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Research Article

Characterization of Salt Tolerant Phosphate and Zinc Solubilizing *Bacillus* Isolates for Plant Protection and Plant Stimulation use in Sustainable Agriculture in Myanmar

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Abstract

The use of rhizobacteria associated with plant roots in extreme environments could be a promising strategy to overcome the limitations of crop production induced by soil salinity. In the present study, salt tolerant-phosphate and zinc solubilization bacteria were isolated from the salt-affected rhizospheric soil samples of various agricultural sites. Among 13 isolates could solubilize phosphate, the three most efficiency isolates P-10, P-11 and P-14 were selected based on zinc and phosphate solubilization potential. The partial sequence of 16S rDNA genes and phylogenetic tree indicated that P-10 related to *Bacillus subtilis*, P-11 related to *Bacillus pumilus* and P-14 related to *Bacillus safensis* respectively. These three isolates could survive and solubilize inorganic phosphate and zinc up to 2M NaCl. Regarding percent solubilization index (PSI) and quantitative measurement, P-11 was found as the most potent isolate for both phosphate and zinc solubilizing potential. This is first report for *Bacillus safensis* which could solubilize tricalcium phosphate and zinc oxide under salt-stress condition. In addition the stress tolerant genes of *Ots*A and *Ots*B for P-14 were isolated.

INTRODUCTION

Agriculture is the most important economic sector in Myanmar as it is essential for national food security and a major source of livelihood for its people. The country's wide agro-ecological diversity enables farmers to grow more than 60 different crops which include tropical and temperate varieties. The predominant food crop is rice which is cultivated in approximately 50% of Myanmar's agricultural land. As global warming is being felt all over the world, Myanmar also suffers from the adverse effects of climate change such as scarcity of rainfall, irregular rainfall, heat stress, drought, flooding, sea water intrusion, land degradation, desertification, deforestation and other natural disasters. Abiotic stresses, such as drought and salinity, are among the major environmental constraints that limit growth, productivity and quality of crops [1]. The United Nations Food and Agriculture Organization estimated that approximately 20% irrigated lands are affected by increasing salinity [2]. In Europe, twenty-six countries have reported cases of salinization with higher frequency in Mediterranean coastal areas [3]. Salinity and drought primarily cause disruption of ionic and water homeostasis of plant cells with consequent deleterious effects on general growth and eventually plant death [4]. Therefore, identifying and developing eco-friendly strategies that can ameliorate plant growth in response to abiotic stresses are an immediate need in agricultural systems that have to cope with the jeopardies of climate change increasingly. Microorganisms or active natural compounds (bioeffectors) provided to the plant root-zone may directly or indirectly affect plant performance under both favorable and unfavorable environments. Bioeffectors (BE) include plant growth-promoting rhizobacteria (PGPR) that have been proven to improve plant performance based on biological functions occurring at the soil-plant-microbe interfaces. The PGPR can benefit plant development through multiple mechanisms of action. They can operate directly through the production of substances which promote growth and increase nutrient availability in soil and their uptake, or indirectly through the suppression of plant pathogens [5].

Phosphorus (P) is one of the major essential macronutrients of plants which regulates protein synthesis and plays an important role in biological development. Along with these essential

functions, P is also associated with complex signal transduction, macromolecular biosynthesis, energy transformations, respiration and nitrogen fixation in legumes in the plant [6]. Most of the P (95-99%) present in the soil is part of insoluble compound and hence cannot be utilized by plants [7]. Since P is a stable element in soils, it does not form a gas (such as ammonia), therefore cannot move far from where it is applied. The reason for the stability of phosphate compounds in soils is that they are highly reactive and reacts rapidly with other compounds (such as Al^{3+}, Ca^{2+} and Fe^{3+}), which become increasingly insoluble in the soil. Therefore, the release and mobilization of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability. To overcome this problem, most of the farmers regularly use chemical phosphate fertilizers which get incorporated into the soil. This applied phosphorus easily transforms into an insoluble and stable form with limited availability to plants and only 5% or less of the total amount of P in soil is available for plant nutrition [8,9]. Due to the negative environmental impacts of chemical fertilizers and their increasing costs, the use of Phosphate Solubilizing Bacteria (PSB) is advantageous in the sustainable agricultural practices. The use of microbial inoculants possessing P-solubilizing activities in soils is considered as an environmental-friendly alternative to further applications of chemical based P fertilizers [10]. Microbial intervention of PSB seems to be an effective way to enhance the phosphorus availability in soil. The main mechanism of phosphate solubilization is the production of some organic acids. Among the organic acids produced, gluconic, formic acid, 2-ketogluconic, citric, oxalic, lactic, isovaleric, succinic, glycolic and acetic acids produced from P- solubilizing bacteria. These acids are the product of the microbial metabolism [11].

Zinc (Zn) is a key micronutrient, required for all living forms including plants, humans, and microorganisms for their development. Humans and other living organisms require zinc in their lives in little amounts for proper physiological functions. Zinc is a crucial micronutrient for plants which plays various important functions in their life cycle. The deficiency of zinc in the soil is one of the very common micronutrient deficiencies and results in decreased crop production. Majority of the agricultural soil is either zinc deficient or contains zinc in a fixed form which is unavailable to plants, as a result reflecting zinc deficiency in plants and soils. Therefore, to solve the above problem, there is a requirement for alternative and eco-friendly technology such as plant growth-promoting rhizobacteria (PGPR) and organic farming practices to enhance zinc solubilization and its availability to plants. Zinc-solubilizing bacteria (Zn-SB) are promising bacteria to use for sustainable agriculture. Zn-SB have various plant growth-promoting (PGP) properties such as Zn solubilization, P solubilization, K solubilization, nitrogen fixation, and production of phytohormones like kinetin, indole-3-acetic acid (IAA), and gibberellic acid, besides production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and siderophores, hydrogen cyanide, and ammonia. Zn-SB secretes different organic acids that solubilize the fixed form of zinc to available form, which enhances plant growth promotion, yield, and fertility status of the soil [12].

