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Evaluation on multi-trait activities of *Azotobacter* spp. from various water samples in Mandalay environments

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Abstract

Fourteen water samples were collected from different locations of Mandalay and Kyaukse Township. Among them, eight bacterial isolates named A1 to A8 were nominated as *Azotobacter* according to their colonial morphology, microscopic morphology, and pigment production. According to the biochemical characteristics and sequence analysis of isolated bacteria, they were *Azotobacter chroococcum*, *Azotobacter vinelandii*, and *Azotobacter beijerinckii*. The phosphate solubilizing of these isolates was observed from 2.3 to 2.6 SI, and A4 had the highest solubility index. The potassium decomposing of these isolates was observed 2.5 to 3.8 SI, and A6 and A8 had the highest K-decomposing activity. The zinc solubilizing of these isolates was observed at 2.3 to 3.5 SI, and A3 had the highest zinc solubilizing index. Screening methods showed that all eight strains have nitrogen fixing activity and indole acetic acid (IAA) producing activity. The antagonistic activity of all isolated strains was also found against *Pythium* sp. and *Fusarium oxysporum*. The isolated *Azotobacter* sp. can be used as biofertilizer in the agriculture sector, which can increase crop yields and enhance soil fertility according to their plant growth promoting activities.

Keywords: Isolation, characterization, 16S rRNA gene, plant growth, *Azotobacter*, biofertilizer.

Introduction

Azotobacteraceae are heterotrophic gram-negative bacteria and a group of aerobic. The main characteristic of *Azotobacteraceae* is the ability to fix nitrogen in the natural atmosphere, which is vital in the nitrogen cycle. *Azotobacter* species are generally spherical on LB medium or oval on glucose nitrogen free medium and motile (G-NFMM) [1]. *Azotobacter* can live as cysts and produce enormous amounts of capsular slime. They can grow on any medium that has a suitable pH and an organic carbon source [2]. *Azotobacter* produces blue white fluorescent, green, and yellow green pigments [3]. The optimum temperature range of *Azotobacter* is between 20 and 30 °C, and the optimum pH of *Azotobacter* is 6.5 in neutral to alkaline soil [4]. They can be found in the soil of a number of crop plants and vegetative plants [5]. *Azotobacter* species is the preferred organism because they are non-pathogenic and can stay as cysts in soil for many years [6]. Beijerinck studied *Azotobacter chroococcum* isolated from the rhizosphere in Holland in 1901, and it was the first species. *Azotobacter agilis* was also studied by Beijerinck [7]. Lipman found *Azotobacter vinelandii* in 1903 and *Azotobacter beijerinckii* in 1904 from the rhizosphere [8]. *Azotobacter vinelandii* has the ability to produce a yellow-green pigment that gives fluorescence under ultraviolet light. In alkaline soils, marine sediments, and marsh water, *Azotobacter* can be generally found [9]. Biofertilizers, also known as organic preparations or bioinoculants, contain microorganisms that promote nitrogen fixation, phosphate solubilizing, and potassium decomposing for plant growth [10]. *Azotobacter*, *Azospirillum*, and *Rhizobium* are beneficial microbes for crop production to use as biofertilizers [11; 12]. Nowadays, many countries use biofertilizers that give advantages to economic activity. The chemical fertilizers threaten environmental pollution and human health. The research was designed to isolate *Azotobacter* sp. from various water samples, to characterize biochemical tests, identify the isolates by 16S rRNA sequencing analysis, and study the multi-trait activities of isolated bacteria.

Materials and Methods

Study Area and Sample Collection

The bottles of water samples were collected from different places in Mandalay Environs and Kyaukse Township, Myanmar (**Figure 1**). The water samples were taken from 5 to 12 cm of water depth. The plastic bottle (1 liter) was used to collect water samples. Then, the water bottles were carried to the department of biotechnology research to be studied. The samples were named S1 to S14 (**Table 1**). The strains isolated from these samples were named A1 to A8. Their sampling sites and different locations of bacterial isolates were shown in **Table 2**.

Table 1. Water samples collected from different locations in Mandalay and Kyaukse Township.

