



Isolation and Characterization of Efficient Phosphate Solubilizing *Bacillus* (PSB) from Different Agro-ecological Zones of Tigray Soil, Ethiopia

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ABSTRACT

Applications of biofertilizer have great practical importance for increasing fertility of the soil and reducing environmental pollution. Screening and characterizing phosphate solubilizing *Bacillus* (PSB) strains from different agroecologies of Tigray soil and *in vitro* assessment for the adaptability under different abiotic stress would help in selecting the most efficient strain for use as biofertilizer. A total of 64 soil samples were collected from different agro-ecological zones of Tigray and checked for the presence, capability and efficiency of PSB. Out of 64 soil samples 21 of them were phosphate solubilizing. These phosphate solubilizing strains again incubate at 37°C for 48h on pikovskaya medium to see efficacy of phosphate solubilization. Highest efficiency was recorded in MUB28, MUB47 and MUB64 isolates and also tolerant to acidity and alkaline up to pH4 and pH8, respectively. And difference was recorded in saline tolerance among the strains: MUB47 is the most saline tolerance strain which tolerate saline up to 513mM of sodium chloride (NaCl) but MUB28 and MUB64 have tolerated saline up to 340mM of NaCl. The identification of efficient PSB candidate strains with salt & pH tolerant features in the soil sample could have an implication on the end-users to obtain the desired beneficial effect such as maintaining soil healthy, provision of adequate phosphorus from soil to the plant and improving crop yield of Tigray Agriculture. Based on the morphological, microscopic, biochemical and physiological characterization, it may be inferred that the diversity among these efficient PSB strains is low.

Keywords: Biofertilizer, Phosphate solubilizing *Bacillus*, Efficiency, Diversity, Agroecology, Adaptability, Ethiopia.

1. INTRODUCTION

The demand for increasing productivity necessitates provision of adequate nutrients from the soil. However, most agricultural soils of tropics, including Ethiopia are deficient in sufficient phosphorus, besides nitrogen. It is reported that the two nutrients often limit crop production in Ethiopia (Dibabe, 2000).

Tigray, the most northern region of Ethiopia is generally regarded as the most degraded part of the country. This is manifested in the form of soil erosion, deforestation, declining bio-diversity resources, and soil moisture stress (Taffere, 2003). The leading economic sector of the region is agriculture over 85% of the populations are farmers who depends on the high input demanding

varieties and chemical fertilizers in order to improve crop yield (Edwards and Belay, 2003). However, the cost of these inputs is beyond the purchasing power of most farmers. In addition, prolonged use of chemical fertilizers has resulted in a number of negative side effects on the environment (Edwards and Belay, 2003). Long-term application of chemical fertilizer has caused some plant nutrients to be depleted and other to be deposited in excess in the soil, and consequently the acidity of the soil increased (Reddy et al., 1991). Hence, searching alternative strategies to improve soil health and crop yield without causing damage to the soil and environment is a focus of researchers for a number of years.

Biofertilizers are, therefore, gaining importance as they are eco-friendly, nonhazardous and non-toxic (Sharma et al., 2007). Biofertilizer refers to products consisting of selected and beneficial living microbes, which are added to soil as microbial inoculants. These microbes are known to improve plant health by secretion of growth promoting substances and by increasing the availability of micronutrients (Sharma et al., 2007). Inoculation of phosphate solubilizing *Bacillus* (PSB) results in simultaneous increases in crop yields and phosphorous uptake (Datta et al., 1982). Reports also indicated that *Bacillus* inoculants are increasing wheat yield up to 43% (Kloepper et al., 1989).

Phosphate solubilizing *Bacillus* (PSB) can be isolated from soils of different environmental conditions. It is necessary to characterize its efficiency in terms of phosphate solubilizing ability before PSB is used as biofertilizer. PSB can also be characterized under different morphological, physiological and biochemical traits to see their adaptability for different agroecologies and diversity. This would help in selecting the most efficient strain for use as biofertilizer. This study is, therefore, aiming at isolating and characterizing efficient phosphate solubilizing *Bacillus* from different agroecologies of Tigray soil and assesses their suitability.

