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Physicochemical Properties of four Phosphate-solubilizing Bacteria Isolated from the Semi-arid Regions of North Gujarat, India

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ABSTRACT

The rhizosphere of several plants was screened for phosphate solubilizing bacteria (PSB) from eight locations of North Gujrat. Thirty-three PSB were isolated in the study. Four PSB isolates with the highest phosphate solubilization index between 1.55–2 were characterized morphologically and biochemically and identified through 16S rRNA sequencing as *Klebsiella aerogenes, Klebsiella pneumoniae, Kocuria flava and Enterobacter hormaechei*. The growth of isolates was measured by plotting an optical density-based semi-logarithmic growth curve. The isolates were measured for P solubilization in Pikovskaya broth. The isolate *K. flava* LC515414 solubilized maximum Ca_3PO_4 (7.63 µm P mL^{-1}). The acid and alkaline phosphatase activity of the isolates was also measured for 96 h in the growth medium. After 96 h of growth, the isolate *K. flava* LC515414 had the highest final acid and alkaline phosphatase activity of 4.68 U L^{-1} and 5.67 U L^{-1}.

Keywords: Physicochemical properties, phosphate solubilizing bacteria, North Gujarat

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INTRODUCTION

To feed the planet's growing population with limited land resources, the adoption of sustainable agricultural practices is necessary. However, agricultural sustainability cannot be achieved until we opt for energy-neutral or less energy-intensive agricultural practices. Biofertilizer application is a "greener" practice with limitless possibilities in crop improvement. It involves the use of some beneficial microorganisms for plant growth optimization. The bioinoculant application in the agricultural field is known to increase farm income through extra yields and input cost reduction (Barrett and Marsh 2002; Mulongoy et al. 1992; Son et al. 2008). Application of bioinoculants to the host plants serves as a biofertilizer (nitrogen fixation, phosphate solubilization, etc.), bio stimulator (phytohormone production), stress regulator (drought and salinity) and biocontrol agent against phytopathogens (Tallapragada and Seshagiri 2017). Bacteria-based biofertilizers offer a wide range of opportunities to develop better agro-practices. The potential biological fertilizers could play a vital role as sustainable, eco-friendly and cost-effective input (Itelima et al. 2018). Among the bioinoculant traits, P-solubilization is a vital phenomenon associated with several rhizosphere bacteria. The phosphate solubilizing bacteria (PSB) help plant growth through several mechanisms like lowering soil pH by acid production, iron chelation and exchange reactions in the growth environment. As a result, PSBs are emerging as important organisms for soil improvement and plant growth promotion.

MATERIAL AND METHODS

The root system of several plants was collected from eight locations of the Banaskantha district of North Gujarat, India. The plant roots were uprooted in such a way that their root system remains covered with soil. The samples were put in a plastic bag and brought to the laboratory for PSB isolation. The PSBs were isolated from rhizosphere soil through serial dilution on Pikovskaya's medium (Pikovskaya 1948). One g root from each sample was suspended in 9 mL sterilized distilled water in 10 mL test tubes to make 1:9 dilution. Several dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were made. From the 10^{-3}, 10^{-4} and 10^{-5} dilutions, 0.1 mL suspension was transferred onto Petri plates containing Pikovskaya's medium. The bacteria seeded Petri plates were incubated at 30±1°C for three days. The colonies exhibiting surrounding clear zones were selected and purified by streaking on fresh Pikovskaya's agar plates (Ranjan et al. 2013). The bacteria with the highest activity were screened based on the zone diameters through point inoculation (Babu et al. 2017). The phosphate solubilizing index (PSI) was calculated as the ratio of the diameter of halo (cm)/diameter of the colony (cm) (Kumar and Narula 1999). The bacterial cultures were maintained as 15% glycerol stocks containing nutrient broth at -20°C.

Characterization and identification of PSB isolates

The PSB isolates were characterized based on morphological and cultural features of the colonies and through a series of biochemical tests like urease detection, phenylalanine deamination, nitrate reduction, H_2S production and utilization of adonitol, arabinose, citrate, glucose, lactose, lysine, ornithine and sorbitol. For biochemical testing, the 24-hour young cultures of PSB isolates were streaked on every section of HiAssorted™ biochemical test kits. The kit plates were incubated at 30±1°C for 24 h and compared with the provided catalogue. The isolates were 16s rRNA sequenced

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and the sequences were searched on National Center for Biotechnology Information (NCBI) website for homology. The sequences of the identified isolates were submitted to the DNA Data Bank of Japan (DDBJ) database and the accession numbers were obtained.

Growth Measurement of PSB Isolates

Sterile nutrient broth flasks were inoculated with PSB isolates followed by incubation at 30°C for 12 h. The optical density of the inoculated broth was measured spectrophotometrically at 600 nm every 6 hours for 24 h.