No.	Samples	Collection site	Township
1	S1*	Ayeyarwady river jetty3	Mandalay
2	S2	Kandawgyi lake site1	Mandalay
3	S3*	Kandawgyi lake site2	Mandalay
4	S4*	Kandawgyi lake site3	Mandalay
5	S5*	Fish pond in Mahar Muni Image	Mandalay
6	S6*	Taw-dwin Lake	Kyaukse
7	S7*	Tone-long (southern fish lake)	Kyaukse
8	S8*	Tone-lone (northern fish-lake)	Kyaukse
9	S9	SunYe Loch site1	Kyaukse
10	S10	SunYe Loch site 2	Kyaukse
11	S11	SunYe Loch site3	Kyaukse
12	S12*	SunYe Loch site 4	Kyaukse
13	S13	Thamote Canal	Kyaukse
14	S14	Zawgyi River	Kyaukse

Isolation of Bacteria

To isolate bacteria, 5 ml of water was added to the sterile test tube, and 5 ml of 0.9% NaCl solution was mixed with it. The tubes with the water sample were kept standing for about 30 minutes, and 100 μl of water was spread on G-NFMM [13]. After inverting the GNFMM plates, they were incubated at 35 ^{o}C for 48 hours. The bacterial colonies that appeared on G-NFMM were purified by subculturing and incubated at 35 ^{o}C for 2 days. The shapes and sizes of isolated bacteria were characterized by colonial and microscopic morphology. Microscopic morphology was checked by using gram staining method [14]. The single isolated colony was stained and examined with a drop of oil immersion under a microscope at 100 magnifications to check purity.

Biochemical Characteristics of Isolated Bacteria

The isolated bacteria were characterized by using some biochemical tests. Biochemical characteristics of selected bacteria were studied according to Bergey's manual of determinative bacteriology [15]. These tests include the motility test, the catalase test, the starch hydrolysis test, the citrate utilization test, the methyl red test, the Voges-Proskauer test, the indole test, and the triple sugar iron agar test.

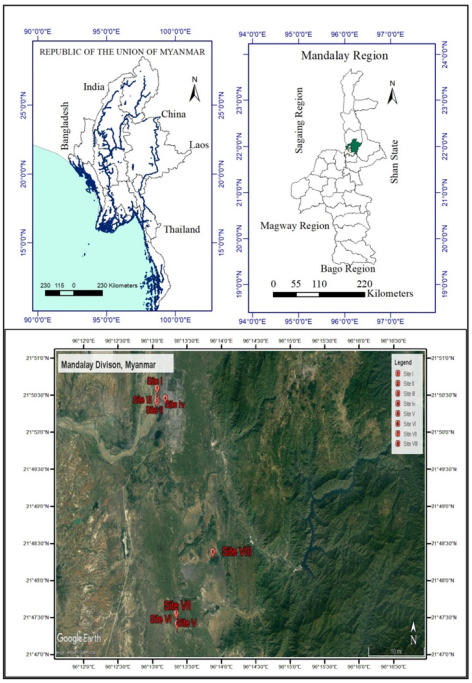

Figure 1. Location map of study area, Mandalay environments, and Kyaukse Township. (Source: Myanmar information management unit and google earth pro)

Molecular Identification of Bacterial Isolates

The purified bacterial isolates were sent to a DNA sequencing service, Eurofins company in Germany, and the sequence data of individual isolates were queried using BLAST in the national center for biotechnology information (NCBI) and GenBank databases. Sequence alignment results showed the maximum identities of each isolate and their relevant accession number. Muscle was used to align sequences that were identified, and a phylogenetic tree was constructed based on the neighbor joining (NJ) and the number of differences method algorithms using the MEGA 11 software. To acquire accession numbers, the nucleotide sequences of isolated *Azotobacter* species were then sent to GenBank. For further application, all isolates were maintained at the microbiology laboratory at the department of biotechnology research.

