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Morphological and Phenotypic Characteristics of Phosphate Solubilizing Bacteria, Isolated from Acidic soils of Tigray, Ethiopia

Aklil Gebremedhin[1*] and Haile Alene[2]

[1]*Tigrai Agricultural Research Institute, Mekelle Soil Research center, Soil Microbiology Researcher, Ethiopia*
[2]*Tigrai Agricultural Research Institute, Humera Agricultural Research Center, Soil Fertility Researcher, Ethiopia*

**Corresponding author*

Abstract

Phosphate solubilise bacteria (PSB) has the ability to convert insoluble phosphates in to soluble phosphates. Characterization of this bacterium as inoculants in acidic soils increases its phosphorus uptake by the respective plant as well as its productivity. This research was conducted on low pH soils (<6.00) of Tsegede area, located in Western zone of Tigray region. The study revealed that a series of morphological and biochemical test were conducted, all the isolated phosphate solubilize bacteria were indicated gram negative, rod shaped and mucous producing. All of the strains were grew well at pH 6 and 7, temperature 15-35 ^{o}C and at 4% salt concentration. A positive response of the tested isolates was shown for 1.5 mg/l and 3.0 mg/l Mn SO_4 and 0.25 mg/l Zn SO_4. The anticipated significant response of antibiotic resistance was recorded. Hundred percent of the isolates were utilize L-arginine as N source as well as D-glucose, D-fructose and Lactose as of carbon sources. The highest solubilization index (SI) was observed from isolate ESB (2.21) followed by ESE (2.18) and the smallest SI was recorded from CHB, ITA and ESC (1.67). The highest colony diameter was obtained from Chegargudo at soil pH of 5.30 and from Endaslasea at soil pH of 4.92. The lowest colony diameter (4.00mm) was found at soil pH of 5.70 and 5.76. The tested phosphate solubilize bacteria had also a significant response after they grow on PVC broth media, accordingly the highest PS was obtained from FSA (30.70 ESB mg L^{-1}) followed by ITA (29.96 mg L^{-1}). The potential of the collected PSB to release phosphate indicates that a drop down of pH values from7.0 to 4.9.

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Introduction

Phosphorus is one of the major limiting nutrients for plant growth next to nitrogen (N) being abundant in soil in both organic and inorganic forms (Fernandez *et al.*, 2007). Unlike nitrogen, this element is not acquired through biochemical fixation while comes from other sources to meet the agricultural production. Synthetic P fertilizer is the main source of plant available in most agricultural soils, but the majority (75-90%) of the added chemical P fertilizer is fixed by chemical complexes that exist in the soil system like iron (Fe), aluminium (Al) and calcium (Ca) (Turan *et al.*, 2006). Moreover the efficiency applied chemical P fertilizers rarely exceeds 30% due to P fixation, either in the form of iron phosphate or aluminium phosphate in acidic soils (Norrish and Rosser 1983) or in the form of calcium phosphate in neutral to alkaline soils (Lindsay *et al.*, 1989). According to Beyene (1982) more than 70-75% of Ethiopian highland soils are characterized by P

deficiencies, the deficiency is very sever in the acidic soils of the southern, south-western and western regions. Areas with high in Al^{+3} and Fe^{+3} are totally incriminated with high P fixation (Sertsu and Ali, 1983). A greater part or approximately 95 to 99% of soil organic and inorganic phosphorus is present in the form of insoluble phosphates which is easily bound by the existing Al or Fe in acidic soils, or Ca and Mg in alkaline soils which cannot be utilized by the plants (Yang *et al.,* 2010). With high levels of exchangeable Ca, available P ions react with solid phase CaCO3 and precipitate on the surface of these particles to form Ca-P minerals (Lindsay *et al.*, 1989). Converting insoluble phosphates (both organic and inorganic) to a form available for plants is a necessary goal to achieve sustainable agricultural production. Several reports show the ability of diverse bacteria to solubilize inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate (Sashidhar and Podile, 2010). Hence, a large amount of chemical phosphorus fertilizer has been used to alleviate agricultural production or plant growth, and has negative impact in respect to environment and economy.