2. MATERIALS AND METHODS

2.1. Description of the study area

Tigray region found at northern part of Ethiopia is bordered by Eritrea to the north, Sudan to the west, Afar region to the east and Amhara region to the south. Based on the 2007 Census conducted by the central statistical agency of Ethiopia (CSA), Tigray has an estimated total population of 4,316,988 (CSA, 2013). The leading economic sector of the region is agriculture over 85% of the populations are farmers. However, farming practices are backward and

traditional: farmers ploughing their fields by a pair oxen using the local plough called “Mahresha”, sow and harvest by hand, production increases resulted from expanding cultivated area and not from increasing yield (Taffere, 2003). The agroecological zones and niches of Tigray are diversified with distinct vegetation cover, soil and other natural resources.

2.2. Study Design

PSB was isolated from soils of different environmental conditions and selects the most efficient strain to recommend for the use of bio-fertilizer. Further the isolates were characterized under different morphological, physiological and biochemical traits to see their adaptability for different agroecologies and diversity of the isolates.

2.3. Soil Sample collection

A total of 64 soil samples were collected from different agro-ecology zones of Tigray. The soil samples were collected from the surface of (0-30cm) several spots of each sampling sites and mixed (homogenized). The composite soil samples were then sub-sampled and transported in sterilized polythene bags to Mekelle University, Department of Biology, Microbiology laboratory.

2.4. Isolation of phosphate solubilizing *Bacillus* (PSB)

Bacillus strains were isolated from the soil using a heat treatment at 100°C during 25-30 min. One gram of soil from each sample was suspended in 9 ml of sterilized water to make 1:10 dilution. These were shaken on shaker for 30 minutes to break clogs. Then series of dilution were made (10^{-4} – 10^{-9}). Pikovskaya medium which was autoclaved at 121°C and pressure 15psi for 15 minutes was used to isolate PSB. From the diluted soil sample 0.1ml of suspensions were transferred on Petri dishes containing 15 ml pikovskaya medium. The suspensions were spread uniformly on Petri dishes using glass rod spreader and incubated for 5 days at 30±2°C. Colonies showing clear zone around them were picked and subculture for purification (Pikovskaya, 1948).

2.5. Solubilizing efficiency

All phosphate solubilizing *Bacillus* were spot inoculated on pikovskaya medium and incubated at 37°C for 48 h for detection of their efficacy. Halos surrounding the colonies were measured and the solubilizing efficiency (SE) was calculated as follow (Pikovskaya, 1948):

$$\text{Solubilizing efficiency (SE)} = \frac{\text{Solubilization diameter} \times 100}{\text{Growth diameter}} \text{(Equation - 1)}$$

2.6. Morphological and microscopic characterization of efficient PSB strains

Colonies were characterized on the basis of color, elevation, shape, margin and surface of the colonies. Simple staining was performed to determine cell shape and cellular arrangement. A culture of 24 and 48 hour old efficient PSB strains were gram stained and spore stained, respectively (Nanda, 2005; Prescott et al., 1999).

2.7. Motility test

The motility test determines the presence of flagella, external appendages used by the efficient PSB for movement. An isolate of PSB was inoculated in a tube containing Sulfide, Indole, Motility (SIM) medium with sterile transfer needle. The needle was inserted and with drawn in a straight line in the center of the medium. It was incubated for 48 hours at 30-32°C. Growth away from the line of inoculation indicates that the organism is motile. PSB without flagella do not spread away from the line of inoculation so they are non-motile (Michael and Burton, 2011).