Screening on Nitrogen Fixing Activity by Indophenol Method

The bacterial strains were injected in the bottles that contain G-NFMM broth and incubated for one week at 35^{o}C. Then 1 ml of sample was centrifuged at 10000 rpm for 20 minutes. Ammonium concentration was measured by indophenol method by taking the supernatant. Firstly, 0.5 ml of EDTA solution was added to supernatant. Then 0.5 ml of p-buffer was added. After adding 2.5 ml of nitroprusside reagent, 2.5 ml of hypochlorite solution was added to the supernatant in a small bottle. The reaction mixture was placed at room temperature for 3 hours and ammonium was estimated by the color of the sample.

Table 2. Bacteria isolates from different locations in Mandalay environments and Kyaukse Township.

No.	Samples	Strain	Collection Site	pH	Temperature
1	S1	A1	96°03 21.745" E and 21°58 13.576" N	7.8	25°C
2	S3	A2	96°03 21.709"E and 21°57 07.664" N	7.3	25°C
3	S4	A3	96°03 21.470" E and 21°56 49.277"N	7.4	25°C
4	S5	A4	96°04 48.758" E and 21°57 09.017"N	7.6	25°C
5	S6	A5	96°07 44.652" E and 21°32 32.639" N	8.2	25°C
6	S7	A6	96°07 34.727" E and 21°33 40.007"N	8.4	25°C
7	S8	A7	96°07 34.588" E and 21°33" 40.333" N	8.3	25°C
8	S12	A8	96°13 28.758" E and 21°40 36.516" N	8.2	25°C

In this method, 0 mg/ml of ammonium concentration will show yellow color, and 3 mg/ml ammonium concentration will show green color.

Screening on Phosphate Solubilizing Activity

To determine the phosphate solubilizing activity of isolated bacteria, national botanical research institute phosphate (NBRIP) media was used. The single colony of bacteria was placed on NBRIP media. After five days of incubation at 35°C, the colony showed a zone of clearance around it. The halo-zone diameter was recorded by the phosphate solubilizing index [16]. The solubilizing index (SI) can be determined using the following formula:

$$SI = \frac{\text{colony diameter + halozone}}{\text{colony diameter}}$$

Screening on Potassium Decomposing Activity

To determine the decomposing of potassium, the eight bacterial strains were tested on K decomposing medium by plating method. Pure colonies were spotted on K medium and incubated at 35°C. After five days, the clear zone around the colony was observed, and the diameters of the clear zone were recorded [17]. The solubilizing index (SI) was calculated using the same formula as the phosphate solubilizing activity.

Screening on Zinc Solubilizing Activity

To determine the solubilizing of zinc, zinc solubilizing medium was used to test the eight bacterial strains. The single colony of bacteria was dotted on medium at 35°C. After incubating them for five days, clear zones around the colonies were recorded [18]. The SI was measured and calculated using the above formula.

Screening on IAA Producing Activity

The production of IAA by the isolated strains was determined calorimetrically using Salkowski's reagent mixture [35% perchloric acid (50 ml); 0.5 M $FeCl_3$ (1 ml)]. To test IAA quality, isolated strains were aseptically cultured in G-NFMM plates containing 0.5 mg L-tryptophan per ml, and then incubated at 35°C for 3 days. After incubating the plates, 2 ml of Salkowski's reagent and one drop of orthophosphoric acid were added into the plate medium at room temperature. The color turned pink, which indicated IAA production [19; 20].

Table 3. Biochemical characterization of isolates

Biochemical Test	A1	A2	A3	A4	A5	A6	A7	A8	C
Gram Staining	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	-
Starch Hydrolysis	-	-	-	-	-	-	-	-	-
Citrate Utilization	+	+	+	+	+	+	+	+	-
Motility	+	+	+	+	+	+	+	+	-
Methyl Red	+	+	+	-	-	+	-	-	-
Voges-Proskauer	+	+	+	+	+	+	+	+	-
Indole	-	+	-	-	-	-	-	-	-
TSI agar									
glucose	+	+	+	+	+	+	+	+	-
lactose	+	+	+	+	+	+	+	+	-
sucrose	+	+	+	+	+	+	+	+	-
gas	-	-	-	-	-	-	-	-	-
H_2S	-	+	-	+	+	-	+	-	-
Solubility Index									
Phosphate	2.5	2.5	2.3	2.6	2.3	2.3	2.3	2.3	0
Potassium	2.5	3	3	3	3.5	3.8	3.5	3.8	0
Zinc	2.8	2.3	3.5	2.3	2.3	2.3	2.3	3	0