To minimize the environmental impact and farmer's economy phosphate solubilise bacteria as an alternative source of chemical fertilizer is important in the sustainable agricultural production. Use of microbial inoculants possessing P-solubilization in soils is environmental-friendly as an alternative to further application of chemical P fertilizers (Zaidi *et al.,* 2009).

Among the bacterial genera reported to express phosphate solubilization are Pseudomonas, Bacillus, rhizobia, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium and Erwinia have been isolated from soils (Rodriguez and Fraga, 1999). These bacteria can grow in media containing calcium phosphate complexes as the sole source of P, solubilize and assimilate a large proportion of P, and release P in high amounts. Phosphate is solubilized via organic acid synthesis and released by microorganisms (Puente *et al.,* 2004). This reaction, appearing as a halo or clear zone on the plate, is used to assess the P-solubilizing activity of these bacteria. Undoubtedly, the selection of considerably efficient PSB strains as possible inoculants will be a promising way to release large amount of P from soil to improve the current status of extensive chemical fertilizer usage. Therefore this research was determined and characterizations of phosphate solubilize bacteria as an alternative source of fertilizers to acidic soils.

Materials and Methods

Sample site and Collection

The study site is located in the Western Zone of Tigray Region between 37° 16'0" to 37°30'0" East longitude and 13° 18' 40"- 13°22' 0" North latitude and in the range of 2319 to 2939 masl altitudinal range with highland agro-ecological zones. It is characterized by diverse physio geographic features with high and rugged mountains, flat topped plateau, deep gorges, incised river valleys and rolling plains.

A total of 60 sub-soil samples were collected from an oxen plough depth by inserting sterilized auger to protect contamination and polyethylene plastic bags prior to pyisco-chemical and biochemical characterization containing 30, 15 and 15 sub samples from Endassilasie, Chegarkudo and Intabela kebeles respectively. Out of these ten (10) composite soil samples were collected and transported to Mekelle Soil Research Center, Soil Microbiology Laboratory. The collected soil samples were coded, labelled and backed by their pH level in polyethylene plastic bags to determine the *Rhizobial* population or bacterial growth. Off the collected soil samples two samples from Chegargudo coded as CH-A and CH-B, Three samples from Intabela (IT-A, IT-B and IT-C) and the remaining Five samples from Endaslasea (ES-A, ES-B, ES-C, ES-D and ES-E) were stored at 4^{O}c refrigerator for farther biochemical test and the remaining soil samples air dried, powered, well mixed to represent a single sample and grounded to pass through 2mm and 0.5mm sieve. Details of the physical and chemical properties of the study site were listed in Table 1.

Isolating Phosphate solubilising bacteria

One gram (1gm) of soil were weighed from the bulk soil samples and dispersed in 9 ml of autoclaved distilled water. One ml (1ml) of the dispersed solution was transferred to 9 ml of sterilized distilled water to form 10^{-2} dilution. Similarly 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} serials were made for each soil sample. The serially diluted (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) soil samples were plated (0.1 ml) on *Pikovaskya's* agar medium containing tricalcium phosphate (TCP) as phosphate source. The *Pikovaskaya's* agar medium contains 10 g glucose, 0.5 g yeast extract, 0.5 g NH_2SO_4, 0.1 g $MgSO_4$ $7H_2O$, 5 g $Ca_3(PO_4)_2$), 0.2 g $NaCl_2$, 0.2 g KCl_2, 0.001 g $MnSO_4$ $2H_2O$, 0.001 g $FeSO_4$ $7H_2O$, 15 g Agar and dissolved in 1000 ml distilled water. Prior to isolating *Pikovaskya's*