2.8. Biochemical characterization of efficient PSB strains

2.8.1. Starch hydrolysis

Starch agar (20 ml soluble starch (10%), 100 ml melted nutrient agar) was inoculated with young culture of efficient PSB strains and incubated at 32⁰C for 3 - 5 days. The culture was then flooded with Lugol's iodine solution. The enzymatic hydrolysis of starch by amylases was indicated by clear zone around the colony. Unchanged starch gave a blue color (Collins and Lyne, 1970).

2.8.2. Catalase test

Catalase activity was checked by flooding fresh 3% hydrogen peroxide on to 18-24 hour old efficient PSB culture on agar slant the enzymatic hydrolysis of hydrogen peroxide was indicated by bubbles of oxygen, for non-catalase producing bacteria no oxygen bubbles are observed (Michael and Burton, 2011).

2.8.3. Citrate utilization test

Simmon citrate agar was streak plated with a young efficient PSB culture and incubates at 32⁰C for 24 hours. Utilization of citrate was indicating blue color in the medium(Michael and Burton, 2011).

2.8.4. Methyl red test (MR test)

Test tubes containing 5 ml MRVP broths were inoculated with 24 hours old efficient PSB culture and incubate at 30±2⁰C for 2-3 days. Then 5 drops of methyl red indicator were added and gently

rolled between the palms to disperse the methyl red. The development of red color indicates a positive reaction (Pacarynuk and Danyk, 2005).

2.8.5. Vogues proskauer test (VP test)

A test tube containing 5 ml MRVP broths were inoculated with 24 hours old efficient PSB cultures and incubate at $30\pm 2^{\circ}\text{C}$ for 2-3 days. Then one ml of VP reagent-1 (6g of alpha naphthol dissolved in 100ml of 96% ethanol) and one ml of VP reagent-2 (16g KOH dissolved in 100ml double distilled water) were added to the MRVP broth respectively. The formation of pink or red color represents positive VP test or acetone production. A yellow or brown color is a negative result (Pacarynuk and Danyk, 2005).

2.8.6. Urea test

Test tubes containing 5ml urea broth were inoculated with efficient PSB culture and incubate for 48 hours at 35°C . If ammonium is produced as a result of urea hydrolysis, the increase in pH will develop pink colour (Michael and Burton, 2011).

2.8.7. Indole production test

Test tubes containing 5ml SIM medium were inoculated with efficient PSB culture and incubate for 48 hours at $30-32^{\circ}\text{C}$. Subsequently five drops of kovac's reagent was added. The test tubes were allowed to stand. The development of a red color in the top layer will indicate a positive result for indole production and no red color development represents a negative test (Michael and Burton, 2011).

2.8.8. Hydrogen sulphide (H_2S) test

Test tubes containing 5ml SIM medium were inoculated with efficient PSB culture and incubate for 48 hours at $30-32^{\circ}\text{C}$. When H_2S is produced, the strip turns from brown to black (Rangaswami and Bagyaraj, 2005).

2.9. Physiological Characteristics

2.9.1. Salt tolerance

Three strains PSB1, 2 and 6 had significantly increased the germination percentage of the tomato seeds at salt concentration between 0 to 60 mM (Anjali et al, 2013). Accordingly, Nutrient agar (20ml/petriplate) was amended by 171mM, 257mM, 340mM, 513mM, and 856mM of Sodium Chloride (NaCl). A loop full of young efficient PSB culture was inoculated and then incubated at 32°C for 3 days. Bacteria that were able to grow on 171mM-856mM NaCl amended nutrient agar medium was considered as salt tolerant.

2.9.2. pH tolerance

A pH meter was calibrated with buffer solution having pH 4.01, pH 7 and pH 10.01. And then nutrient agar media was adjusted to pH 4, 5, 6, 8 by adding 0.1N of HCl and 0.1N NaOH. Each petri dish containing nutrient agar (20ml/petri plate) having pH 4, 5, 6, 8 was inoculated with a loop full of young efficient PSB culture and incubate at 32°C for 3 days. The pH values of the soil sample from the different agro-ecological zone of Tigray were measured and it was within the range of 5.5-7.5. In vitro the efficient PSB strains had shown a tolerance to acidity and alkalinity beyond the P^H range of the environment (5.5-7.5). For this reason, we considered it as pH tolerant.

