.

Determination of mineral elements of the bio-extract

The nitrogen, phosphorus, potassium, calcium, magnesium, iron, copper, and zinc contents were determined. The nitrogen concentration was determined using the Kjeldahl method (Sáez-Plaza et al. 2013). The bio-extract sample was oxidized and decomposed with concentrated sulfuric acid (25 mL) using a mixed catalyst ($CuSO_4$: $NaSO_4$ = 1:20) heated at 400 °C until the solution was clear. The potassium concentration was measured following the procedure of Bray II (Chien et al. 2018) using a double beam spectrophotometer (UH5300, Hitachi, Japan), whereas concentrations of potassium, calcium, magnesium, iron, copper, and zinc were assessed using an atomic absorption spectrophotometer (AAS) (Spectra Ad 55B, Varian, Australia) (APHA 2017).

Determination of plant growth regulators of the bio-extract

Concentrations of indole-3-acetic acid (IAA), gibberellic acid (GA3), and cytokinin (zeatin, zeatin riboside, N6-[Δ2-isopentenyl] adenine, N6-[Δ2-isopentenyl] adenosine) after fermentation were determined using a high-performance liquid chromatography-photodiode array (HPLC-PDA) (1260 Infinity II, Agilent, United States) (Szkop and Bielawski 2013) at the Central Laboratory, Faculty of Agriculture, Chiang Mai University.

DNA extraction and direct 16S rRNA gene amplification

Bio-extracted microbiome DNA was extracted using a DNeasy PowerSoil Pro kit (QIAGEN), qualified, and sent for library construction. Next, 16S rRNA gene sequencing libraries were prepared using a KAPA HiFi HotStart DNA Polymerase and Nextera XT Index kit according to Illumina's instructions, with the target region of V3V4 using the primers Bakt 341F/805R. The primers 341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3') were used (Lee et al. 2021; Han et al. 2022). Polymerase chain reaction (PCR) was performed using the program; 95°C for 3 min; 25 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; and a final extension for 5 min at 72°C. To check the size, the PCR product was tested on a Bioanalyzer DNA 1000 Chip. The expected size of the PCR amplicons was approximately 550 bp. The prepared library was sequenced using the Illumina MiSeq platform (Macrogen, Inc.) (South Korea).

Bioinformatics analysis

The 16S rRNA amplicon sequences were analyzed. Short reads were filtered, and extra-long tails were trimmed. Filter reads were clustered with 100% identity using the CD-HIT-DUP. Chimeric reads were identified and secondary clusters were recruited into the primary clusters. Noise sequences with <97% similarity index were removed. The remaining representative reads from non-chimeric clusters were clustered into operational taxonomic units (OTUs) using a greedy algorithm into OTUs using CD-HIT-OUT (https://sites.google.com/view/cd-hit) and rDnaTools. Taxonomic assignment and diversity statistic including Shannon index, Chao 1 index, Gini-Simpson index and Good's coverage value were analyzed by quantitative insights into microbial ecology bioinformatics software (QIIME) v1.8.0 (http://qiime.org/1.8.0/).

Investigation of plant growth promoting bacteria in the bio-extract

To determine plant growth promoting bacteria (PGPB) of the bio-extract, microbial concentration was determined by counting the colonies on agar plate. The selected colonies were taken to assess nitrogen fixation using Bruk's N-free medium, phosphate solubilization using Pikovskaya's agar medium (Pikovskaya 1948), IAA production using Salkowski's reagent (Saengsanga 2018) as well as ammonia production (Cappuccino and Sherman 1992).

Effects of bio-extract on growth of rice seedlings

A complete randomized design (CRD) was used to compare the effects of five different concentrations of bio-extract on the growth of Thai jasmine rice (*O. sativa* L. var.