agar media was weighed using sensitive balance, added to volumetric flask, filled with deionised distilled water and adjusted its pH to neutral (7.0), sterilized in autoclave and then after purred to sterilized Petri plates. After overnight cooling of each petri plate's isolation was done using the standard procedures of ten folds serial dilution and spread plate method. One gram (1g) of soil sample was weighed from each and suspended in to 9 ml test tubes having sterilized distilled water, soaked overnight and thoroughly shakes using electrical mixer (vertox). One millilitre (1ml) of the suspension was again transferred to 9 ml of autoclaved and sterilized distilled water to form 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} was made serials to each sample. 0.1 ml of each dilution was spread on newly prepared *Pikovaskaya's* agar medium containing insoluble Tricalcium phosphate (TCP) and incubated at 27-30^{0}C for 7 days. After 7 days of incubation period colonies showing halo zones were picked and purified by 5 times subculture method on *Pikovskaya's* (PVK) agar medium for studying colony morphology. The pure halo zone forming colonies were stored at 4^{O}c refrigerator by inserting them in to a slant test tube for farther biochemical characterization.

Morphological and Physiological Characteristics

A loop full of the purified culture were streaked on newly prepared Pikovaskaya's media to determine the morphological and physiological characteristics of each phosphate solubilize bacteria.

Gram Stain Test

The pure cultures of bacterial isolates were kept for their gram staining to quantify specific identification of the isolates either gram negative or gram positive. The staining was done in laminar air flow hood under light microscope.

Colony Morphology

The morphology of isolates was examined on YEM agar plates using log phase culture.. After 2-3 days incubation period at 30 ^{O}C, individual colony was characterized on the basis of colony- shape, size, colour, texture and Gram stain reaction.

Acid-Base Production Test

The newly prepared yeast extract manitol agar (YEMA) was enriched with 25µgml^{-1} Bromothymol blue to selectively identify the fast and slow growing *Rhizobia*. All the isolated bacterial strains were subjected to pass on BTB test in triplicates. After incubation of the tested isolates on BOD incubator at 28±2 ^{o}C for 48 hours a color change was observed for their fast or slow growers.

Temperature, salinity and pH resistance

Of these isolates were tested by incubating them at the 10-45^{O}C temperature range with 5 ^{O}C interval, pH ranges from 4.0 to 9.5 with 0.5 interval and 0.5%, 1%, 2%, 3%, 4% and 5% (w/v) of NaCl respectively in triplicates,, Control plates were incubated at 28±2 ^{O}C for 3-5 days.

Phenotypic and Biochemical test

To evaluate the phenotypic and biochemical characteristics of the selected phosphate solubilize bacteria recent microbiology methods and a series of procedures were used by triplicate each isolate.

IAR

The resistance of the rhizobial isolates to specified IAR were determined by streaking on solid YEM medium containing filter sterilized the selected antibiotics using 0.22 µm size membrane filters (mg/L) including Ampicillin (5 and 10), Erythromycin (5 and 10), Rifampicin (5 and 10) and Streptomycin (5 and 10mg/l) (Beynon and Josey, 1980). Except Erythromycin others were dissolved in sterilized distilled water (Somasegaren and Hoben, 1994) and the stock solutions were added to autoclaved YEMA media cooled approximately to 45ºC.

Metal Salts

All the isolates were tested for their sensitivity to metal salts in triplicates. Freshly prepared YEM agar plates were amended with metal salts $MnSO_4$ $7H_2O$ at 1.5, 3.0, 6 and 12 mg/l and $ZnSO_4$ $7H_2O$ at 0.25, 0.5, 1 and 2mg/l. Effect of metal salts was determined by *Rhizobial* growth after incubating the plates at 30 ^{O}C for 3-5 days on BOD incubator.

Carbon and Nitrogen source utilization

Rhizobial isolates were further investigated for their ability to utilize different carbon and nitrogen sources. Loop full of 72 hours old YEM broth culture of each rhizobial isolate was streaked, on YEMA medium containing D-glucose, D-fructose and lactose as well as Glycine, Alanine, L-arginine and Urea as carbon and

nitrogen source (Amargar *et al.,* 1997). The plates were incubated at 28±2 ^{o}C for 5-7 days and growth was recorded as (+ve) for presence of growth or (-ve) absence of growth in relation to the positive control of YEMA plates.

Phosphate Solubility

The quantitative and qualitative analysis of phosphate solubility was conducted by plate and broth culture method.