Estimation of Inorganic Phosphorus from the Growth Medium

Inorganic P in the growth medium was estimated through Fiske-Subbarow method. Aliquots of standard P solution were pipetted into a series of tubes. Each tube (including blank) was added with 0.4 mL 10% trichloroacetic acid or 60% perchloric acid, 0.4 mL ammonium molybdate solution, 0.2 mL of amino-naphthol-sulfonic acid (ANSA) reagent and 4 mL double-distilled water. The tubes were mixed well and kept for 5 minutes to see the development of blue color in the solution. The solution in the tube was filled in cuvettes and optical density (OD) was measured at 640 nm. A standard graph was prepared with P concentrations and OD values on the x and y-axis.

Acid and alkaline phosphatase assay

Phosphatase enzyme was extracted using 10 mL sterilized nutrient broth contained in 20 mL sterilized test tubes. The test tubes containing nutrient broth were inoculated with 1 mL bacterial culture in three replicates and incubated at 37°C for 48 h. After incubation, the tubes were withdrawn and centrifuged at 5,000 rpm for 10 min at 4°C. The cell-free supernatants were withdrawn after 24, 48, 72 and 96 h from centrifuge tubes and assayed for acid and alkaline phosphatase activities.

The acid phosphatase endpoint assay was determined as per the method of (Bergmeyer and Bernt 1974) by using p-nitrophenyl phosphate (pNPP), a colorless substrate that produces a colorimetric product p–nitrophenolate (light yellow color). The 0.5 mL 90 mM acetate buffer (pre-equilibrated at 37°C at 10 min, 8.8 pH) was mixed with 0.5 mL 15.2 mM substrate. The alkaline phosphatase endpoint assay was determined as per the method of Bernt (1974). The 0.5 mL 100 mM glycine NaOH buffer (pre-equilibrated at 37°C for 10 min at 8.8 pH) was mixed with 0.5 mL of 15.2 mM pNPP substrate. The 0.1 mL cell-free supernatant was mixed with acidic or alkaline phosphatase-specific buffer and incubated at 37°C for 10 min. After incubation, the reaction was stopped by adding 4 mL NaOH, followed by spectrophotometric observation of absorbance at 410 nm and the OD values were extrapolated.

RESULTS AND DISCUSSION

PSB screening

Thirty-three PSB isolates were screened from the rhizosphere soil of several plants (Fig. 1 **and Table 1**). The PSBs were screened on Pikovskaya's agar plates. The medium displayed translucent P solubilization zones around the bacterial colonies due to the extracellular secretion of gluconic acid (Gaur 1990; Stephen and Jisha 2011). Out of 33 microbial isolates, four with the highest PSI between 1.55 – 2 were selected for further analysis. In previous studies, several species of P solubilizing *Bacillus* and *Pseudomonas* were isolated from the rhizosphere of chickpea, cluster bean okra, chilli, tomato, cotton and eggplant (Baliah and Begum 2015; Baliah et al. 2016; Midekssa et al. 2016).