KDML105). Bio-extract was prepared by diluting with water, and rice seeds were soaked with diluted bio-extracts at concentrations of 0.1, 0.5, 1.0, and 1.5% (v/v), along with the control. Rice seeds (20 seeds per replicate) were germinated in the dark in a 14 cm Petri dish immersed in 15 mL of the ready-made solutions. The germination percentage was recorded for 4 days when the radicle had elongated to 2 mm. Rice shoot and root lengths were measured after 9 days, and the seedling vigor index was calculated according to the method proposed by Teixeira et al. (2021).

Vigor index = %germination × (shoot length + root length)

Statistical analysis

All data were analyzed to assess the significance of the experiments. The results are expressed as mean ± standard deviation (SD) of three replicates. Analysis of variance (ANOVA) and multiple comparisons using Duncan's multiple range test (DMRT) were performed to determine the significance level at the 5% level of probability.

RESULTS AND DISCUSSION

Mineral elements, plant growth regulators and chemical properties content of the bio-extract

Bio-extracts are a combination of existing organic wastes and molasses, a dark brown waste product of sugar production. The utilization of fermented bio-extracts decreases the need for additional chemicals to promote plant growth and control pests. A fermented bio-extract could improve soil fertility and promote the quality of the crop and productivity for various crop plants and vegetables (Ji et al. 2017). The bio-extract, which is made from rice bran and molasses, has a pleasant smell and dark brown appearance in the absence of gas bubbles. The chemical composition of the bio-extract was determined at the end of fermentation. The various amounts of the macronutrients and micronutrients were detected (Table 1). Calcium had the greatest concentration of 2100.75±14.12 mg L^{-1}, followed by magnesium which was 732.68±5.45 mg L^{-1}. The amount of nitrogen, phosphorous, potassium and iron were 248.56±4.23, 124.48±2.10, 8.00±1.02, and 112.60±3.32 mg L^{-1}, respectively. Electrical conductivity (EC) and pH values were 14.96±2.45 ds m^{-1} and 3.5±0.2, respectively. Additionally, the bio-extract was demonstrated to be a source of plant growth regulators, including IAA (0.26 mL L^{-1}), and total cytokinins (1.02 mL L^{-1}). Bio-extract fermentation transforms primary raw materials into organic acids, thereby lowering the pH levels. The result showed a low pH of 3.4 and was considered to be acidic, which was encouraging the uptake of some elements in plants, such as iron, copper, and boron. Organic nutrients in the bio-extract are released gradually as microorganisms transform organic waste into soluble forms. The intended nutrients were observed, particularly nitrogen, potassium, calcium, magnesium, and iron. The availability of nutrients in the bio-extract in the form of nitrogen (NO_3^- and NH_4^+), phosphorus ($H_2PO_4^-$ and HPO_4^{2-}), and potassium (K^+) could promoted plant growth and development (Phibunwatthanawong and Riddech 2019). The EC value of the bio-extract was high indicating large amount of minerals and inorganic substances. In addition, it has properties that are consistent with the ideal qualities of stable bio-extract such as less alcoholic and acidic odors and the absence of gas bubbles (Chooklin et al. 2021).

Bacterial diversity of the bio-extract

The extracted DNA passed the quality test with a DNA concentration of 1.225 ng μL^{-1}, total volume of 35 µL, and total concentration of 0.043 µg. The total number of bases, reads, and the %GC, %Q20, and %Q30 were calculated. In the bio-extract, 117,503 reads were produced and the total read bases were approximately 53 M bp. The GC content was 54.05%, Q20 and Q30 were 99.22 and 97.05%, respectively (Table 2).

Fermentation of compost and bio-extracts supports a diverse community of bacteria that specialize in the breakdown of lignocellulosic plant materials (Wang et al. 2016). In this work, the rarefaction curves for the OTUs recorded an adequate coverage depth, where the samples reaching the asymptote were 39,892 sequences, and 32 OTUs were obtained (Figure 1) from the V3V4 region of the 16S rRNA gene sequences of the bio-extract sample after a series of quality control steps. The biodiversity index values, including Shannon, Chao 1, Gini-Simpson, and Good's coverage indices, were 2.68, 32, 0.74, and 1.0, respectively (Table 3). The amplicons of hypervariable V3-V4 region of the 16S rRNA gene revealed diverse microbial species. The hypervariable V3-V4 region is generally accepted that it can provide information on the profile of the bacterial community more accurately than the hypervariable V1-V3 region (Teng et al. 2018).