Screening on Antagonistic Activity

To determine the antagonistic activity, a total of eight bacterial isolates were tested with *Pythium* sp. and *Fusarium oxysporum* on potato dextrose agar (PDA) medium by plating method. Dual cultures (bacteria and fungus) were spotted separately on opposite sides on potato dextrose agar medium and incubated at 25°C. After three weeks of incubation, an antagonistic effect was observed. The zone diameter of inhibition on fungal growth was determined and recorded.

Results

Study Area and Sample Collection

Collection sites and township of S1 to S14 samples are shown in **Table 1**, and the results of strains A1 to A8 from 14 samples

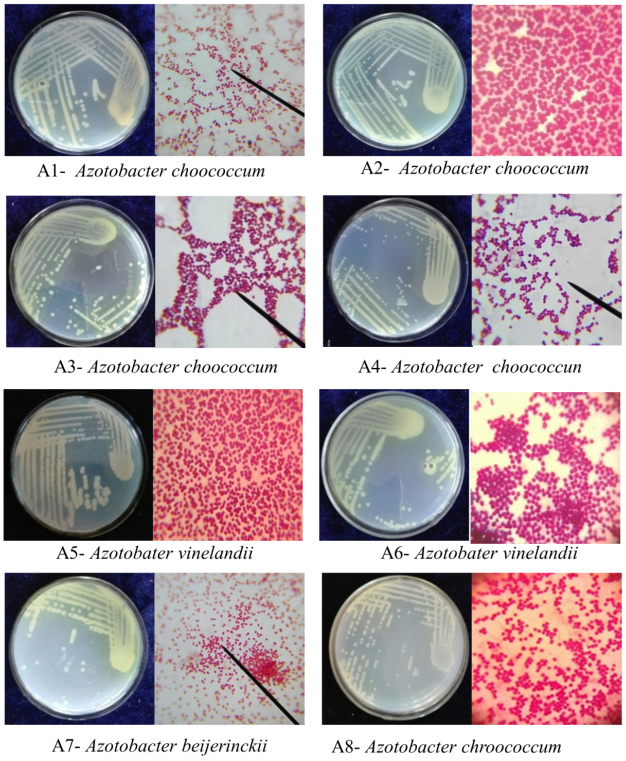

Figure 2. Colonial and Microscopic Morphology of Isolated Strains.

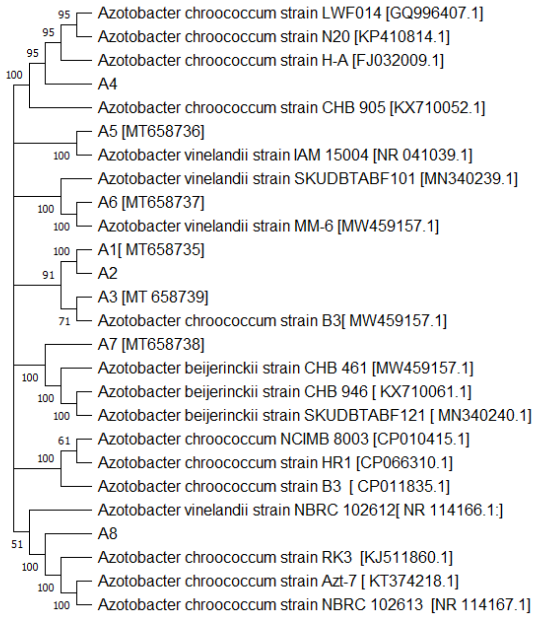

Figure 3. Phylogentic tree of 16S rRNA gene sequences of the bacterial eight isolated strain from water samples.

are shown in **Table 2**. The samples showed a pH range of 7-9 and a temperature of 25°C.