Trehalose, is a non-reducing glucose disaccharide synthesized by *otsA* and *otsB gene*, occurs in a wide variety of organisms, from bacteria and archaea to fungi, plants and invertebrates [13]. Three pathways for trehalose biosynthesis have been identified in bacteria. (a) The *otsA-otsB* pathway is found in a wide range of microorganisms including *E. coli* and *Sacchromycease cerevisiae*. (b) The *treY-treZ* pathway is used by *Arthrobacter, Rhizobium sp*. and *Sulfolobus acidocaldarius*. (c) The *treS* pathway has been characterized in *Pimelobacter sp.* and in *Thermus aquaticus*. It was found to constitute the shells that are secreted by various insects positioned on tree leaves in the Middle East [14]. Trehalose plays a crucial role in metabolic homeostasis and abiotic stress tolerance in various organisms [15]. A remarkable feature of trehalose is its ability to enable a variety of organisms to endure extreme physiological stresses such as dehydration, high salt concentrations, heat and freezing. The sugar is accumulated to high levels in anhydrobiotic plants, fungi and bacteria that tolerate complete desiccation and resume metabolic activity upon rehydration. Trehalose is the major compatible solute with protective effects at high osmolarity. Accumulation of endogenous trehalose in response to salt stress enables **E. coli** to sustain a concentration of 0.5 M NaCl [16]. The major aim of this research work is to apply the isolated phosphate and zinc solubilizing *Bacillus* strains as biofertilizer and biocontrol agent in agriculture effectively under abiotic stress to reduce the chemical fertilizers, improve soil quality, increase nutrient availability leading to increase crop yields and to improve farmers' profit and to preserve the sustainable environment in Myanmar as well as industrial enzyme production.

MATERIALS AND METHODS

Sample Site and Collection

Soil samples were collected from different agricultural areas of Tawtween region (21.555231 N 96.126646 E) in Kyaukse, Myanmar. The plants were dug out, the excess bulk soil was removed by gently shaking, and the soil adhering the root was considered rhizosphere soil [17] and collected in sterilized plastic bags.

Isolation of Salt Tolerant Phosphate Solubilizing Bacteria

Each one gram of soil samples was placed into the sterile test tubes. Nine milliliter of normal saline was added to the test tube and vigorous shaking was made. Then the test tube was stand for one hour for setting down all the heavy materials from the sample. 0.5 ml of the upper portion of the sample solution was taken and inoculated into 500 ml of the sterile Pikovskaya's liquid medium with 6%NaCl and incubated at 30˚C (the pH of the medium was adjusted to 7 before inoculating of the sample)[22]. The Pikovskaya's medium consisted of yeast extract 0.50 (g/l), dextrose 10.00 (g/l), calcium phosphate 2.00 (g/l), ammonium sulfate 0.50 (g/l), potassium chloride 0.20 (g/l), magnesium sulfate 0.10 (g/l), manganese sulfate 0.0001 (g/l), ferrous sulfate 0.0001 (g/l), and dissolved in 1000 mL distilled water. After 5 days incubation, 100ul of the broth culture was taken and spread onto the Pikovskaya's solid medium (agar 15 (g/l), with 6% NaCl and incubated at 30ºC for 5 days and colonies with a clear halo were marked positive for phosphates solubilization and were considered as PSB. These selected colonies were sub-cultured for 3 times by striking method till the pure cultures were obtained on

the same PKV media when grown at 30ºC. Isolated bacteria were kept on PKV agar slant at 4ºC and in 40% glycerol at -80ºC for further study. The isolated strain was checked from the different medium by Gram's staining. All the media used in this studied were sterilized at 121ºC for 20min.

Genomic DNA Isolation, PCR Amplification and Sequencing of 16S rDNA Gene

For identification of the isolated strains by 16S rRNA sequencing method, total genomic DNA was extracted by boiling 1 colony at 95ºC for 5min in 50ul nuclease-free water. The cell lysates were centrifuged and 1ul of the supernatant containing the genomic DNA was used to PCR amplify the 16S rRNA gene. PCR reaction was performed in a thermal cycler (Thermo Fisher Scientific). The universal primers (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-TACGGYTACCTTGTTACGACT-3') were used for the amplification of the 16S rDNA gene fragment. PCR amplifications were carried out in 50ul PCR reaction mixture consisted of 5ul 10 x Thermopol buffer, 1ul dNTP Mix, 1ul of both forward and reverse primers, 1ul of Tag polymerase, 1ul of template DNA and the rest were Milli Q water. The amplification cycle consisted of an initial denaturation step of 3min at 93ºC, followed by 35 cycles of 30 sec at 95ºC (denaturation), 30 sec at 60ºC (annealing) and 30 sec at 72ºC (extension), with a final extension step for 3min at 72ºC. PCR products were electrophoresed using 1% agarose stained with ethidium bromide (0.5 ug/ml), and visualized using a Gel luminax 312. PCR products of isolates were purified using PCR purification kit (ThermoFisher Scientific) according to the manufacturer's instructions and the amplified product was sequenced on Sanger sequencing platform at Eurofins for sequencing. All the bacterial isolates were classified by BLAST analysis of their respective 16S rRNA gene partial sequences. For the determination of closest type strains NCBI Blast was used [18]. The final sequence was submitted to GenBank [19].

Salt Tolerance of Isolates

Cultures were tested for their tolerance to sodium chloride (NaCl) at 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 M using LB agar (10g tryptone, 5g yeast extract, 10g NaCl, 18g agar, and 1000mL distilled water) and Pikovskaya's agar media. Growth of colonies on the plate was recorded after 3 days of cultivation. Every treatment was repeated three times.

Analysis of Phosphate Solubilizing Activity

The qualitative as well as quantitative analysis of phosphate solubilizing activity of the selected isolates was conducted by plate screening method and broth culture method, respectively.