Illumina NGS technology is the most widely used technique for evaluating microbial diversity and functional analytical profiles in metagenomic research (Fidien et al. 2021). Microbial consortia play an important role in organic transformations and secondary metabolite production. The bacterial diversity in the observed bio-extract was belonged to the kingdom Monera. The results demonstrated that 3 bacterial phyla, five classes, six orders, eight families, 14 genera, and 27 species were identified (Figure 2). Variations in the relative number of dominant bacteria indicated that the primary phyla across all samples were Actinobacteria (5.1%), Firmicutes (49.4%), and Proteobacteria (45.5%).

Table 1. Mineral elements, plant growth regulators and chemical properties content of the bio-extract

Mineral elements	**Values (mg L^{-1})**
Nitrogen	248.56±4.23
Phosphorous	124.48±2.10
Potassium	8.00±1.02
Calcium	2100.75±14.12
Magnesium	732.68±5.45
Iron	112.60±3.32
Zinc	5.15±1.20
Copper	<1.00
Plant growth regulators	**Values (mL L^{-1})**
IAA	0.26±0.03
GA3	Nd.
Cytokinin	
- Zeatin	0.19±0.02
- Zeatin riboside	0.18±0.01
- N6-(Δ2-isopentenyl) adenine	0.38±0.03
- N6-(Δ2-isopentenyl) adenosine	0.27±0.02
Chemical properties	
pH	3.5±0.2
EC (ds/m)	14.96±2.45

Note: Nd- not detected.

Table 2. Sequencing results indicating the total bases, read count, GC%, Q20% and Q30% of the bio-extract

Parameters	**Count**
Total bases	53,117,135
Read count	117,503
%GC	54.05
%Q20	99.22
%Q30	97.05

Table 3. Bacterial diversity indices

Diversity indices	**Values**
OTUs	32
Shannon	2.68
Chao 1	32
Gini-Simpson	0.74
Good's coverage	1.0

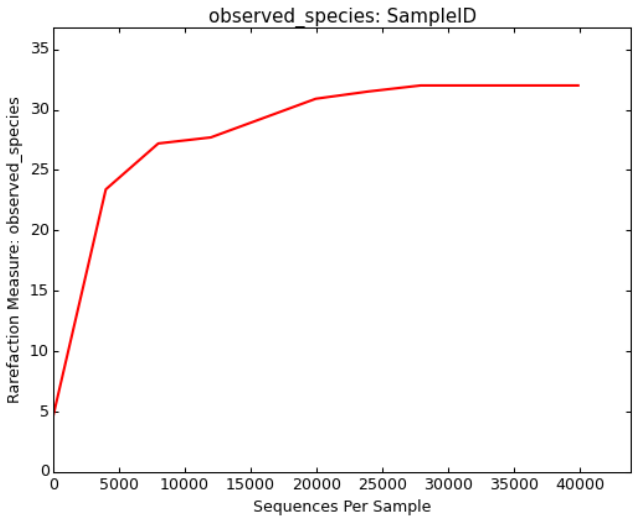

Figure 1. The number of sequences and operational taxonomic units (OTUs) in the bio-extract