Isolation and Biochemical Characterization of Bacteria

The purified bacteria were observed for their colonial morphology and microscopic morphology such as color, shape and size (**Figure 2**). The isolated colonies were checked by gram staining method under microscope that revealed and characterized as *Azotobacter* species. They were pale-yellow and mucoid according to their morphology and colony sizes were 2 to 4 mm. According to gram straining, they were gram negative, cocci and their sizes were 2 to 4 μm. The isolated bacteria, A1 to A8 were identified by biochemical characterization tests shown in **Table 3**.

Identification of *Azotobacter* spp. by 16S rRNA gene sequencing

Detection of 16S rRNA genes of eight isolated bacteria by gel electrophoresis was shown in **Figure S1**. The isolated strains belonged to the genus *Azotobacter* according to molecular analysis. The strain A1 showed 100% identity with *Azotobacter chroococcum* in NCBI BLAST search while the strain A2 showed 99.77% identity with the *Azotobacter chroococcum*, respectively. The strain A5 revealed 100% similarity with *Azotobacter vinelandii* strain IAM 15004 (NR 041039.1) (**Figure 3**). The 16S rRNA gene sequences of A6 showed 99.52% similarity with *Azotobacter vinelandii*, A7 showed 98.87% with *Azotobacter beijerinckii*, A8 showed 99.58% with *Azotobacter chroococcum* and A3 exhibited 99.50% with *Azotobacter chroococcum*, respectively. The accession numbers were MT658735 for A1, MT658739 for A3, MT658736 for A5, MT658737 for A6 and MT658738 for A7, respectively, after submitting to the GenBank. The closely related sequences were aligned the number of differences method by using MEGA 11 and the neighbor-joining tree was created [21]. The bootstrap (1000 replication) was used for a statistical support for the nodes in phylogenetic tree constructed with the 16S rRNA similarities (%). **Figure 3** showed that the phylogenetic tree analysis involved 26 nucleotide sequences was united through Muscle using MEGA 11 software. Bootstrap values below 50% are not indicated, fewer than 5% alignment gaps. A total of 757 positions in the final data were involved.

Screening on Nitrogen Fixing Activity by Indophenol method

When the activity of isolated strains that fix nitrogen was screened by culturing in the G-NFMM broth, all isolates changed the color (**Figure 4**). The potential of nitrogen fixation was indicated by their color changing of all isolates.

Screening on Phosphate Solubilizing Activity

A1, A2, A4 gave higher halo zone diameter than A5 in NBRIP media. Strain A3, A5, A6, A7, and A8 showed the same clear zone in phosphate solubilizing activity test (**Figure 5**).

Screening on Potassium Decomposing Activity

In K-decomposing test, strain A6, A8 exhibited 3.8 mm clear zone and the strains A5, A7 showed the same 3.5 mm clear zone.

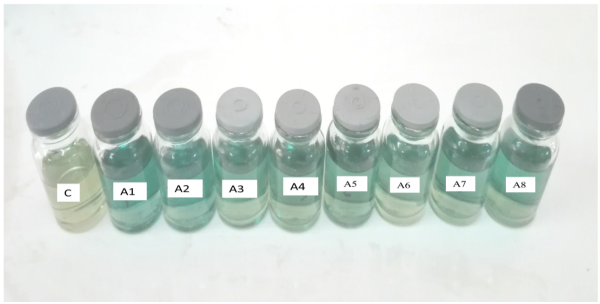

Figure 4. Screening of *Azotobacter* species nitrogen fixing activity.

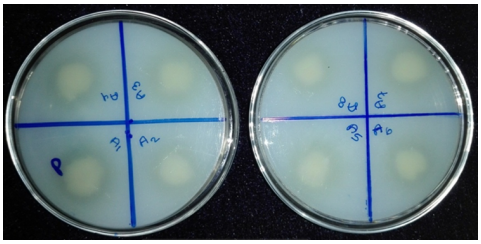

Figure 5. Screening on phosphate solubilizing activity of *Azotobacter*.

Strains A2, A3, A4 showed 3 mm clear zone strain A1 showed 2.5 mm clear zone (**Figure 6**).

Screening on Zinc Solubilizing Activity

All strains showed clear zone around their colonies on zinc agar plates after 5 days of incubation at temperature 35°C. Among eight strains, A3 showed the maximum clear zone, and followed by A8 and A1. Strains A2, A4, A5, A6, and A7 showed the same clear zone (**Figure 7**).