2.9.3. Data analysis

Morphological, microscopic, biochemical and physiological character was used to determine the matrix similarity index of the efficient PSB strains using Adansonian method (Sneath and Sokal, 1962).

$$S = \frac{Ns}{Ns+Nd} \times 100 \quad (\text{Equation - 2})$$

Where Ns is the number of similar (positive) characteristics and Nd is the number of different characteristics (positive in one and negative in the other).

3. RESULTS AND DISCUSSION

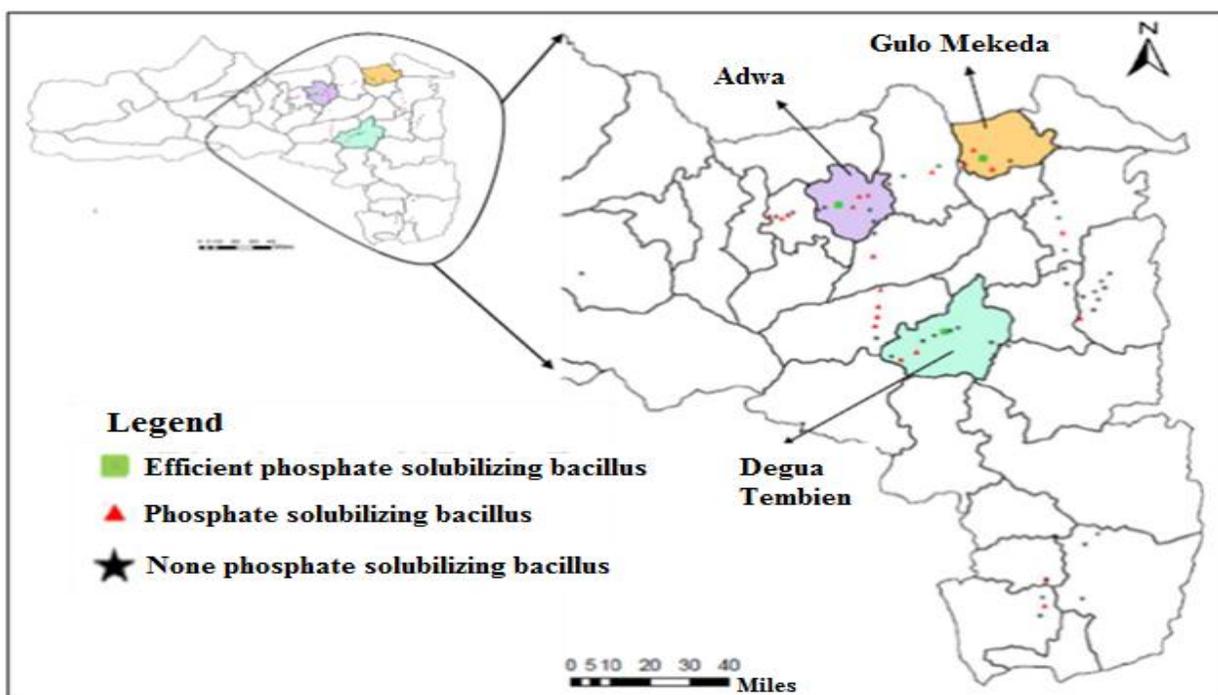
A total of 64 soil samples were obtained from different agro-ecological zones of Tigray. These soil samples were checked for the presence of efficient PSB. It was found that 21 of them were able to form clear zone on pikovskaya medium which indicated that 33% of them were phosphate solubilizing (particularly, calcium phosphate solubilizing) and again 14% of the PSB(3 out of the 21 PSB) were efficient in phosphate solubilizing ability i.e. MUB28, MUB47, MUB64 (Table 1). The specific places where MUB28, MUB47, MUB64 isolates found are “Adigolagul” at an altitude of 2527 m above sea level (asl), “Maydeleata” at an altitude of 1896 m (asl) and “Duhutsa” at an altitude of 2289 m asl, respectively (Fig 1), which are high and moderate land areas of Tigray (Taffere, 2003).