At the class level, the majority of the Proteobacteria phylum were categorized as Alphaproteobacteria (45.5%), whereas the Firmicutes phylum was divided into Bacilli (41.0%) and Clostridia (8.4%). At the order level, the bio-extract was dominated by Rhodospirillales (45.5%) and Lactobacillales (36.5%), whereas Eubacteriales, Bifidobacteriales, and Bacillales had the values of 8.4%, 5.1%, and 4.6%, respectively. Taxonomic classification at the family level divided bacterial diversity into eight major families (Figure 2). The predominance of Acetobacteraceae (45.5%), Lactobacillaceae (36.5%), Clostridiaceae (8.4%), and Bifidobacteriaceae (5.1%) were found. Furthermore, a total of 17 genera were identified as *Aeriscardovia*, *Bifidobacterium*, *Oceanobacillus*, *Brevibacillus*, *Laceyella*, *Risungbinella*, *Thermoactinomyces*, *Lacticaseibacillus*, *Lactobacillus*, *Lentilactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, *Liquorilactobacillus*, *Schleiferilactobacillus*, *Clostridium*, *Schnuerera*, and *Acetobacter*. Among them, *Acetobacter* and *Lactobacillus* were the most abundant at 45.5% and 27.8%, respectively. In addition, 27 bacterial species were discovered in the bio-extract (Figure 2). The dominant species were *Acetobacter ghanensis* (45.5%) and *Lactobacillus kitasatonis* (18.2%).

As a result, Firmicutes have been reported to be the most dominant taxa contributing to sugarcane processing plants (Sharmin et al. 2013). Kim et al. (2021) reported that Firmicutes and Actinobacteria have many functional genes related to plant growth promotion, development, and decomposition. Proteobacteria, belonging to the Alphaproteobacteria order Rhodospirillales, are well recognized for their agricultural applications. In this study, abundant species in the bio-extract were *Acetobacter ghanensis* and *Lactobacillus kitasatonis*. The genus *Acetobacter* has also been recorded as a diazotrophic species capable of fixing atmospheric nitrogen into plants (Reis and Teixeira 2015) and synthesize plant growth promoting substances such IAA and GA to encourage root proliferation plant development (Keswani et al. 2020). Bacteria in the genus *Lactobacillus*, or LAB, have been employed in agricultural systems for decades to improve soil quality, control disease, and promote plant growth. LAB isolated from a variety of sources have been demonstrated to be efficient biofertilizers, biocontrol agents, and biostimulants (Lamont et al. 2017).

Investigation of plant growth promoting bacteria in the bio-extract

PGPB in bio-extract was assessed for plant growth promoting traits such nitrogen fixation, phosphate solubilization, IAA production and ammonia production. The results demonstrated that the bacterial concentration by total plat count were 3×10^5 CFU mL^{-1}. Base on their morphology, 112 colonies were selected and subjected to determine the plant growth promoting traits. Out of them, 87 (80.55%) and 96 (88.89%) isolates were belonged to nitrogen fixer and ammonium producer respectively. Ninety-one isolates (84.26%) were able to solubilize insoluble phosphate (Tri-calcium phosphate). The IAA producer in the bio-extract was 74 isolates (68.52%). IAA is one of the most of auxin that affect various aspects of plant development (Keswani et al. 2020).

The beneficial microbes in bio-extract may improve soil fertility and the nutrient uptake *via* nitrogen fixation, phosphate solubilization, and provide IAA to enhance plant development (Phibunwatthanawong and Riddech 2019). These characteristics of PGPB are recognized as efficient biofertilizers, biocontrol agents and biostimulants (Pirapak et al. 2022). Some PGPB, however, exhibited one or more of the aforementioned characteristics (Ambrosini and Passaglia 2017).