Screening on IAA Producing Activity

IAA producing activities of all isolated strains were screened by using GNFMM containing 0.5 mg/ml L-tryptophan and incubated at 35°C for 3 days. After incubating the plates, Salkowski's reagent was added into the culture plates. IAA producing activity was indicated by the development of pink color (**Figure 8**).

Screening on Antagonistic Activity

The antifungal activity of isolated strains A1 to A8 showed good antifungal activity against *Pythrium* sp. and did not show

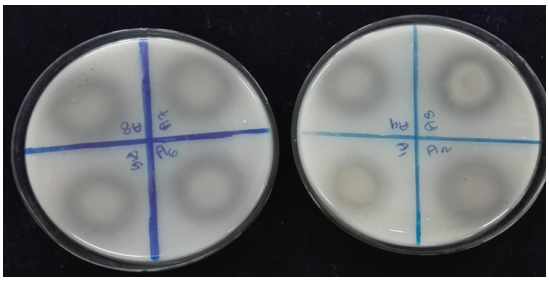

Figure 6. Screening on zinc solubilizing activity of *Azotobacter*.

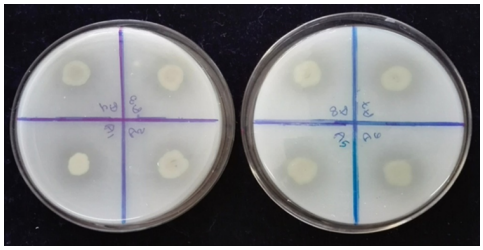

Figure 7. Screening on potassium decomposing activity of *Azotobacter*.

distinct activity against *Fusarium* sp. after 3 weeks incubation (**Figure S2**).

Discussion

Azotobacter can be found in the rhizosphere of vegetables and plantation crops [22]. All plants need nitrogen, phosphate, potassium and zinc for its growth. However, plants can absorb ammonia converted from nitrogen. *Azotobacter* is proficient to convert nitrogen into ammonia, making it readily available for plant absorption [23]. *Azotobacter* is beneficial nitrogen fixer. *Azotobacter* bio-fertilizer benefits in agriculture to integrate nitrogen management. *Rhizobium*, *Azotobacter*, and *Azospirillum* enrich nitrogen nutrition in N-deficient soils [24]. After the death of the *Azotobacter* cell, cell proteins are mineralized in soil and contribute to the nitrogen accessibility of crop plants [25]. Gibberellic acid and IAA produced by *Azotobacter* encourage plant growth development. Antimicrobial compounds produced by some species of *Azotobacter* are able to inhibit plant pathogens and prevent economic losses [26]. In this research, eight isolated *Azotobacter* strains were obtained from 14 water samples. The bacteria were characterized by 16S rRNA gene sequencing method to know exactly about the genus of *Azotobacter* sp. They were *A. chroococcum*, *A. beijerinckii*,and *A. vinelandii*. Eight strains were cultured in G-NFM medium containing BTB as indicator and showed changing of the color from green to blue that was an indication of nitrogen fixing activity. The isolated strains A1 to A8 showed nitrogen-fixing activity on the nitrogen-free medium. All eight strains showed phosphate solubilization activity on NBRIP medium. The widest clear zone diameter was found in *A. chroococcum* of all isolated strains in phosphate solubilization. To convert insoluble phosphorus to an accessible form, orthophosphate, the capacity of some microorganisms is an important trait in a PGPR for increasing crop yields. *Azotobacter* can also convert insoluble phosphate to orthophosphate that is soluble for plants to absorb [27]. Insoluble inorganic phosphate can be absorbed by the roots of plants with the help of bacteria that can solubilize phosphate [28]. Eight isolates showed the clear zone around the colonies in potassium decomposition.