Qualitative Measurement of Phosphate Solubilization

Bacterial isolates were screened for their tricalcium phosphate solubilizing activity on PKV plates. Isolates were spot inoculated on the center of agar plate aseptically. All the plates were incubated at 30ºC for 5 days. A clear zone around a growing colony indicated phosphate solubilization and was measured as phosphate solubilization index (SI). SI was calculated as the ratio of the total diameter (colony + halo zone) to the colony diameter [20]. All the observations were recorded in triplicate. Strains developing clear zones around their colonies could easily be identified as PSBs.

Quantitative Measurement of Phosphate Solubilization

Quantitative estimation of solubilized P by bacterial isolate was done by the vanadomolybdophosphoric yellow color method [21] in Pikovskaya's broth containing 2000 mg/ml tricalcium phosphate. The phosphate solubilizing ability of each isolate was tested using insoluble tricalcium phosphate [$Ca_3(PO_4)_2$] as sole P source in Pikovskaya's medium. 10 ml of Pikovskaya's broth containing 2000 mg/ml P in the form of tricalcium phosphate was inoculated with 0.1ml of bacterial culture (inoculum adjusted to $2x10^8$ CFU/ml) at 30ºC up to 12 days. After incubation, 1 ml of the supernatant was taken out on 3rd, 6th, 9th and 12th day. The supernatant was obtained by centrifugation at 10,000 rpm for 20min and was passed through a 0.20 uM millipore filter and then 0.1 ml of the supernatant (filtered) was mixed with 0.25 ml of Barton's reagent and volume was made up to 5 ml with double distilled water (ddw). After 10min, the intensity of yellow color was read on UV-VIS Spectrophotometer at 430nm and the amount of P-solubilized was extrapolated from the standard curve. The experiments were conducted in triplicates and values were expressed as their mean. A standard curve was prepared by dissolving 0.02195 gm of potassium dihydrogen orthophosphate (dried at 60ºC for 1h and then cooled in desiccators) in 100 ml of double distilled water (ddw) and labeled as stock P solution, 50 ppm. Aliquots of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of working solution and 0.25 ml of Barton's reagent were added to each flask and the volume was made up to 5 ml with distilled water. After 10min, the intensity of yellow color developed was read at 430 nm spectrophotometrically. Standard curve was prepared by plotting absorbance at 430 nm *vs* concentration of P.

Analysis of Zinc Solubilization

Zinc solubilization was checked using zinc oxide as insoluble zinc source. Spot inoculation of the isolates was done in the center of the Zinc solubilizing agar medium (Dextrose 10(g/l), $(NH_4)_2$ SO_4 1(g/l), KCl 0.2(g/l), K_2HPO_4 0.1(g/l), $MgSO_4$ 0.2(g/l), ZnO 1.0(g/l), Agar 15(g/l)) (HIMEDIA). These plates were then incubated at 30ºC for 48 to 72 h. Zinc solubilization was checked in the form of a clear halo formed around the colony representing the production of organic acids as a possible mechanism of the zinc solubilization. Quantitative zinc solubilization was carried out in Zinc solubilizing liquid medium in 250 ml flasks by Zinc Assay Kit (MAK032-1KT, SIGMA-ALDRICH) for 3, 6, 9 and 12 days.

Screening of Nitrogen Fixation Activity

The visual detection of nitrogen fixation activity of the isolated strain was observed by using nitrogen free glucose mineral medium and adjusted to pH 7. Individual purified colony growing on the nitrogen free glucose mineral medium was taken and inoculated into NFG medium. After incubation, 1ml of the supernatant was taken out on 3rd, 6th, 9th, 12th and 15th day and measured by Ammonium cuvette test kit (LCK 305, HACH-UK) [22].

Screening of Siderophore Producing activity

Siderophore production was checked by using Chrome azurols (CAS) agar medium by the method described by Schwyn and

Neilands, [21]. Actively growing cultures were spot inoculated on the CAS blue agar plate. These plates were then incubated at 30ºC for 48 to 72 h. Formation of yellow-orange halo around the colony indicated production and release of the siderophores on the agar plate.

Detection of Antagonistic Activity

The antagonistic abilities of the isolates were determined in dual-plate confrontation assays against the plant pathogens *Fusarium oxysporum* (DSM 841, DSM 2646, DSM 62045, DSM 62308) from DSMZ (German Collections of Microorganisms and Cell Cultures) using PYG medium. After one week and two weeks incubation, the growth inhibition around the bacterial isolates was recorded.

Indole Acetic Acid production

Auxin production was studied in LB medium. Bacteria were grown in 50ml LB broth supplemented with 50 mgL^{-1} of L-Tryptophan and incubated in dark on orbital shaker at 200rpm for 72h. Hormone production was checked in supernatant using Salkowsky's reagent method [23].

Molecular Detection of Trehalose Biosynthesis Marker genes, otsA and *otsB*

According to the published sequences of *otsA* and *otsB* of *E.coli* in the Genebank, 1.2-kb sequence encodes trehalose 6-phosphate synthase and 800-bp encodes trehalose 6-phosphate phosphatase. The respective primers were designed to amplify for *otsA* and *otsB* from chromosomal DNA of isolated *Bacillus strains*. The primers *otsA-Fwd* (GTCGTAGTATCTAACCGG) and *otsA-Rev* (CTGCAACTTCGTCACGATC) were used to amplify the *otsA* gene and *otsB-Fwd* (GTGACAGAACCGTTAACC) and *otsB-Rev* (GACTAAACGACTCATAGTC) were used to amplify the *otsB* gene of isolated strains. The PCR was carried out for both *otsA* and *otsB* gene, in a final volume of 50 ul containing 1 pmol of each forward and reverse primer, 16 ThermoPol reaction buffer (New England BioLabs), 0.2 mM dNTPs (Thermo scientific), 1.25 U Vent DNA polymerase (New England BioLabs) and DNA template (obtained via colony PCR). The thermal programme consisted of 3min at 94ºC for initial denaturation, 32 cycles of 30s at 94ºC for denaturation, 1.5min at 55ºC for annealing and 45 s at 72ºC for extension; 7min at 72ºC was used for a final extension [24].