Soil inoculation with phosphate solubilizing bacteria showed improvement in solubilization of fixed soil phosphorus and resulted in higher crop yield (Freitas et al., 1997, Nautiyal, 1999).

Table 1. Phosphate solubilizing abilities of the efficient *Bacillus* strains on calcium phosphate.

Efficient PSB strains	After 48 hours of incubation at 37°C		
	Solubilization diameter (cm)	Growth diameter (cm)	Solubilizing Efficiency (Eq. 1)
MUB28	0.62	0.3	206
MUB47	0.6	0.4	150
MUB64	0.8	0.5	160

Pseudomonas fluorescens and *Bacillus megaterium* strains shows greater than 180 phosphorous solubilizing ability, which was considered as high phosphorous solubilizing efficiency (El-Komy, 2005). Accordingly, MUB28 strain which was isolated from *Hordeum vulgare* (barley) rhizosphere soil recorded higher solubilization efficiency up to 206, while MUB64 and MUB47 isolated from *Hordeum vulgare* (barley) and *Eleusine coracana* (millet) rhizosphere soil showed relatively medium solubilization efficiency up to 160 and 150 respectively (Table 1). For this reason MUB28, MUB47 and MUB64 were identified as efficient phosphate solubilizing strains. It could be then inferred that these efficient isolates were highly promising biofertilizers in increasing agricultural productivity.

Figure 1. Map of Tigray region showing the sampling site for the efficient phosphate solubilizing *Bacillus*.

These efficient PSB strains were further subjected to microscopic, morphological, biochemical and physiological characterization: all the strains were Gram positive, spore forming, rod shaped with circular end and their cellular arrangement was single. The morphological characteristics also clearly indicated that all the strains have circular shape, mucoid surface, flat elevation and with entire margin (Table 2).

Table 2. Microscopic and colony characteristics of the efficient PSB strains.

<i>Efficient PSB strains</i>	<i>Microscopic characteristics</i>					<i>Morphological(Colony) characteristics</i>			
	<i>C. S</i>	<i>C.A</i>	<i>M.T</i>	<i>G.R</i>	<i>S.F</i>	<i>Shape</i>	<i>Surface</i>	<i>Elev.</i>	<i>Margin</i>
MUB28	Rod	Single	+	+	+	C	M	F	E
MUB64	Rod	Single	+	+	+	C	M	F	E
MUB47	Rod	Single	+	+	+	C	M	F	E

Note: C.S= cell shape, C.A= Cellular arrangement, M.T= Motility test, G.R= Gram reaction, S.F= Spore formation, Elev.= Elevation of colony, C= Circular, M=Mucoid, F= Flat, E= Entire

Citrate and starch were utilized as source of carbon. Moreover, difference among the isolates in production of catalase was recorded. The result showed that MUB28 and MUB64 were catalase positive, and MUB47 was catalase negative (Table 3).

Table 3. Biochemical characteristics of the efficient PSB strains.

<i>Efficient PSB strains</i>	<i>Biochemical Characteristics</i>							
	<i>S.H</i>	<i>C.P</i>	<i>U.H</i>	<i>H₂S</i>	<i>I.P</i>	<i>MRT</i>	<i>VPT</i>	<i>C.U</i>
MUB28	+	+	-	-	-	-	+	+
MUB64	+	-	-	-	-	-	+	+
MUB47	+	+	-	-	-	-	+	+

Note: S.H= Starch hydrolysis, C.P= Catalase production, U.H= Urea hydrolysis, H₂S=Hydrogen sulphide test, I.P= Indole production, MRT= Methyl red test, VPT= Vogues proskauer test, C.U= Citrate utilization.