Effects of bio-extract on growth of rice seedlings

The germination percentage (93-95%) of the rice after 4 days of soaking showed no significant difference ($p > 0.05$) between the groups (Table 4). However, this bio-extract had a beneficial effect on the germination rate when diluted to appropriate concentrations. For root and shoot elongation, the highest root length was 7.64±0.63 cm, which was equivalent to a 36.4% increase, relative to the control, when 0.1% (v/v) of bio-extract was applied. The highest shoot length was 7.78±0.33 cm when 0.1% (v/v) bio-extract was applied. Again, this was the most effective in increasing the vigor index of rice seedlings, with the highest value of 1,466±66, which was equivalent to a 15.2% increase relative to the control. As expected, rice seedlings exposed to 0.1% (v/v) bio-extract had the highest biomass (Figure 3). On the other hand, increasing the bio-extract concentration (0.5-1.5%, v/v) significantly decreased root and shoot elongation, as well as the vigor index of rice seedlings (Table 4). These results indicated that the application of the bio-extract at appropriate concentrations could promote plant growth. In this study, an adequate dilution (0.1%, v/v) of the bio-extract had a positive effect on rice development at an early stage. However, higher concentrations (>0.5%) of the bio-extract may cause adverse effects on the growth of rice plants because the fermentation process produces organic acids and therefore increases the acidity of the bio-extract. This might be due to toxic compound produced by insufficient degradation. Understanding how bacterial diversity affects bio-extracts is critical for understanding their production and applications in organic agriculture.

