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Characterization of Plant Growth Promoting Bacteria from Sugarcane (*Saccharum officinarum* L.) Rhizosphere of Wonji-Shoa Sugar Estate and Farmers Landraces of Ethiopia

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ABSTRACT
Application of chemical fertilizers apart from its low use efficiency, environmental impact and soil quality degradation, was not cost effective in the Ethiopian sugar sector. Thus, looking attractive opportunities which are environmentally friendly approaches and cost effective for the sustainability of the sector like use of biological fertilizers is of paramount importance. Therefore, the objective of this study was to preliminarily screen effective plant growth promoting bacteria from sugarcane fields. One hundred sixteen rhizosphere isolates was taken from sugarcane fields. The isolates were characterized and tested under laboratory condition for their phosphate solubilizing characteristics. Moreover, 23% and more than 60% of the isolates could produce indole acetic acid and ammonia, respectively. Isolate HWR27 showed significantly ($p \leq 0.05$) the highest solubilization index (3.32) on solid Pikovskaya media. The lowest solubilization index was recorded for the isolate HWR8 (2.33). Three different isolates (HWR24, HWR27 and HWR48) having plant growth promoting characteristics were evaluated for their solubilization efficiency under three different liquid media (tricalcium phosphate, rock phosphate and bone meal). Among these isolates, HWR48 solubilized the highest amount of phosphate (88.41 mg L^{-1}) from tricalcium phosphate and the same isolate solubilized 33.57 mg L^{-1} of phosphate from rock phosphate. The solubilization of phosphorus was complimented with reduction in pH. Further research to evaluate the effectiveness of the isolates on growth of sugarcane under greenhouse and field condition would be recommended.

Key words: Plant growth promoting bacteria, sugarcane rhizosphere, phosphate solubilizing

INTRODUCTION

Ethiopia was one of the countries with highest sugarcane yield in the world (208.9 t ha^{-1} (ha^{-1}) in 1978 cropping season), however, the yield declined to 126.9 t ha^{-1} in the 2010 cropping season (FAO., 2010). Moreover, the juice phosphate content of sugarcane in the factories is below the standard <300 mg kg^{-1} (Sundara, 2000) and declining from time to time. While in the Estates, fertilizer is applied at a level of 69-276 kg N ha^{-1} in the form of urea plus 50 kg P ha^{-1} in the form of Di-Ammonium Phosphate (DAP) (Ambachew and Abiy, 2009a, b; Ambachew and Ademe, 2009).

Despite the high level of chemical fertilizer application, the use efficiency is low. Less than 50% of the applied urea is used by plants (Halvorson *et al*., 2002) and 75-90% of added P is rapidly precipitated as insoluble forms and becomes unavailable to plants (Goldstein, 1986). In addition, the long-term conventional cropping practices in general, cane

burning, excessive tillage and over use of chemicals in particular, have substantially degraded the major soil quality indicators of the soils of sugarcane plantations (Alemayehu, 2010). In addition, the prolonged use of chemical fertilizers to enhance soil fertility and crop productivity has often negative effects on the complex system of the biogeochemical cycles (Steinshamn *et al*., 2004). The use of chemical fertilizers can cause leaching and run-off of nutrients, especially N and P, leading to environmental degradation (Gyaneshwar *et al*., 2002).

To alleviate such problems, the use of biological fertilizer sources is promoted in many parts of the world (Avis *et al*., 2008; Leach and Mumford, 2008). Therefore, microorganisms in the soils are critical to the maintenance of soil function, because of their involvement in key processes such as soil structure formation, decomposition of organic matter, toxin removal and the cycling of carbon, N, P and sulfur (Van Elsas, 1997). In addition, microorganisms play key roles in suppressing soil borne plant diseases and in promoting plant growth and changes in vegetation (Doran *et al*., 1996). Thus, soil microorganisms with beneficial activities on plant growth and health; provide an attractive alternative to conventional agriculture (Antoun and Prevost, 2006). In this regard, application of Phosphate Solubilizing Bacteria (PSB) indicates beneficial effects on sugarcane growth, yield and juice quality. For instance, 25% reductions in the amount of P fertilizer and 12.6% increment in cane yield were obtained when P fertilizer was used in combination with PSB (Sundara *et al*., 2002).