Qualitative Measurement of PS

One over ten mL of each PSB culture preserved in sterile distilled water was placed on *Pikovskaya's* agar plates containing insoluble tricalcium phosphate 2.5 g, glucose 10g, $(NH_4)_2SO_4$ 0.5 g, NaCl 0.2 g, $MgSO_4.7H_2O$ 0.1 g, KCl 0.2g, Yeast Extract 0.5 g, $MnSO_4$ trace, $FeSO_4.7H_2O$ trace, Agar 15 g, pH adjusted to 7.2 and dissolved in 1000 ml distilled water.

Three day old culture isolates with 10^{-8} viable cells ml^{-1} were streaked on newly prepared and sterilized *Pikovskaya's* agar plates then incubated at 28 ± 2^{O}C for 7 days. After 7 days of incubation, the clear halo zone and colony diameter were measured and the phosphate solubilisation index (SI) was calculated following the formula indicated in (Edi-Premono *et al.,* 1996).

$$SI = \frac{Colony\ Diameter + Hallo\ zone\ diameter}{\text{Colony Diameter}}$$

Quantitative Measurement of PS

100ml of *Pikovskaya* broth was prepared without phosphate source and dispensed in 250 ml Erlenmeyer flasks. In each flask, about 0.5g of tri-calcium phosphate (Ca_3 $(PO_4)_2$) was added and sterilized at 121ºC at 15 psi for 15 minutes. Then after 1ml of culture containing about 10^8 cells ml^{-1} suspensions of each isolates was inoculated into the medium and kept at 28 ± 2ºC in shaker incubator for about 12 days.

All the experiments were carried out in triplicate. 10 ml of each isolate was withdrawn at regular intervals of 3 days and was examined for soluble phosphate and pH changes using spectrophotometer and digital pH meter, respectively, following the method cited in (Subba Rao, 1993).

Results and Discussion

Isolation and Identification of Phosphate Solubilize bacteria (PSB)

In the present study the collected soil samples were subjected to phosphate solubilizing bacteria in *Pikovaskaya's* solid and liquid media. A total of Eight (8) phosphate solubilize bacterial colonies were isolated on *Pikovaskaya's* agar medium having tri-calcium phosphate (TCP) from agricultural soil. Upon 3-7 days of incubation period the largest halo zone (5.67 mm) were recorded from isolates ESA and ESB followed by ESE (4.33 mm); while the smallest halo zone diameter (2.67 mm) was measured from isolate ESC. Similarly, the highest solubilization index (SI) was observed from isolate ESB (2.21) followed by ESE (2.18) and the smallest SI was recorded from CHB, ITA and ESC (1.67) (Table 1). Variations of the halo zone formation and SI could possibly the difference in; isolates type, natural environment, soil pH, soil management and agricultural practices (Table 1), therefore, the possibility of isolating indigenous strains from diverse environment could avoid the competition with indigenous microbes (Haile *et al.,* 2016). According to Supriya *et al.,* (2017) report bacterial colonies showing solubilisation index ≤7mm were selected for P quantification and the current result agreed to this conclusion. The colonies showing clear halo zones around the microbial growth were considered as phosphate solubilizing bacteria. Different studies stated that halo zone formation around the bacterial colonies was created due to the production of organic acids, polysaccharides or due to the activity of phosphatase enzymes of phosphate solubilizing bacterial (Paul and Sinha, 2013).

Cultural and Growth Characteristics of Isolates

The growth characteristics of the tested isolates were taxonomically classified based on their cultural and morphological properties. Soil pH, gram stain and colour are some of the cultural properties and colony morphology and diameter are some of the morphological properties. Accordingly, all the tested isolates were gram negative, rod shaped motile bacteria with round colony margin on solid media, yellow colour after they grow on BTB, no colour absorbance after they grow on YEMA having congo red (Table 1). All of the tested isolates were shown large mucoid colony morphology except ITB and ESC. According to the research indicated by Mideksa *et al.,* (2015) and Mulata (2009) most agricultural soils had more gram negative than gram

positive. The current study is in line to this research. As indicated in table 1 the colony diameter and solubilisation index were ranged from 3.67 to 5.33 mm and 1.67 to 2.21 mm respectively. The highest colony diameter (5.33 mm) was obtained from CHB (Chegargudo) at soil pH of 5.30 and from ESA (Endaslasea) at soil pH of 4.92.