RESULTS AND DISCUSSION

Isolation and Identification of Phosphate Solubilizing Bacteria (PSB)

PSB is a phosphate-solubilizing bacteria which can be routinely screened by a plate assay method using Pikovskaya's medium. The bacteria will grow on this medium and form a clear zone around the colony [25,26]. These bacteria can convert tricalcium phosphate in the medium from insoluble to soluble forms [27]. Phosphate solubilization potential has been attributed to the strains ability to reduce pH of the surroundings, either by releasing organic acids or protons [28]. Organic acids, such as gluconic acid, formic acid, oxalic acid, and citric acid, secreted by PSB can directly solubilize mineral phosphate as a result of anion exchange or indirectly chelate both Fe and Al ions associated with phosphate. This leads to increased P availability, which ultimately increases plant P uptake.

In the present study, soil samples from the different agricultural sites were selected for isolation of phosphate solubilizing bacteria. These habitats were chosen due to greater possibility of occurrence of phosphate solubilizing bacteria. Panhwar found considerably higher number of PSB population in the rhizosphere in comparison with non-rhizospheric or bulk soil [29]. Barea also screened several rhizospheric bacteria for phosphate solubilizing potential [30]. In this study a total of 13 phosphate solubilizing bacterial colonies were isolated on PKV agar medium containing tricalcium phosphate (TCP) and 6% NaCl. Isolation sources, soil pH, percent identity and accession number of isolated bacterium were shown in (Table 1). Even though the isolation sources were the agricultural soils, among 13 isolated bacteria, P-1, P-4, P-5, P-13, P-15, P-16 and P-17 showed high similarity with marine bacterium. It could be assumed that the collected soil samples were from salt-affected agriculture soil and the use of 6% NaCl in the isolation medium. Out of 13 bacterial isolates, 3 isolates (P-10, 99.93% similarity with *Bacillus subtilis*; P-11, 99.70% similarity with *Bacillus pumilus*; and P-14, 100% similarity with *Bacillus safensis*) were chosen for this research work because they were found to be both potent phosphate solubilizers and zinc solubilizers showing clear halo zone and yellow halo zone around its colony when using PKV medium and Zinc solubilizing medium with bromothylmol blue indicator. The yellow halo zone formation around the bacterial colonies could be due to the production of organic acids or due to the production of polysaccharides or due to the activity of phosphatase enzymes of phosphate solubilizing bacterial strains [31-34]. Although P-2, P-6 and P-8 showed clear zones on both PVK and Zinc medium, they were discarded for this research work because they were high similarity of *Enterobacter sp*. and they could not be used as biofertilizer due to their opportunistic pathogenic effects. The three selected isolates could grow up to 2M NaCl but well growth was found up to 1.8M NaCl containing medium. The cultural and some biochemical characteristic of selected isolates were shown in (Table 2). According to the cultural and some biochemical characteristic of isolated bacterium, it was found that the three isolates showed similar results. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequence of P-10, P-11, P-14 isolates were described in (Figure 1).

Analysis of Phosphate Solubilization

Phosphorous (P) is an essential nutrient element after nitrogen. It is unavailable to plants because in the soil it is mostly present in the fixed form. Werra, Borah described that phosphate-solubilizing bacteria identified as *Pantoea aananatis*, *P. putida*, *Brevibacillus agri*, *B. subtilis,* and *Bacillus megaterium. P. fluorescens* and isolated from *Oryza sativa* were reported to solubilize phosphate, promote the growth of various crop plants, and protect against root diseases caused by pathogenic fungi. Li and Amanda D.M. Matos have demonstrated that *Bacillus subtilis* strain SEM-9 and *Bacillus pumilus* exhibited the ability to release P from inorganic phosphate sources, including calcium hydrogen phosphate, aluminum phosphate, calcium phosphate and ferric phosphate [46,47], but the reports of phosphate solubilization by *Bacillus safensis* were not found. However, in this study, it was

Table 1: Isolation sources, soil pH, percent identity and accession number of isolated bacterium.

No.	Sample Code	Soil Source	Soil pH	Description	% Identity	Accession Number
1	P-1	Sesame	9	*Bacillus marisflavi*	100	MN 902140
2	P-2	Sesame	9.1	*Enterobacter hormaechei*	99.01	MN 902141
3	P-4	Corn	8.8	*Bacillus oceanisediminis*	99.93	MN 902142
4	P-5	Rice	8.6	*Bacillus marisflavi*	99.78	MN 902143
5	P-6	Rice	8.2	*Enterobacter cloacae*	99.93	MN 902144
6	P-8	Chilli	8.3	*Enterobacter cloacae*	99.79	MN 902145
7	P-10	Rice	8	*Bacillus subtilis*	99.93	MN 902146
8	P-11	Green gram	8	*Bacillus pumilus*	99.70	MN 902147
9	P-13	Chilli	8.2	*Bacillus marisflavi*	100	MN 902148
10	P-14	Chilli	8.2	*Bacillus safensis*	100	MN 902149
11	P-15	Rice	8.2	*Bacillus aquimaris*	99.79	MN 902150
12	P-16	Rice	8.2	*Bacillus marisflavi*	99.79	MN 902151
13	P-17	Green gram	8	*Bacillus marisflavi*	99.65	MN 902152

Table 2: Cultural and Biochemical characteristics of selected bacterial isolates.

Isolates	Grams' reaction	Colony morphology	Microscopic morphology	Sugar fermentation			Gas production	H2S production	Starch hydrolysis	Protease activity	Catalase test
				Glucose	Lactose	Sucrose					
P-10	+	Rough, opaque, cream colour	rod	+	-	-	-	-	+	+	+
P-11	+	Rough, opaque, cream colour	rod	+	-	-	-	-	+	+	+
P-14	+	Rough, opaque, cream colour	rod	+	-	-	-	-	+	+	+

Table 3: Salt tolerant activity of selected isolates.