The economic benefit of sugarcane production in Ethiopia is dwindling from time to time due to the rising cost of fertilizer. Moreover, there is no information on the use of biofertilizers for sustainable sugarcane production in the country. Therefore, this study was initiated to preliminarily screen the existence of effective Plant Growth Promoting Bacteria (PGPB) from sugarcane fields for future application with the specific objectives:

- To isolate plant growth promoting bacteria and characterize isolates based on cultural, morphological, biochemical and physiological features and
- To evaluate the phosphate solubilizing capacity and antagonistic effect of the isolates

MATERIALS AND METHODS

Description of the study areas and sample collection: Sugarcane rhizosphere soil samples were collected from commercial sugarcane fields of Wonji-Shoa and from non-commercial growing areas of Yloubancho, Kereyu Harzuna and Yelen Kebeles. Wonji-Shoa is located at 8°31' North latitude and 39°12' East longitude at an altitude of 1500 masl. Yloubancho Kebele is located at 6° 56.06' North and 38°22.793' East at an altitude of 2042 masl. Kereyu Harzuna Kebele is located at 7° 42.648' North and 39° 35.844' east at an altitude of 1848 masl. Yelen Kebele is located at 10° 04.978' North and 39°53.606' East at an altitude of 1235 masl. Samples were labeled and kept individually in plastic bags and preserved in ice boxes to prevent from any contamination and damage.

Isolation of rhizosphere bacteria: One gram of rhizosphere soil was suspended in nine milliliter of sterilized distilled water. An aliquot (100 μL) from 10^7 serial dilutions were inoculated on King's B medium and incubated at 28°C for 48 h (King *et al*., 1954).

Purification, preservation and characterization of isolates: The cultures were purified by picking a single well isolated colony of the rhizosphere bacteria, transferred into the King's B medium and sub-cultured on separate plates until uniformity were maintained. The purified cultures were transferred to slants and when sufficient growth was observed stored at 4°C (King *et al*., 1954). The cultures were coded based on their place of identification (Haramaya), sample source (Wonji-Shoa, Yloubancho, Harzuna and Yelen Kebele) and sampling area (Rhizosphere) by taking the first letters except for the two Kebeles (Yloubancho and Yelen takes the first two letters) (HWR, HYlR, HHR and HYeR) and numbered based on the number of isolates of each sample source. Isolates were characterized on the basis of the cultural, morphological, biochemical and physiological characteristics.

Production of indole acetic acid: Indole acetic acid production was detected as described by Bric *et al*. (1991). The supernatant (2 mL) obtained from fully grown for 72 h was mixed with two drops of orthophosphoric acid (H_3PO_4) and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid ($HClO_4$), 1 mL 0.5 M $FeCl_3$ solution). The development of pink color indicates IAA production.

Production of ammonia: Freshly grown cultures were inoculated in 10 mL peptone water in each tube and incubated for 48 h at 36±2°C. Nessler's reagent (0.5 mL) was added in each tube. The development of brown to yellow color indicated a positive test for ammonia production (Cappuccino and Sherman, 1992).

Antagonistic test: Bacterial isolates were screened for their antagonistic activity towards the major economical sugarcane disease *Ustilago scitaminea* by dual inoculation *in vitro* assay on Waksman agar medium (Berg *et al*., 2002). Whips of the pathogen were collected from Wonji-Shoa infected sugarcane plants, preserved in clean paper bags and dried in the laboratory. The spore was separated from impurities by sterilized 150 μm sieve and grown on malt extract agar medium for 48 h. An exact 6 mm diameter of agar plug with mycelium of the pathogen was placed on the center of the Waksman agar medium plat by using cork borer. The composition of the medium was protease peptone 5 g, glucose 10 g, meat extract 3 g, NaCl 5 g, agar 20 g and distilled water 1000 mL (Berg *et al*., 2002). Forty eight hours old bacterial isolates were spotted on the same plate, approximately 3 cm

distance from the pathogen. The plates were incubated at 28±2°C and checked for zone of inhibition of the fungus growth after five days of incubation. The measurement of growth inhibition zone was done by using the following equation (Panhwar *et al*., 2012).

$$\text{Inhibition in radial growth (\%)} = \frac{r_1 - r_2}{r_1} \times 100$$

where, r_1 is the radial fungus growth in control and r_2 is the radial fungus growth in treatment.