The lowest colony diameter (3.67 mm) was found from ESE at soil pH of 5.26 followed by ESC and ITC at soil pH of 5.70 and 5.76 respectively which is in the range of moderately acidic. The highest SI (2.21) were obtained from ESB at soil pH of 5.26 followed by ESE (SI= 2.18) at similar soil pH, this is also similar to the above pH classification as moderately acidic (Tekalign, 1991).

Similarly, the lowest SI (1.67) was obtained from soil pH of 5.30, 5.32 and 5.76 respectively, which is in the same pH range (Moderately acidic). According to Kumar *et al.,* (2018) and Satyaprakash *et al.,* (2017) report the principal mechanism for solubilisation of soil P is lowering of soil pH by microbial production of organic acids. Strong correlation was observed between solubilisation indexes and releasing of organic acids or protons (Alam *et al.,* 2002).

Physiological characteristics

All the screened phosphate solubilise bacteria were subjected to various physiological characteristics such as resistance to salinity, pH and temperature in triplicates. Accordingly, all the tested isolates were highly resistance to $NaCl_2$ concentration starting from 0.5 % to 2.5 %, pH 7.5 to 9.5, as well as 15-35 ^{O}c temperature levels (Table 2). As the concentration of NaCl rises beyond 3.0 % except two strains the remaining tested strains were insignificant growth and none of them were grown at 5 % NaCl concentration and pH 4.

Biochemical Test

The selected isolates were farther investigation on various biochemical properties to indicate their capability of resistance and their ability to utilize different carbon and nitrogen sources. Accordingly; all the tested isolates had shown positive response to 1.5 mg/l and 3.0 mg/l Mn SO_4, 0.25 mg/l Zn SO_4, 10 mg/l Rifampicin and 5 mg/l Ampciline, and hundred percent of the isolates were utilize L-arginine as N source as well as D-glucose, D-fructose and Lactose of carbon sources. None of the isolates were utilizes L-alanine and urea from the selected N sources (Table 3). ITB had grown well at the given Mn SO_4, Zn SO_4 and relatively highly resistance to the selected antibiotics.

Qualitative Test

Qualitative phosphate solubilisation potential was likely observing the largest clear halo zones around the bacterial growth on Pikovaskaya agar media and PSI was calculated. Phosphate solubilisation index (PSI) of the selected isolates was in the range of 1.67 to 2.21 at soil pH range of 4.92 - 5.76 similar results were obtained from *Vicia faba* L. of Ethiopian soils, with soil pH (4.8 - 6.3) as well as SI in the range of 1.25 to 2.10 (Girmaye *et al.,* 2014). In contrary to this Gebremedhin *et al.,* (2019) also obtained SI in the range of 1.10 to 2.67 at soil pH of moderately acidic to moderately alkaline (5.90 to 8.11) which is the optimum pH for growth of the isolates. Of the collected isolates three of them ESA, ESB and ESE were recorded > 2.00 SI; these isolates had collected or originated from the same field management and similar soil pH (4.92-5.26). Highest SI was obtained from ESB isolate (2.21) followed by ESE isolate (2.18) at similar soil pH of 5.26 which is moderately acidic and the lowest SI (1.67) was obtained from CHB, ITB and ESC isolates respectively. Superior solubilisation index was obtained by Alia *et al.,* (2013) from roots of vegetables that elevates from 1.8 to 5.0.

Quantitative Test

Quantitative PSB was measured using PVC broth culture containing tricalcium phosphate $(Ca_3 (PO_4)_2$ with 3 days interval, and evaluated by measuring the soluble P (mg L^{-1}) and the changes in pH as indicated in Table 4. As shown in figure one all the measured values were extracted from P standard curve.