Isolates	NaCl concentrations (Molarity, M)					
	1	1.2	1.4	1.6	1.8	2
P-10	+ + +	+ + +	+ + +	+ +	+ +	+
P-11	+ + +	+ + +	+ + +	+ +	+ +	+
P-14	+ + +	+ + +	+ + +	+ +	+ +	+

+ + + Very well growth, + + Well growth, + Poor growth

observed that all three *Bacillus* isolates could solubilize both insoluble phosphate and zinc.

Data regarding percent phosphate solubilization index (PSI) revealed that the three bacterial isolates exhibited different sorts of PSI which were ranged from 220.05 to 252.5 for without NaCl, 212.5 to 240.05 for 3% NaCl and 198.4 to 225.0 for 6% NaCl containing medium, respectively (Figure 2). Maximum PSI was observed in case of inoculation of isolate P-11. Minimum PSI was reported by inoculation of rhizobacterial isolate P-14. These were subjected to inoculation in broth amended with tricalcium phosphate for quantification of phosphate solubilization. Quantitative phosphate solubilization revealed that tested rhizobacterial isolates on solid media were also positive for phosphate solubilization in liquid media amended with insoluble phosphate (Figure 4). The selected isolates also showed variation among the solubilization of phosphate which was ranged from 54.63 to 76.99 ppm for 3 days, 60.34 to 104.88 ppm for 6 days, 83.25 to 118.90 ppm for 9 days and 98.04 to 120.38 ppm for 12 days incubation. The P-11 showed maximum solubilization of phosphate followed by P-14 and P-10 as they solubilized phosphate up to120.38, 115.89 and 98.04 ppm after 12 days incubation, respectively. Inoculation of these isolates also caused drop in pH of Pikovskaya's broth compared to control. It was revealed from data that all the isolates caused drop in pH of broth

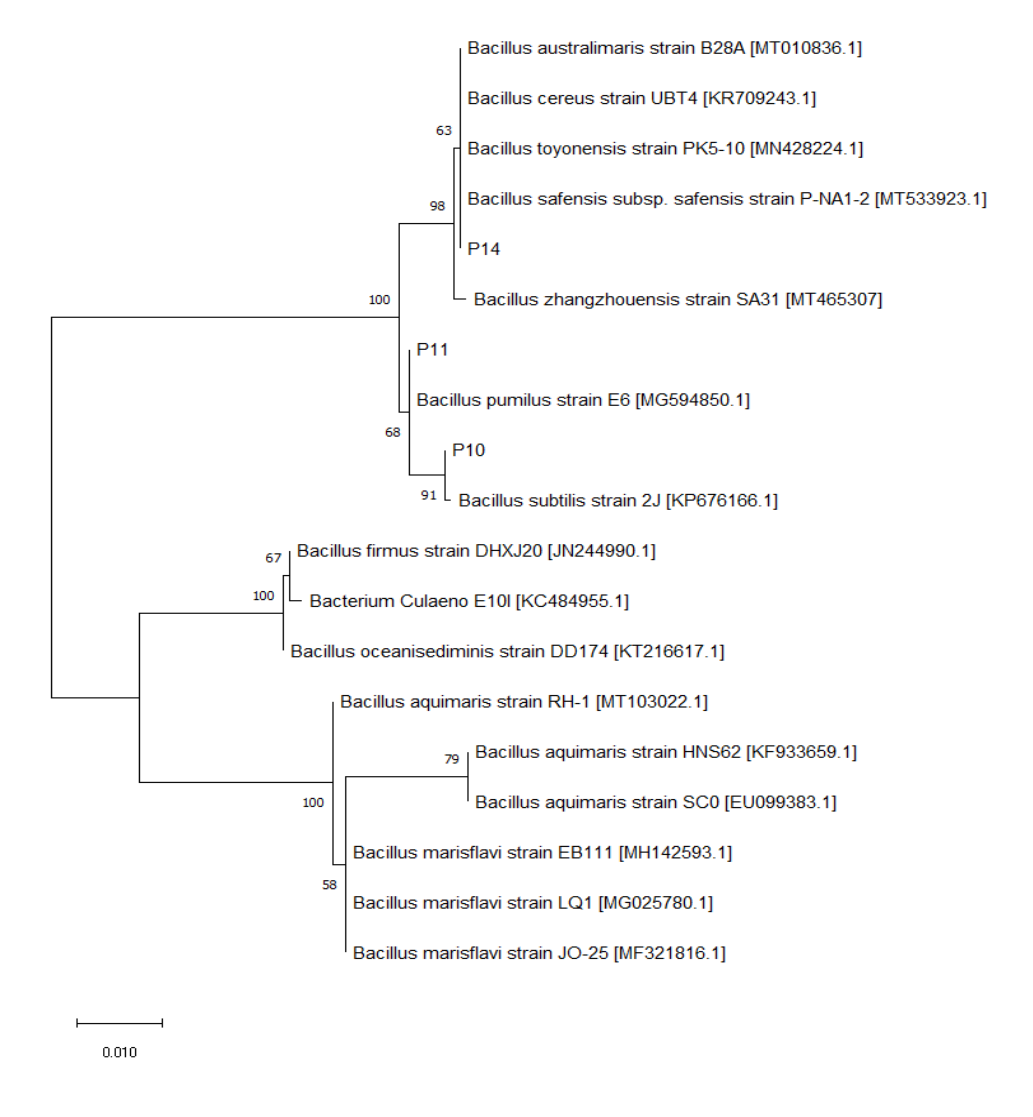

Figure 1 Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequence of P-10, P-11, P-14 isolates.