Evaluation of isolates for phosphate solubilizing capacity: Isolates were evaluated on solid and three different phosphate source liquid media for their phosphate solubilizing capacity in triplicates.

Preliminary screening of isolates using solid media: Phosphorus solubilizing capacity of isolates was carried out on Pikovskaya medium containing $Ca_3(PO_4)_2$ as insoluble P source. The medium was composed of: The 10 g glucose, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.001 g $MnSO_4$. The $7H_2O$, 0.001 g $FeSO_4.7H_2O$, 0.5 g yeast extract, 15 g agar and 5 g $Ca_3(PO_4)_2$ in one liter distilled water (Pikovskaya, 1948). The isolates were incubated at 30°C for seven days. Isolates showing clear zones around the colonies within seven days were coded positive for P solubilization. The solubilization index was measured by calculating the ratio of the total diameter (colony+halo zone) and the colony diameter (Premono *et al*., 1996).

Evaluation of effective isolates for phosphate release in liquid media: One hundred milliliter of Pikovskaya broth was prepared without phosphate source and dispensed in 250 mL Erlenmeyer flasks and to this, 500 mg of tricalcium phosphate $Ca_3(PO_4)_2$, 2.4 mg of rock phosphate and 400 mg of bone meal was separately added to each flask. The flasks were sterilized at 121°C and 15 psi for 15 min. Then 1 mL of culture containing about 3×10^7 cells mL^{-1} suspensions of each isolates was inoculated into the medium. The flask were kept on shaker and incubator for about 16 days. The P solubilizing rates of each isolates was monitored at 4, 8, 12 and 16 days interval and pH changes were recorded on similar days using digital pH meter (Jenway brand). Soluble phosphorus was determined following the method cited in Rao (1993). The insoluble material was filtered through Whatman filter paper number 42 and filtrate was centrifuged at 6,000 rpm for 30 min. Ten milliliter of the filtrate was taken into 50 mL volumetric flask to which 2.5 mL of Barton's reagent was added and the volume was adjusted to 50 mL with distilled water. The optical density of yellow color developed after 10 min was measured at 430 nm by using spectrophotometer and the concentration of available phosphorus (mg kg^{-1}) was calculated from standard phosphorus (KH_2PO_4) curve.

Statistical analysis: Data on colony diameter, halo zone diameter, solubilization index, pH of culture media and P concentration were subjected to analysis of variance by using statistical analysis computer software version 9.1. Duncan's Multiple Range Test was used to compare means of phosphate solubilization efficiency of isolates on solid media and least significant difference test was used to compare means of phosphate solubilization efficiency of isolates in liquid media at $p<0.05$.

RESULTS AND DISCUSSIONS

Isolation and characterization of isolates: One hundred sixteen rhizosphere bacterial isolates were identified from the sugarcane rhizosphere soil samples. Based on the characteristics described in the Bergey's Manual of Determinative Bacteriology (Krieg and Holt, 1984), attempts were made to classify them in to their respective genera. Accordingly the rhizosphere isolates were found to belong to the genus *Pseudomonas*.

Production of indole acetic acid: All of the 116 isolates were tested for IAA production, of which 27 of the rhizosphere isolates were able to produce IAA (Table 1). Khalid *et al*. (2004) reported that approximately 80% of the rhizosphere bacteria can produce IAA. Similarly Kumar *et al*. (2012) also found most of the rhizosphere bacteria can produce plant growth promoting hormone i.e., IAA. Indole acetic acid is one of the most important phytohormone and function as important signal molecule in the regulation of plant development. It has been reported that IAA production by PGPB can vary among different species and strains and also influenced by culture conditions, growth stage and substrate availability (Mirza *et al*., 2001).

Production of ammonia: Among the 116 isolates only seventy five isolates were able to produce ammonia (Table 1). Ammonia production is an important trait of PGPB that indirectly influences plant growth. Kumar *et al*. (2012) found almost all efficient isolates could be able to produce ammonia.