Accordingly, the amount of solubilised P released by the isolates exhibited wide variation ranging from 18.13 to 30.70 mg L^{-1}, with a significant drop in pH from 7.00 to 4.75 (Table 4). Ca_3 $(PO_{4)2}$ containing medium solubilisation is slow in the first three days of incubation and exponentially increase in the next two days (day 2 and day 3) (fig. 3); while it decreases at day 4 or after the coming 3 days of incubation period (fig. 3). The highest solubilisation was observed from isolate ESB (25.97 mg L^{-1}) and ITC 25.55 mg L^{-1}) in the first 3 days of incubation. The mechanisms of solubilisation were indicated lowering of pH values by production of organic acids or releasing protons (fig.4). In the 12th day of, pH in the culture medium decline to 4.75 from an initial pH of 7.00.

Table.1 Growth, morphological and cultural characteristics of PSB

PSB isolate	Soil pH	pH (Tekalign, 1991)	G. CR	G. BTB	Gram stain	C. Morphology	CD. (mm)		
							C	H	SI
CHB	5.30	MA	CL	Y	-	LM	5.33	3.67	1.67
ITA	4.98	SA	CL	Y	-	LM	4.67	4.00	1.86
ITB	5.32	MA	CL	Y	-	LW	5.00	3.33	1.67
ITC	5.70	MA	CL	Y	-	LM	4.00	3.67	1.92
ESA	4.92	SA	CL	Y	-	LM	5.33	5.67	2.06
ESB	5.26	MA	CL	Y	-	LM	4.67	5.67	2.21
ESC	5.76	MA	CL	Y	-	LW	4.00	2.67	1.67
ESE	5.26	MA	CL	Y	-	LM	3.67	4.33	2.18

Where; MA moderately acidic, SA strongly acidic, CL color less, Y Yellow, LM Large mucoid, LW Large watery, CD colony diameter, C colony, H, halozone, SI solubilisation index.

Table.2 Physiological characteristics of phosphate solubilize bacteria on solid media

Physiological Characters		Phosphate Solubilize Bacterial Isolates								% of resistance
		CHB	ITA	ITB	ITC	ESA	ESB	ESE	ESC	
NaCl levels	0.5%	+	+	+	+	+	+	+	+	100
	1%	+	+	+	+	+	+	+	+	100
	1.5%	+	+	+	+	-	-	+	+	80
	2%	+	+	+	+	+	+	+	+	100
	2.5%	+	+	+	+	+	+	+	+	100
	3.0%	-	-	+	-	+	-	-	-	20
	3.5%	-	-	+	-	+	-	-	-	20
	4.0%	-	-	+	-	+	-	-	-	20
	4.5%	-	-	+	-	+	-	-	-	20
	5%	-	-	-	-	-	-	-	-	0
pH levels	4.0	-	-	-	-	-	-	-	-	0
	5.0	-	-	+	+	+	-	+	-	40
	6.0	-	+	+	+	-	-	+	-	40
	7.5	+	+	+	+	+	+	+	+	100
	8.5	-	+	+	+	+	-	-	-	40
	9.5	+	+	+	+	+	+	+	-	90
Temperature	15 ^{O}c	+	+	+	+	+	+	+	+	100
	20 ^{O}c	+	+	+	+	+	+	+	+	100
	25 ^{O}c	+	+	+	+	+	+	+	+	100
	30 ^{O}c	+	+	+	+	+	+	+	+	100
	35 ^{O}c	+	+	+	+	+	+	+	+	100