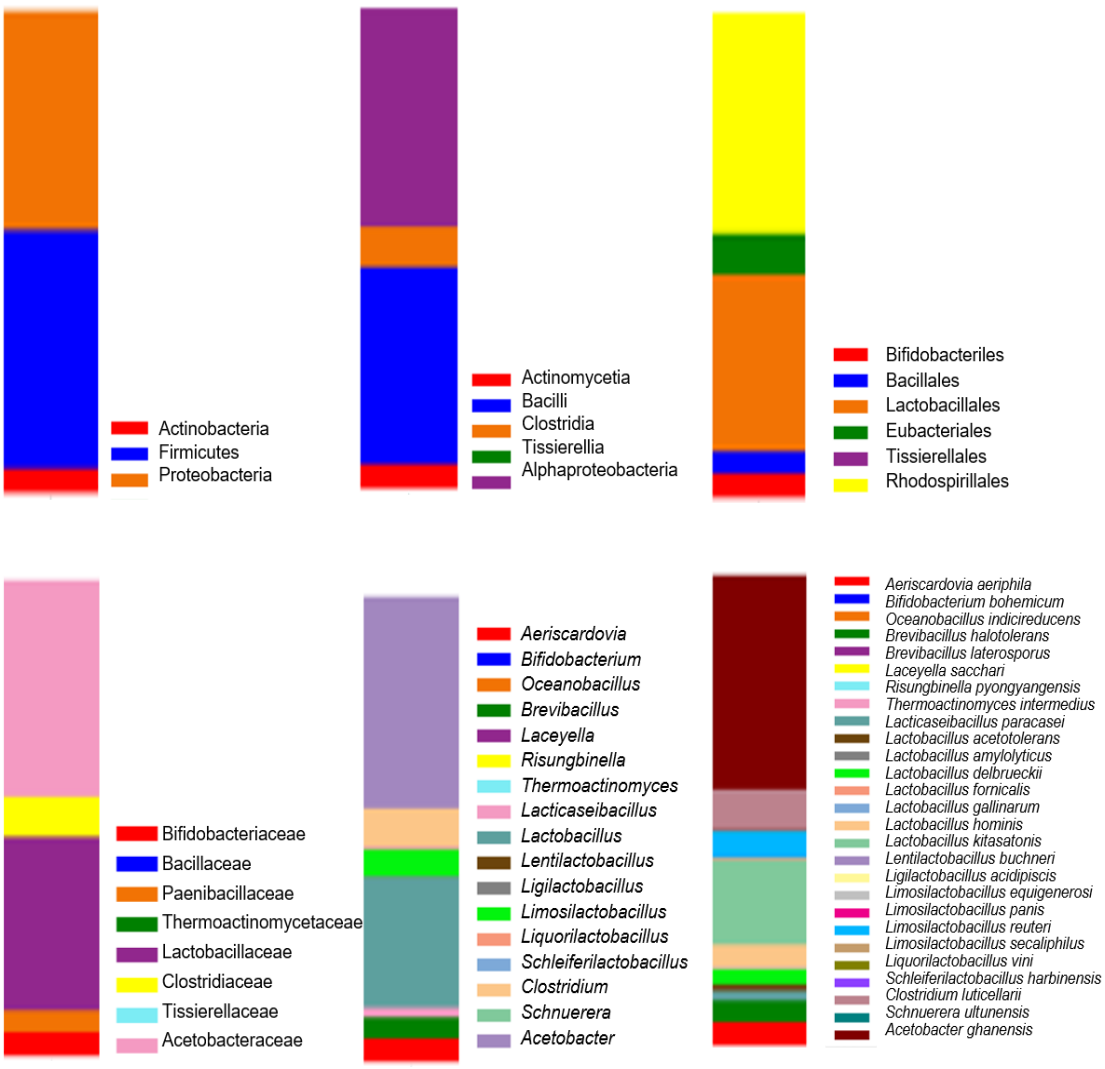

Figure 2. The relative abundance of bacterial community compositions at the phylum, class, order, family, genus, and species levels

Table 4. Effects of bio-extract on growth of rice (*Oryza sativa* L. var. KDML105) seedlings

Concentration (%)	Germination (%)	Root length (cm)	Shoot length (cm)	Total length (cm)	Vigor index
Control	93.3	5.60 ± 0.64	7.72 ± 0.56*	13.32 ± 1.18	1273 ± 110
0.1	95.0	7.64 ± 0.63*	7.78 ± 0.33*	15.43 ± 0.69*	1466 ± 66*
0.5	95.0	4.33 ± 0.51	6.67 ± 0.50	10.99 ± 0.28	1044 ± 27
1.0	93.3	4.19 ± 0.74	5.99 ± 0.35	10.18 ± 1.02	951 ± 95
1.5	95.0	4.67 ± 0.41	6.10 ± 0.15	10.77 ± 0.26	1023 ± 25

Stars (*) indicate statistically significant differences at $p < 0.05$ (DMRT). Values in the parentheses indicate the average percentage increase (+) or decrease (-) relative to the controls.

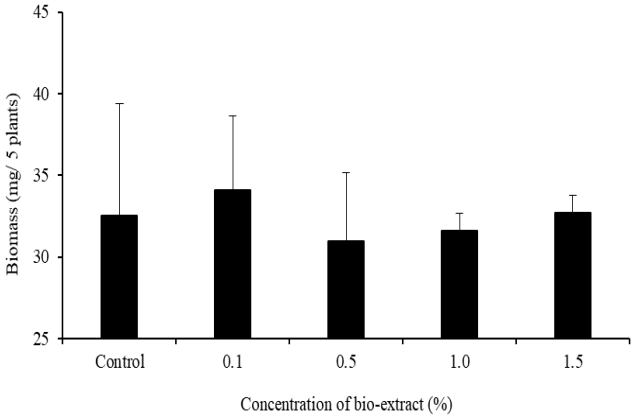

Figure 3. The biomass of rice seedlings after treatment with the bio-extract. Values are the mean ± SD of three replicates (5 seedlings per replicate)

In conclusion, the 16S rRNA gene was studied to determine the bacterial diversity of a local bio-extract produced by a farmer in Nakhon Ratchasima, Thailand, and how the microbiota affected the chemical properties and plant growth-promoting activities of the bio-extract. The result suggests that the bio-extract contains an adequate level of plant nutrients and phytohormones to increase rice plant growth. Variations in bacterial diversity were revealed using OTUs and diversity indices. A total of three bacterial phyla, five classes, six orders, eight families, 17 genera, and 27 species were identified. Firmicutes (49.4%), Proteobacteria (45.5%), and Actinobacteria (5.1%) were the dominant phyla. An appropriate dilution (0.1%, v/v) of the bio-extract significantly increased rice plant development. Further investigation of other properties, such as antioxidative enzymes produced by bacteria that have beneficial effects on plants, is required to fully understand the metabolic capacity of bacteria in this bio-extract.

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