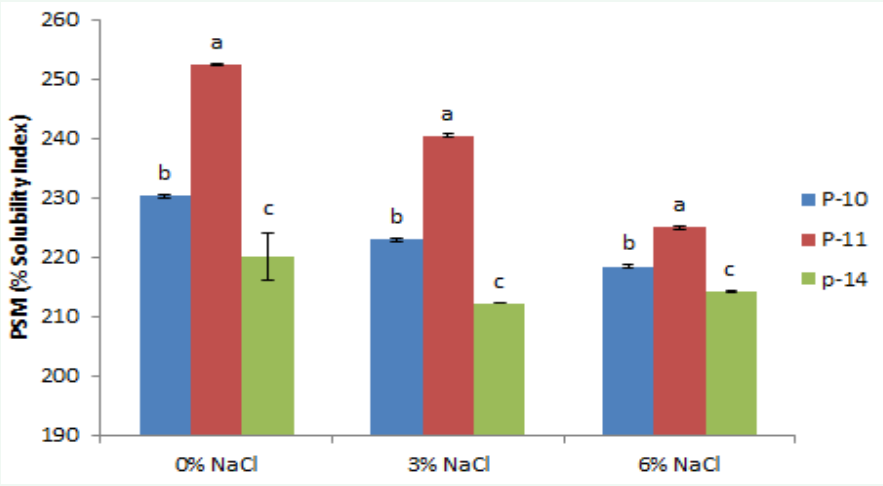

Figure 2 PSI (Percent Solubility Index) of isolated bacteria on PKV medium.

and solubilized insoluble phosphate. Maximum drop in pH was observed with inoculation of P-11, followed by P-10 and P-14.

Analysis of Zinc Solubilization

Results obtained from in vitro screening of selected isolates for their ability to solubilize ZnO, percent zinc solubilization index (PSI) revealed that the three bacterial isolates exhibited different sorts of PSI which were ranged from 228.34 to 235.04 for without NaCl, 220.02 to 225.05 for 3% NaCl and 207.07 to 218 for 6% NaCl containing medium, respectively (Figure 3). The positive rhizobacterial isolates for solubilization of zinc in solid media were subjected to inoculation in broth amended with ZnO for the determination of quantitative Zn solubilization. The results revealed that all the tested rhizobacterial isolates were also positive for solubilization of Zn in liquid media (Figure 5). The selected isolates also showed variation among the solubilization of zinc which was ranged from 9.05 to 9.82 ppm for 3 days, 10.29 to 12.68 ppm for 6 days, 12.25 to 13.60 ppm for 9 days and 13.58 to 15.27 ppm for 12 days incubation, respectively. The results of quantitative solubilization assay revealed that maximum solubilized concentration of insolubilize Zn was shown by rhizobacterial isolates P-11 as it solubilized insoluble zinc up to 15.27 ppm. Minimum quantity of 13.58 ppm solubilized Zn was obtained with the inoculation of isolates P-14.

Zinc is an imperative micronutrient required for optimum plant growth. Zinc solubilizing bacteria are potential alternatives for zinc supplementation and convert applied inorganic zinc to available forms. Various PGPR have found to be effective zinc solubilizers. These bacteria improve the plant growth and development by colonizing the rhizosphere and by solubilizing complex zinc compounds into simpler ones, thus making zinc available to the plants. Hussain reported that Bacillus sp. has shown enhanced growth and zinc content when inoculated in plants [45]. However, reports of zinc solubilization by *Bacillus safensis* were not found.

Screening of Nitrogen Fixation Activity

The selected three bacterial isolates were also cultured on G-NFMM solid and broth medium and incubated. The culture was

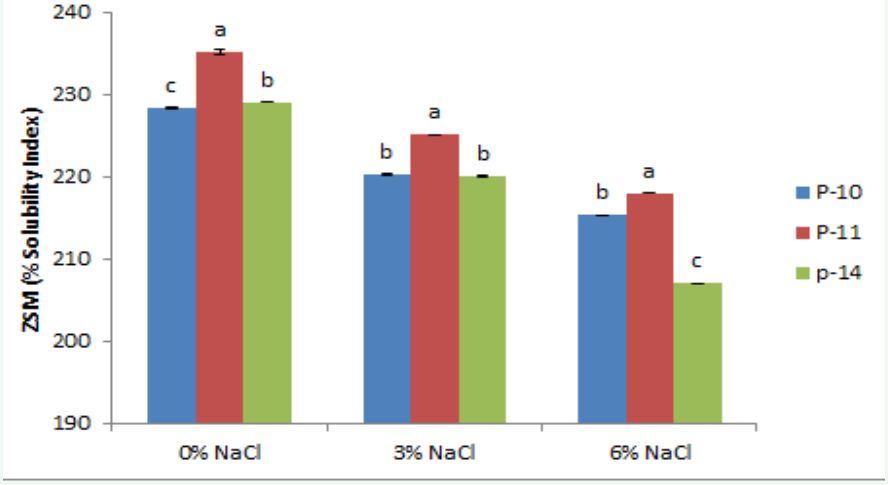

Figure 3 PSI (Percent Solubility Index) of isolated bacteria on Zinc solubilizing medium.

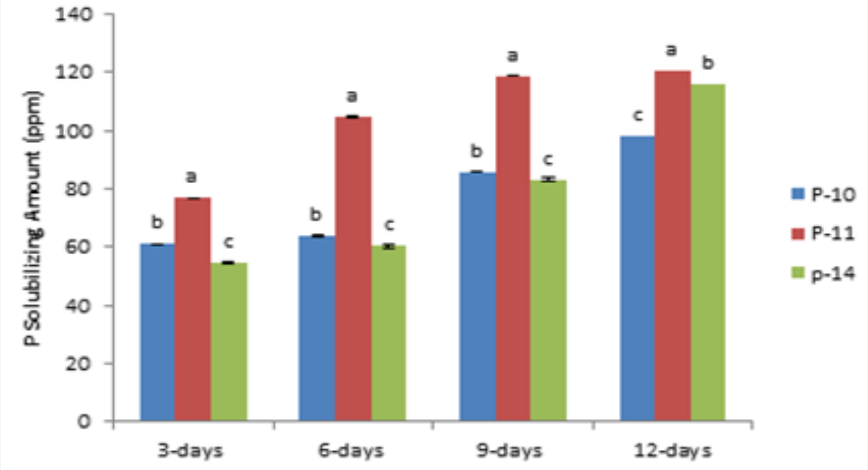

Figure 4 Quantitative measurement of phosphate solubilization amount of isolated bacteria by Vanadomolybdophosphoric yellow color method.