Table.3 The efficacy of the isolated PSB on different biochemical test

Biochemical Characters		Phosphate Solubilize Bacterial Isolates							
		CHB	ITA	ITB	ITC	ESA	ESB	ESE	ESC
$MnSO_4$ levels (mg/l)	1.5	+	+	+	+	+	+	+	+
	3.0	+	+	+	+	+	+	+	+
	6.0	+	+	+	-	-	+	+	-
	12.0	+	-	+	-	+	-	-	-
% of resistance isolate		100%	75%	100%	50%	75%	75%	75%	50%
$ZnSO_4$ levels (mg/l)	0.25	+	+	+	+	-	-	+	-
	0.5	-	+	+	+	-	-	+	-
	1.0	-	-	+	-	-	-	+	-
	2.0	-	+	+	-	-	-	-	+
% of resistance isolate		10%	75%	100%	50%	0%	0%	75%	10%
Antibiotics levels (mg/l)	5 Eritrimycine	+	-	+	-	+	+	+	+
	10 Eritrimycine	+	-	+	-	+	+	+	+
	5Striptomycine	-	-	+	-	-	+	+	+
	10 Striptomycine	-	-	+	-	-	-	+	+
	5 Rifampicin	+	-	-	+	-	+	+	+
	10Rifampicin	+	-	+	+	+	-	+	+
	5 Ampciline	-	+	+	+	+	+	+	+
	10 Ampciline	+	-	-	+	-	+	+	+
% of resistance isolate		62.5%	10%	75%	50%	50%	75%	100%	100%
N sources	Glycine	-	-	+	-	-	-	+	-
	Alanine	-	-	-	-	-	-	-	-
	Arginine	+	+	+	+	+	+	+	+
	Urea	-	-	-	-	-	-	-	-
% of resistance isolate		10%	10%	50%	10%	10%	10%	50%	10%
C source	Glucose	+	+	+	+	+	+	+	+
	Fructose	+	+	+	+	+	+	+	+
	Lactose	+	+	+	+	+	+	+	+
	Galactose	+	+	+	+	+	+	+	+
	Dextrose	+	+	+	+	+	+	+	+
	Sorbitol	+	+	+	+	+	+	+	+
% of resistance isolate		100%	100%	100%	100%	100%	100%	100%	100%

Table.4 Quantitative estimation of phosphate solubilization bacteria in broth media

PSB	3 days incubation period		6^{th} day Incubation period		9^{th} day incubation period		12^{th} day incubation period	
	PS (mg/l)	pH	PS (mg/l)	pH	PS (mg/l)	pH	PS (mg/l)	pH
ESB	25.997	5.75	27.493	5.60	0	0	0	0
ESC	18.134	5.75	10.922	5.43	19.008	5.44	17.137	4.75
ITC	25.55	5.40	26.969	5.33	27.419	5.40	20.329	5.00
ENB	25.118	5.73	27.493	5.62	22.004	5.42	13.53	5.06
ESA	23.942	5.82	30.701	5.71	1.444	5.66	8.873	5.12
CHB	23.236	5.87	28.052	5.57	26.908	5.48	15.582	4.93
ITB	24.319	5.66	24.319	5.52	26.421	5.60	14.679	4.89
ITA	24.319	5.34	29.961	5.21	4.966	5.20	17.551	5.31