Antagonistic test: Among the 116 isolates of the rhizosphere bacteria, only two isolates (HYeR12 and HYlR1) were found to inhibit the growth of *Ustilago scitaminea* (sugarcane smut disease) after five days of dual inoculation *in vitro* assay on Waksman agar medium (Table 1). The isolate HYeR12 inhibits 71.4% of the growth of the fungus whereas the isolate HYlR1 inhibits 69.8% (data not shown). Fatima *et al*. (2009) and Noori and Saud (2012) found 55-99 and 51-65% inhibition of the two pathogens *Rhizoctonia solani* on wheat and *Pyricularia oryzae* on rice, respectively.

During their growth period, isolate HYlR1 was at first covered by the fungus after 72 h of incubation, later from the 96 h onwards the isolate form clearance zone around the bacterial cultures. The dimension of inhibition circle was ranged from 0.7-1.0 cm in the two isolates, while the control plates were fully covered by the fungus.

Table 1: Plant growth promoting characteristics of isolates

IAA positive isolates	Ammonia positive isolates	Antagonistic isolates	PSB positive isolates
HWR2, HWR10	HWR24, HWR33	HYeR12 and HYlR1	HWR2, HWR4
HWR48, HWR53	HWR2, HWR3, HWR4, HWR5, HWR6		HWR8, HWR16
HYeR3, HYeR15	HWR8, HWR10, HWR11, HWR12		HWR22, HWR24
HHR4, HWR4	HWR15, HWR16, HWR17, HWR18		HWR26, HWR27
HWR14, HWR27	HWR24, HWR26, HWR27, HWR28		HWR29, HWR30
HWR36, HWR50	HWR29, HWR30, HWR31, HWR33		HWR31, HWR32
HWR56, HYeR5	HWR35, HWR36, HWR38, HWR39		HWR42, HWR44
HYlR4, HHR15	HWR43, HWR44, HWR48, HWR49		HWR48, HWR56
HWR7, HWR22	HWR50, HWR53, HWR54, HWR55		HWR59, HYeR2
HWR29, HWR40	HWR57, HWR58, HWR60, HWR61		HYeR5, HYeR6
HWR51, HWR58	HWR62, HYeR3, HYeR6, HYeR9		HYeR7, HYeR11
HYeR10, HYlR18	HYeR12, HYlR1, HYlR2, HYlR4		HYlR3, HYlR9
and HHR18	HYlR6, HYlR7, HYlR10, HYlR11		HYlR11, HYlR12
	HYlR12, HYlR13, YlR15, HYlR16		HYlR14, HHR4
	HYlR17, HHR1, HHR2, HHR7, HHR9		HHR12, HHR17
	HHR11, HHR12, HHR16, HHR19		
	HHR20		

IAA: Indole acetic acid, PSB: Phosphate solubilizing bacteria

Preliminary screening of isolates using solid media: From the total of 116 isolates, 30 rhizosphere isolates were positive for phosphate solubilization on solid Pikovskaya medium (Table 1). Out of the positive isolates 15 isolates were effective having clearance zone of >1 mm (Premono *et al.*, 1996). Colony diameter, halo zone diameter and solubilization index (based on colony and halo zone diameter) for effective isolates are presented in Table 2. The highest colony diameter was recorded by isolate HHR17 (6.67 mm) followed by isolates HYlR14 (6.33 mm), HYeR7 and HWR2 (6.00 mm), HWR42, HYeR2 and HYlR11 (5.67 mm) and HWR59 (5.33 mm) but they are not statistically different from each other. The smallest colony diameter was also recorded by isolate HWR24 (3.67 mm). The highest halo zone diameter was recorded by the isolate HWR27 (10.67 mm) followed by isolates HHR17 (9.33 mm) and HYlR14 (9.00 mm), however, the smallest halo zone diameter was recorded by isolate HWR24 (6.00 mm). The halo zone as criteria is not enough for PSB selection, as many isolates that could produce small halo zones on solid media could conversely mobilize significant amount in liquid media (Gupta *et al.*, 1994). However, while screening a large number of microorganisms this method can be regarded as generally reliable for isolation and preliminary characterization of PSB (Rodriguez and Fraga, 1999). Isolate HWR27 had significantly ($p \leq 0.05$) different and higher solubilization index (3.317) than the other effective isolates. Even if isolate HWR24 had smallest colony and halo zone diameter, it had higher solubilization index (2.69) next to HWR27 (3.317) but not statistically different from other isolates. The smallest solubilization index (2.33) was recorded by isolate HWR8 (Table 2). Alam *et al.* (2002) also found solubilization index ranging from 1.69-3.29 from maize rhizosphere isolates.