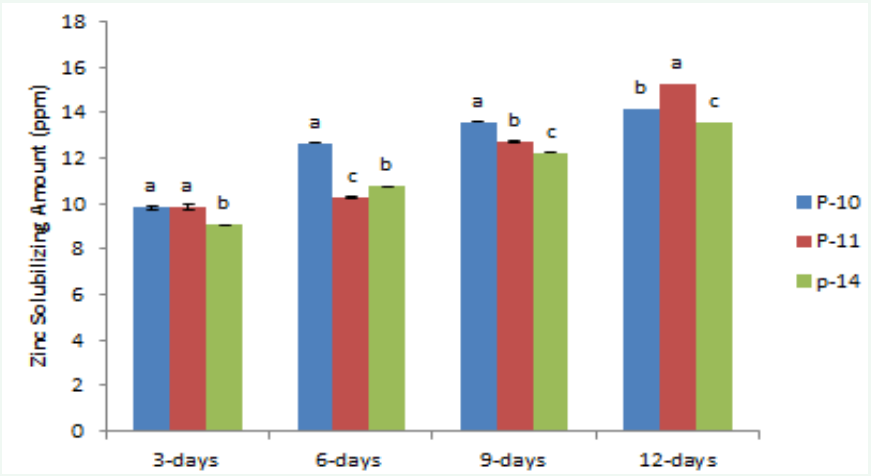

Figure 5 Quantitative measurement of zinc solubilization of isolated bacteria by zinc test kit.

taken after 3, 6, 9 and 12 days incubation for screening of nitrogen fixing activity by test kit. According to the test kit results, it was found that all three isolates could not be detected the nitrogen-fixing activity even though they can grow in the nitrogen free mineral medium.

Siderophore Producing Activity and Antagonistic Activity

Thomashow and Weller (1991) stated that it is important to understand the mechanisms involved in the interactions between microorganisms, pathogens, and host plants for the better use of natural resources in crop productivity management [40]. There are siderophores production, and the mechanisms of antagonism and biocontrol agents Sinha and Parli that has been proved in plant growth-promoting bacteria Yang [41,42]. The siderophore production was assessed by placing 10 ul of overnight culture of selected bacteria on Chrome Azurol S (CAS) solid media prepared by Schwyn and Neilands [21] and the plates were incubated at 30ºC under dark conditions for 7 days. After incubation, all three isolates showed orange halo zone around the bacterial colony on blue colored agar medium (Figure 6).

Ferreira revealed that different *Bacillus* spp. can produce 66 types of antibiotic compounds against bacteria and fungi such as bacillomycin, fengycin, mycosubtilin, and zwittermicin, which are effective in suppressing the growth of target pathogens in vitro [43,44]. The antifungal activity of bacteria isolates was determined by dual culture assay using virulent plant pathogen *Fusarium oxysporum.* In this study, four *F.oxysporum* strains were provided from culture collection of the Department of Life Sciences and Chemistry, Jacobs University Bremen, Germany. It was found that the three isolates could inhibit the growth of fungal DSM 62308 which strain was isolated from *Mathiola incana*, vascular bundle of wilting plant in green house until after 2 weeks incubation, but these isolates could not inhibit the growth of other fungal strains such as DSM 841, DSM 2646 and DSM 62045.

Indole Acetic Acid Production

Indole Acetic Acid (IAA) is a naturally occurring auxin produced by PGPR through L-tryptophan metabolism pathway. IAA increases root growth and length, expands the root surface area, facilitates the absorption of soil nutrients by plants [36]. Bacillus is a spore forming bacteria commonly found in soil and is resistant to unfavorable growing conditions or stress factors, such as temperature, chemicals, etc. Bacillus sp. is qualified to be PGPR since it is able to synthesize various beneficial substances [38]. Several researchers have isolated the genus *Bacillus*, which are able to produce IAA, from soil and rhizosphere, including *B. megaterium, B. thuringiensis, B. pumilus, B. weihenstephanensis, B. cereus, B. toyonensis* and *B. subtilis* [37,39]. *B. safensis* possesses some plant growth-promoting traits and also has promising biotechnological applications due to its ability to produce various industrial enzymes and industrially applicable secondary metabolites. *Kothari reported that B. safensis* affects plant growth, since it is a powerful plant hormone producer, and it also acts as a plant growth-promoting rhizobacteria, enhancing plant growth after root colonization [35]. But, in this study, it was found that the three bacillus isolates could not produce IAA.

Molecular Detection of Trehalose Biosynthesis Marker Genes, otsA and *otsB*

In this case, the primers for *OtsA* and *OtsB* were designed using the *OtsA* and *OtsB* gene sequences of **Escherichia coli str. K-12 substr. MG1655**. Although the designed primer for *OtsA* worked for the three isolates, only P-14 isolates showed the actual size for *OtsB* gene. Therefore it was observed that the designed primer for *OtsB* did not work properly for P-10 and P-11. After PCR amplification, the isolated gene fragments were checked by electrophoresis and all the two gene fragments for P-10, P-11 and P-14 showed the correct sites compared with the Gene Ruler DNA Ladder Mix at 1.0-kb for *OtsA* and only P-14 showed the correct sites at 600-bp for *OtsB* (Figure 6).

CONCLUSION

In the present study, three salt tolerant *Bacillus* rhizospheric soil bacterial strains were isolated from salt affected agricultural soil of tropical area of Myanmar and selected based on both their phosphate solubilization and zinc solubilization potential.