Successful agricultural development results in a significant reduction of poverty and an improvement in food security. To this effect, the construction of dams and development of irrigation schemes provides Tigray farmers with greater food security. Regular monitoring under different agro-ecological zones of Tigray soil for salinization and P^H could help in developing a method for rating efficient phosphate solubilizing bacteria for a particular environment. Therefore, MUB28, MUB47 and MUB64 were evaluated in vitro for their ability to tolerate salt and pH (Table 4). Additionally, the pH values of the soil sample from the different agro-

ecological zones of Tigray were measured and it was within the range of 5.5 – 7.5. In vitro the efficient PSB strains had a tolerance to acidity and alkalinity beyond the P^H range of the environment (5.5 - 7.5). MUB28, MUB64, MUB64 were tolerant to acidity and alkaline up to pH4 and pH8, this makes them highly tolerant to acidic and alkaline environment of Tigray. Thus, these kind of acid and alkaline pH tolerant strains of PSB will have paramount importance as biofertilizers in Tigray.

Table 4. Physiological characteristics of efficient PSB strains.

<i>Efficient PSB species</i>	<i>Salt tolerance</i>					<i>p^H tolerance</i>			
	<i>171mM</i>	<i>257mM</i>	<i>340mM</i>	<i>513mM</i>	<i>856mM</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>8</i>
MUB28	+	+	+	-	-	+	+	+	+
MUB64	+	+	+	-	-	+	+	+	+
MUB47	+	+	+	+	-	+	+	+	+

Difference was also recorded in salinity tolerance among the strains. MUB47 is the most saline tolerance strain which tolerate saline up to 513mM of NaCl but MUB28 and MUB64 had an ability to tolerate saline up to 340mM of NaCl (Table 4). High salinity tolerance organisms can be used for preparation of biofertilizer (Rajankar et al., 2007). Hence the strains could be ideal as phosphate bio-fertilizer.

Table 5. Matrix of Similarity Index among the efficient PSB strains.

<i>PSB Strains</i>	<i>MUB28</i>	<i>MUB64</i>	<i>MUB47</i>
MUB28	100		
MUB64	86.66	100	
MUB47	90	83.33	100

Organisms with a high similarity index, eighty percent (80%) or more, are grouped together (Sneath and Sokal, 1962). The matrix of similarity index of these efficient phosphate solubilizing *Bacillus* strains was more than 80% (Table 5), and this was high similarity index, making it difficult to differentiate them. So, it may be inferred that the diversity among the groups is low.

4. CONCLUSION

Phosphate solubilizing *Bacillus* (PSB) can be isolated from soils of different environmental conditions. It is necessary to characterize its efficiency in terms of phosphate solubilizing ability

before PSB is used as biofertilizer. Out of 64 soil samples 33% of them were phosphate solubilizing this indicates that PSB is common in Tigray soil. Moreover, highest efficiency was recorded in MUB28, MUB47 and MUB64 isolates and also tolerant to acidity and alkaline up to pH4 and pH8, respectively. And difference was recorded in saline tolerance among the strains: MUB47 is the most saline tolerance strain which tolerate saline up to 513 mM of sodium chloride (NaCl) but MUB28 and MUB64 have tolerated saline up to 340 mM of NaCl. These efficient PSB strains showed high similarity index above 80%, it may be inferred that the diversity among these efficient PSB strains is low. The identification of efficient PSB candidate strains with salt & pH tolerant features have an implication on the end-users to obtain the desired beneficial effect such as maintaining soil health, provision of adequate phosphorus from soil to the plant and improving crop yield of Tigray Agriculture. The presence of such efficient PSB strains suggesting to enlist them as candidate strains for further characterization, and application under greenhouse and field condition. This study therefore paves the way for ideal selection of efficient PSB strains with desired features under field condition so as to increase the agricultural productivity of the region.

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