Table 2: Phosphate solubilization efficiency of isolates on solid media

Isolates	Colony diameter (mm)	Halo zone diameter (mm)	Solubilization index
HWR2	6.00^{abc}	8.33^{bc}	2.390^{b}
HWR8	5.00^{bcd}	6.67^{efd}	2.333^{b}
HWR24	3.67^{d}	6.00^{f}	2.690^{b}
HWR26	5.00^{bcd}	7.67^{ecd}	2.533^{b}
HWR27	4.67^{cd}	10.67^{a}	3.317^{a}
HWR42	5.67^{abc}	8.00^{bcd}	2.420^{b}
HWR48	4.67^{cd}	6.33^{ef}	2.367^{b}
HWR56	4.67^{cd}	6.67^{efd}	2.433^{b}
HWR59	5.33^{abc}	8.67^{bc}	2.633^{b}
HYeR2	5.67^{abc}	8.67^{bc}	2.543^{b}
HYeR7	6.00^{abc}	8.00^{bcd}	2.340^{b}
HHR17	6.67^{a}	9.33^{ab}	2.423^{b}
HYlR11	5.67^{abc}	7.67^{ecd}	2.357^{b}
HYlR12	4.67^{cd}	6.67^{efd}	2.433^{b}
HYlR14	6.33^{ab}	9.00^{bc}	2.430^{b}
Mean	5.31	7.89	2.509
CV (%)	13.46	10.69	7.550

Means within a column followed by the same letter are not significantly different at $p>0.05$

Evaluation of effective isolates for phosphate release in liquid media: Three isolates were evaluated in liquid media. The isolates were selected based on their solubilization index result (isolates HWR27 and HWR24), multipurpose isolate (positive for phosphate solubilization, IAA and ammonia production) (isolate HWR48). The value of P (mg L^{-1}) solubilized in the three different liquid media (Tricalcium Phosphate (TCP), Rock Phosphate (RP) and Bone Meal (BM)) and the changes in pH of the corresponding media are presented in Table 3, 4 and 5. In the TCP media, the amount of solubilized P obtained from all of the isolates were significantly ($p \leq 0.05$) different from the un-inoculated control in all incubation period except at the zero days of incubation. Also significant ($p \leq 0.05$) decrease in pH was recorded in all isolates incubation period compared with the un-inoculated control. The highest amount of solubilized P was recorded for the isolate HWR48 (88.41 mg L^{-1}) followed by isolates HWR24 (78.73 mg L^{-1}) and HWR27 (67.94 mg L^{-1}) at 16 days of incubation. The solubilization of P was accompanied by the decrease in pH from 6.85 at zero day of incubation to 4.51 at 16 days of incubation. The lowest pH was recorded for isolate HWR24 (4.51) followed by isolates HWR48 (4.90) and HWR27 (5.33) at the 16 days of incubation (Table 3). Sharma *et al.* (2012) from tea rhizosphere and Qian *et al.* (2010) from shallow eutrophic lake