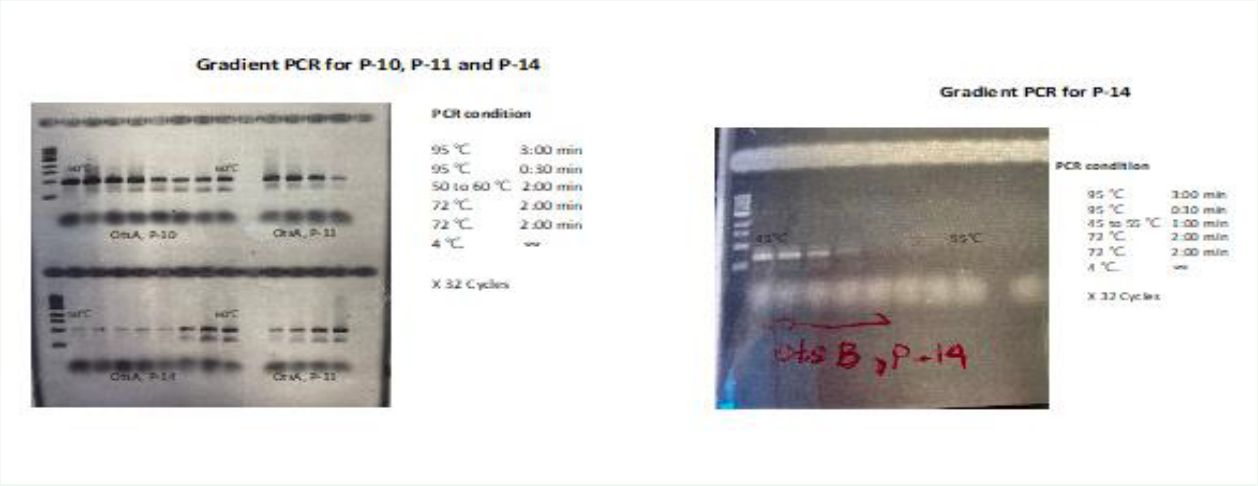

Figure 6 PCR detection of OtsA and OtsB genes from *Bacillus* isolates.

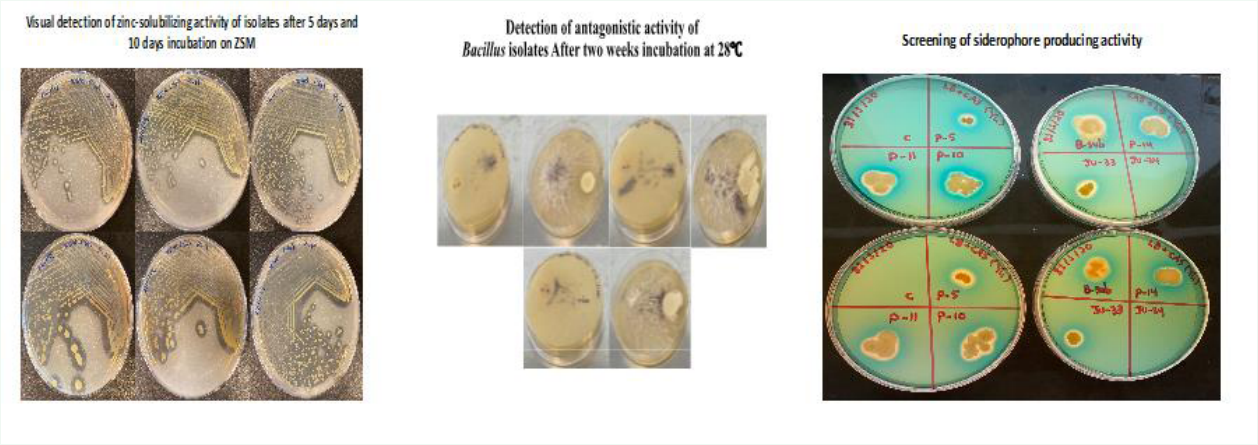

Figure 7 Visual detection of zinc solubilizing activity, antagonistic activity and siderophore producing activity of *Bacillus* isolates.

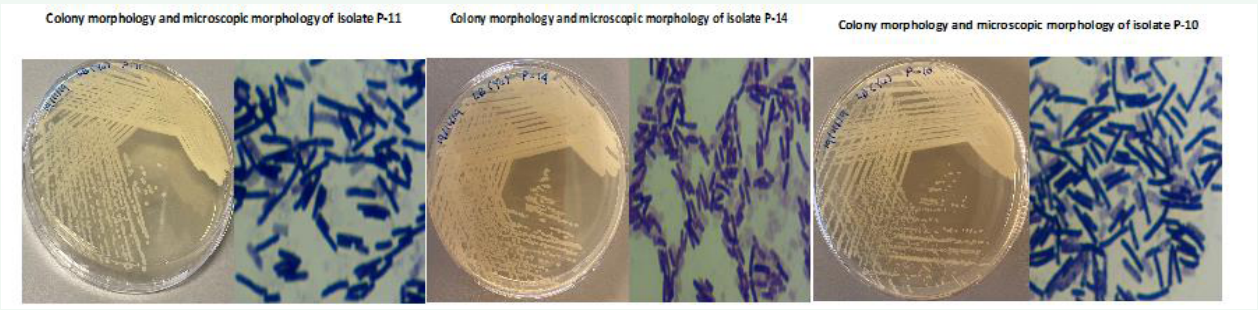

Figure 8 Colony morphology and microscopic morphology (x10) of *Bacillus* isolates.

In addition the *Bacillus* isolates have other PGPR effects such as siderophore producing activity and antagonistic activity. The designed primers of *OtsA* and *OtsB* worked properly for *Bacillus safensis* isolate but not for *Bacillus subtilis* and *Bacillus pumilus* isolates. In the light of these findings, it is obvious that using such useful phosphate and zinc solubilization PGPR as eco-friendly biofertilizer might enhance the growth of economically important crop plants to sustain agriculture under salt stress condition. Further studies are needed to observe their fertilizing effects on the plant and soil conditions.

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CONFLICT OF INTEREST

The Authors declare that they have no conflict of interest.

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