A. vinelandii A6 and *A. chroococcum* A8 showed the highest potassium solubilizing activity among eight strains. The potassium is available in four forms i.e., K solution, K as mineral, K as transferable metal and K as non-exchangeable metal. Mineral K held tightly on the surface of clay. Non-exchangeable

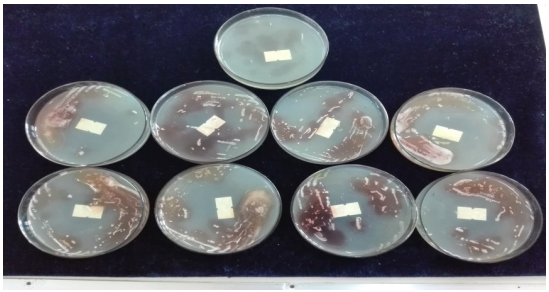

Figure 8. Indole acetic acid (IAA) producing activity of *Azotobacter* sp.

K is a primary factor in determining soil and tightly bound in the crystal lattice. Exchangeable K is loosely bound water in the soil. Soluble K could be measured in a water extract of soil [29; 30]. Zinc solubilizing bacteria showing clear halo zone around their colonies were found in all eight strains. *Azotobacter chroococcum* A3 showed the widest clear zone among the eight strains. A very slight portion of total zinc is present as soluble form in soil solution. The majority of zinc in soil is found in insoluble compounds and minerals [31]. One of the important hormones which promote plant growth is IAA. A positive correlation was detected between tryptophan concentrations and IAA production. All strains produced IAA in screening of IAA production, using tryptophan as carrier. *Fusarium oxysporum* and *Pythium* fungi were found to cause severe diseases in various crops [32]. *Pythium* fungi are water molds, and their spores attack living plants, causing issues such as seedling decay, root and crown rot, and leaf blight [33; 34]. *Fusarium oxysporum* is a fungal pathogen that infect banana, tomato, many other vegetables and ornamentals [35; 36]. In the present study, all strains noticeably showed fungal growth inhibition against the tested pathogen (*Pythium*). The antifungal activity of *A. chroococcum* A1, A3 and A4, *A. vinelandii* A5 and A6, *A. beijerinckii* A7 were occurred against *Pythium* sp. *A. chroococcum* A2 and A8 were fair antifungal activity against *Pythium* sp. after 3 weeks. *A. chroococcum* A4 and A8, the other strains were observed as moderate antifungal activity. As for antifungal activity against *Fusarium oxysporum*, all isolated strains exhibited minimal antagonistic activity, as indicated by the small zone areas. According to the data, eight isolated strains showed good antifungal activity against the *Pythrium* sp. although they have no good activity against *Fusarium* sp. *Azotobacter* can be used as a bio-fertilizer without any significant limitations or challenges, except that it should not be used in conjunction with chemical pesticides and herbicides. The optimum conditions such as pH and temperature of isolated bacteria should be explored for their optimum growth and plant growth promoting activities.

As for future research, isolated *Azotobacter* strains would be used in combination with other bio-fertilizers and would be used as antifungal and bio-fertilizers in agricultural fields. Bio-fertilizers in Myanmar using *Azotobacter* have already been used in agriculture [37]. *Azotobacter* can fix nearly 20 kg N/ha per year to use as a substitute for fertilizer in crop production [38; 39]. Therefore, it can reduce the cost for mineral fertilizers. It has economic impact and safety in farmers.

Conclusion

Eight bacterial isolates were obtained and identified as *Azotobacter vinelandii*, *Azotobacter beijerinckii* and *Azotobacter chroococcum*. Their existence of spherical form is about 2 μm and that of oval form is about 3-4 μm. Their shapes were ranging from cocci which occurred single, in pairs or irregular clusters and some were arranged in chains of variable lengths. They were motile, gram negative and cysts formed. All eight strains showed nitrogen fixing, phosphate solubilizing, potassium decomposing, zinc solubilizing and IAA producing activities. They also have antifungal activity against *Pythium* and *Fusarium oxysporum* fungi. Therefore, *Azotobacte*r strains from this study could be used to develop bio-fertilizer which can enhance soil fertility due to their plant growth promoting activities.

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Supplementary

Figure S1: Detection of 16S rRNA gene from isolated bacteria by gel electrophoresis. Figure S2: Screening on antagonistic activity of *Azotobacter* against *A. Pythium* sp. and *B. Fusarium* sp.

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