Table 3: Tricalcium phosphate solubilization efficiency of isolates

	0 Days		4 Days		8 Days		12 Days		16 Days	
Isolates	pH	P (mg L^{-1})	pH	P (mg L^{-1})	pH	P (mg L^{-1})	pH	P (mg L^{-1})	pH	P (mg L^{-1})
HWR24	6.83	13.17	5.84	66.19 (16.55)	5.55	70.60 (8.83)	5.54	76.35 (6.36)	4.51	78.73 (4.92)
HWR27	6.80	13.21	5.92	59.41 (14.85)	5.89	64.52 (8.07)	5.85	67.14 (5.60)	5.33	67.94 (4.25)
HWR48	6.85	13.33	5.79	45.12 (11.28)	5.78	55.36 (6.92)	5.76	68.89 (5.74)	4.90	88.41 (5.53)
Control	6.85	13.37	6.81	17.74 (4.44)	6.66	21.19 (2.65)	6.56	25.87 (2.16)	6.54	28.10 (1.76)
Mean	6.83	13.27	6.09	47.11	5.97	52.92	5.93	59.56	5.32	65.80
CV (%)	0.92	3.28	1.09	8.93	1.65	4.87	3.15	4.87	7.59	3.50
LSD (0.05)	NS	NS	0.12	7.98	0.18	4.98	0.34	5.47	0.70	4.40

Values in parenthesis are amount of P in mg L^{-1} solubilized per day, CV: Coefficient of variation, LSD: Least significant difference, NS: Not significant at $p>0.05$

Table 4: Rock phosphate solubilization efficiency of isolates

	0 Days		4 Days		8 Days		12 Days		16 Days	
Isolates	pH	P (mg L^{-1})	pH	P (mg L^{-1})	pH	P (mg L^{-1})	pH	P (mg L^{-1})	pH	P (mg L^{-1})
HWR24	6.99	0.95	4.16	2.02 (0.51)	3.93	14.48 (1.81)	3.78	15.20 (1.27)	3.77	22.94 (1.43)
HWR27	6.99	0.91	4.28	6.75 (1.69)	4.24	18.02 (2.25)	4.11	21.39 (1.78)	3.69	29.68 (1.86)
HWR48	6.97	0.91	4.20	2.38 (0.60)	4.27	17.46 (2.18)	4.19	25.48 (2.12)	3.73	33.57 (2.10)
Control	7.01	1.03	6.63	2.58 (0.65)	6.55	5.16 (0.65)	6.49	6.40 (0.53)	6.26	6.11 (0.38)
Mean	6.99	0.95	4.82	3.43	4.71	13.78	4.62	17.11	4.14	23.08
CV (%)	1.38	16.41	3.88	13.14	3.45	10.21	1.26	7.37	6.51	5.53
LSD (0.05)	NS	NS	0.34	1.24	0.30	2.51	0.11	2.52	0.49	2.58

Values in parenthesis are amount of P in mg L^{-1} solubilized per day, CV: Coefficient of variation, LSD: Least significant difference, NS: Not significant at $p>0.05$

Table 5: Bone meal solubilization efficiency of isolates

	0 Days		4 Days		8 Days		12 Days		16 Days	
Isolates	pH	P (mg L^{-1})	pH	P (mg L^{-1})	pH	P (mg L^{-1})	pH	P (mg L^{-1})	pH	P (mg L^{-1})
HWR24	6.94	0.79	6.48	10.75 (2.69)	7.58	4.86 (0.61)	7.47	3.77 (0.31)	7.86	1.94 (0.12)
HWR27	6.95	0.83	6.04	4.96 (1.24)	7.77	1.06 (0.13)	8.23	4.24 (0.35)	8.26	4.09 (0.26)
HWR48	6.95	0.95	6.65	9.72 (2.43)	7.14	4.20 (0.53)	7.25	4.36 (0.36)	7.68	4.84 (0.30)
Control	7.00	0.91	6.92	2.34 (0.59)	6.81	1.81 (0.23)	6.68	1.82 (0.15)	6.89	1.35 (0.08)
Mean	6.96	0.87	6.52	6.94	7.33	2.98	7.41	3.55	7.67	3.06
CV (%)	1.49	17.35	3.90	5.91	0.94	7.71	1.87	7.20	1.85	14.24
LSD (0.05)	NS	NS	0.44	0.92	0.12	0.52	0.25	0.48	0.26	1.03

Values in parenthesis are amount of P in mg L^{-1} solubilized per day, CV: Coefficient of variation, LSD: Least significant difference, NS: Not significant at $p>0.05$

found isolates able to solubilize P (from TCP) ranged from 40.62-136.73 mg P L^{-1} and 4 -170 mg P L^{-1} with the drop of pH, respectively.