Fig.1 Description of the study site

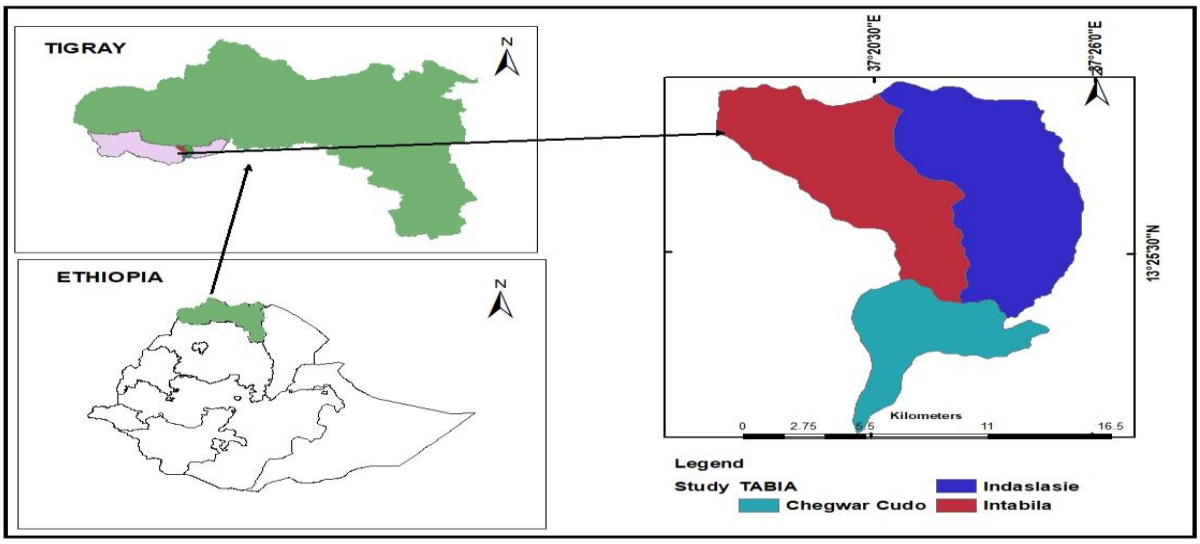


Fig.2 Standard Calibration Curve

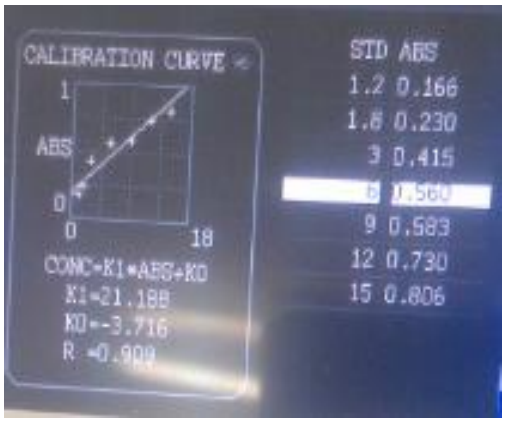


Fig.3 Phosphate solubilise activities

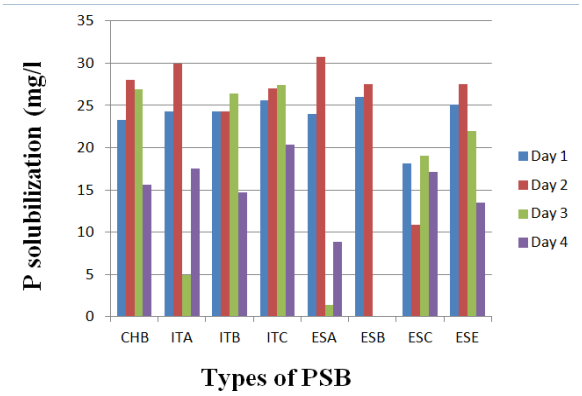
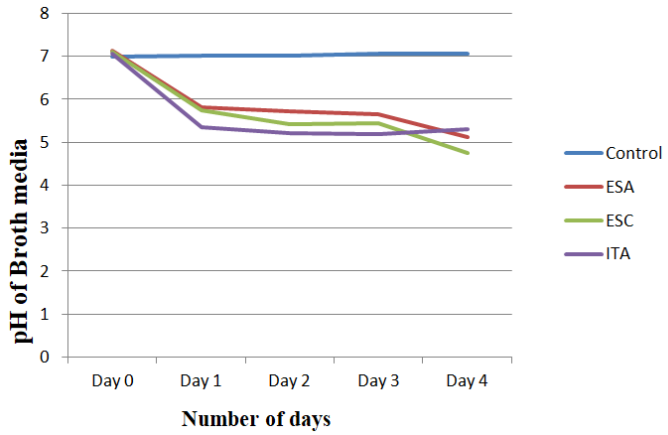


Fig.4 Change in pH during the incubation period



It can conclude that the phosphate solubilizing rhizobia exhibited a broad range of ability of solubilising TCP in *vitro*. Most of the tested isolates were able to tolerate wide range of pH, NaCl, temperature, antibiotics as well as utilized wide range of biochemical testes. Results found an inverse correlation between amount of solubilized phosphate and pH of the culture medium. Isolate that are effective in N_2 fixation and able to solubilise TCP are found to be effective in improving nodulation and plant growth under greenhouse condition. Further research is recommended to investigate its efficacy under field trials in diverse soil types having different amount of soil P.

Conflict of interest

Authors have declared no conflict interests exist.

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