The rock phosphate solubilization efficiency of isolates and their corresponding pH change in liquid media of at different incubation period are presented in Table 4. The highest amount of soluble P was recorded for isolate HWR48 (33.57 mg L^{-1}), followed by HWR27 (29.68 mg L^{-1}) and HWR24 (22.94 mg L^{-1}) at 16 days of incubation. There was significant ($p\leq0.05$) pH change of the media inoculated with the isolates (from 6.99-3.69) compared to the un-inoculated control (7.01-6.26). The lowest pH was recorded for isolate HWR27 (3.69) followed by isolates HWR48 (3.73) and HWR24 (3.77) at the 16 days of incubation. There was significant ($p\leq0.05$) solubilization of bone meal over the un-inoculated control by all tested isolates at 4 and 12 days of incubation and by two isolates at 8 and 16 days of incubation. The highest amount of solubilized P (10.75 mg L^{-1}) was recorded at 4 days of incubation period by the HWR24 isolate. Even if there was significant difference in the amount of solubilized P and pH values compared with the un-inoculated control in bone meal media, the solubilization of P and pH change trend over the incubation periods was not comparable to that of TCP and RP. At 4 days of incubation period all of the test isolates solubilized relatively highest amount of P from BM and also showed lowest pH values. There after the amount of solubilized P was declined at the 8 days of incubation, however, at the 16 days of incubation period the amount of solubilized P was raised to some but the pH values were in increasing trend. The possible reason for this was due to the difference in the solubilization mechanisms of inorganic P and organic P sources and the interactions of orthophosphate and phosphatase. This finding was similar with that of Qian *et al.* (2010) and Keneni *et al.* (2010). Qian *et al.* (2010) noted that in the early stage of the experiment, the activities of alkaline phosphatases were strongly stimulated due to the low contents of orthophosphate but they were gradually inhibited accompanied by an increase of orthophosphate concentration in supernatant. When the mineralization was too weak to balance the assimilation and catabolism of bacteria, orthophosphate content began to decrease while phosphatase activity started to recover.

Evaluation of efficiency of each isolates on the solubilization of the three P sources showed that HWR48 was relatively effective in TCP followed by HWR24. Similarly, isolates HWR48 was relatively effective in solubilization of RP followed by isolate HWR27. All isolates were not effective on solubilization of BM as compared to the TCP and RP

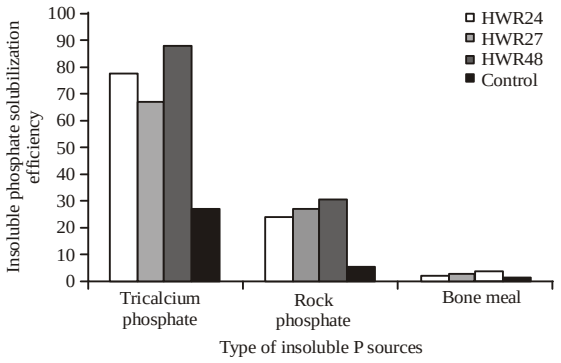

Fig. 1: Insoluble phosphate solubilization efficiency of isolates

(Fig. 1). The highest amount of solubilization was achieved for TCP with isolate HWR48 followed by RP by the same isolate. Even if the amount of solubilized P from BM was lower than the two P source; all the selected isolates solubilized significantly greater amount of insoluble P over the un-inoculated control. There was little amount of phosphorus solubilized at the zero days of the incubation and throughout the incubation period by the control treatment. The possible reason for this was due to the release of PO_4^{-3} during autoclaving. Keneni *et al*. (2010) and Haile *et al*. (1999) found similar results on the zero days.

CONCLUSION

Intensive use of chemical fertilizers over long years degrades the soil qualities which in turn affects the productivity of the sugar sector. Therefore, use of low grade TCP and RP fertilizers supplemented with phosphate solubilizing organisms having also PGP characteristics may partly contribute to soil fertility improvement and for sustainable productivity of the sugar sector. Thus Isolates HWR27, HWR24 and HWR48 for their inorganic phosphate solubilization and Isolates HYeR12 and HYlR1 for their antagonistic effects towards the major sugarcane disease, *Ustilago scitaminea* should be further studied under greenhouse condition.

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