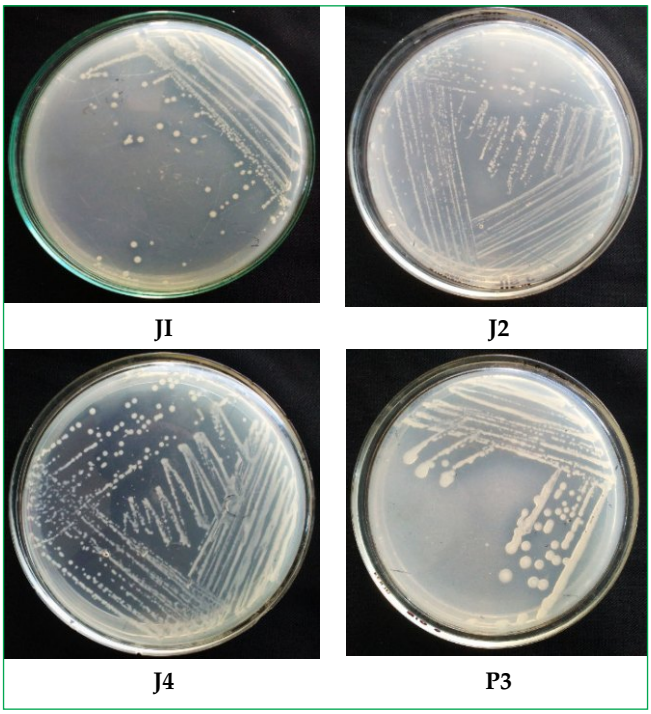


Fig.1: Psolubilization of PSB isolates steaked on Pikovskaya's medium

Some other bacteria, like *Pantoea agglomerans* from the tomato plant (Walpola and Yoon 2013b), *Bacillus megaterium*, *Pseudomonas putida* and *P. fluorescence* from chilli, cotton, eggplant, okra, tomato were isolated and studied (Baliah et al. 2016; Rafi et al. 2019; Singh et al. 2016). In several other studies various PSB types were isolated from the rhizosphere of brassica, capsicum, cauliflower, coriander, cucumber, eggplant, French bean, groundnut, kulfa, lady's finger, onion, potato, salad, spinach, sweet potato and turnip (Alia et al. 2013; Baliah et al. 2016; Dawwam et al. 2013; Taurian et al. 2010).

Characterization and identification of PSB Isolates

Four PSB isolates, namely J1, J2, J4 and P3, with PSI ≥1.55, were morphologically and biochemically unique in the profiles (Table 2).

The colonies of isolate J1 were convexly elevated, round, smooth, translucent and non-pigmented with entire margins. The isolate J2 had flat, round, smooth, translucent and non-pigmented colonies with wavy margins. The colonies of isolate J4 were slightly convex with elevation, round, smooth, translucent and golden yellow-coloured colonies with entire margins. In contrast, P3 colonies were flat elevated, irregular, smooth, translucent and non-pigmented colonies with wavy margins. The isolates were Gram-negative and non-motileFig. 2. The scientific literature reveals the abundance of Gram-negative bacteria in the rhizosphere of many agriculturally important crops (Muleta et al. 2013). Isolates J1 and J2 were capsulated.

Table 1 : List of PSB isolated from various locations of Banaskantha District, Gujarat

Plant sample collection location	Coordinates	Plant rhizosphere	Isolate code	Colony dia. (cm)	Dia. of clear zone (cm)	PSI
College of Basic Science and Humanities, S.K. Nagar	24°19'32", 072°18'34"	*Calotropis procera*	P1	2.99	4.08	1.36
			P2	3.55	5.01	1.41
			P3	**1.39**	**2.15**	**1.55**
			P4	0.91	1.13	1.24
			P5	0.81	1.08	1.33
			P6	0.31	0.37	1.19
			P7	0.45	0.48	1.07
			P8	0.81	1.12	1.38
			P9	0.20	0.24	1.20
			P10	0.31	0.37	1.19
Agronomy Farm, S.K. Nagar	24°19'22", 072°18'19"	*Solanum tuberosum*	C1	2.46	3.45	1.40
			C2	3.42	4.68	1.37
			C3	3.85	5.34	1.39
			C4	1.26	1.48	1.17
Gowswami farmhouse, Deesa	24°15'18", 072°12'41"	*Lantana camera*	D1	1.26	1.48	1.17
			D2	3.22	4.20	1.30
			D3	3.22	4.60	1.43
			D4	1.66	1.69	1.02
			D5	2.47	3.49	1.41
Dantiwada dam, Dantiwada	24°20'34", 072°20'21"	*Cynodon dactylon*	E1	1.66	1.91	1.15
			E2	1.66	1.75	1.05
Dantiwada colony	24°19'32", 072°19'13"	*Cynodon dactylon*	G1	1.81	2.60	1.44
		Cynodon dactylon Web results	G2	3.22	4.20	1.30
Chaturbhuj farmhouse, Siddhpur	23°53'27", 072°22'15"	*Abelmoschus esculentus* *Cynodon dactylon*	H1	1.96	2.42	1.23
			H2	1.53	1.81	1.18
			H3	2.43	2.86	1.18
			H4	1.26	1.66	1.32
Shipu dam, Dantiwada	24°24'51", 072°18'24"	*Senna tora*	I1	0.91	1.20	1.32
			I2	0.81	1.20	1.48
Kamaniya farm house, Palanpur	24°10'37", 072°27'54"	*Solanum lycopersicum*	**J1**	**1.81**	**3.50**	1.93
			J2	**1.52**	**3.01**	1.98
			J3	1.66	2.25	1.36
			J4	**1.26**	**2.52**	2.00

Table 2: Morphological and cultural characteristics of PSB isolates

Characteristics		J1	J2	J4	P3
Gram test		–ve	–ve	–ve	–ve
Morphological	Shape	short rods	short rods	rods	rods
	motility	non-motile	non-motile	non-motile	non-motile
	capsule	present	present	absent	absent
	size	medium	medium	medium	medium
Cultural	shape	round	round	round	irregular
	margin	entire	wavy	entire	wavy
	elevation	convex	flat	low convex	flat
	texture	smooth	smooth	smooth	smooth
	pigment	absent	absent	golden yellow	absent
	opacity	translucent	translucent	translucent	translucent

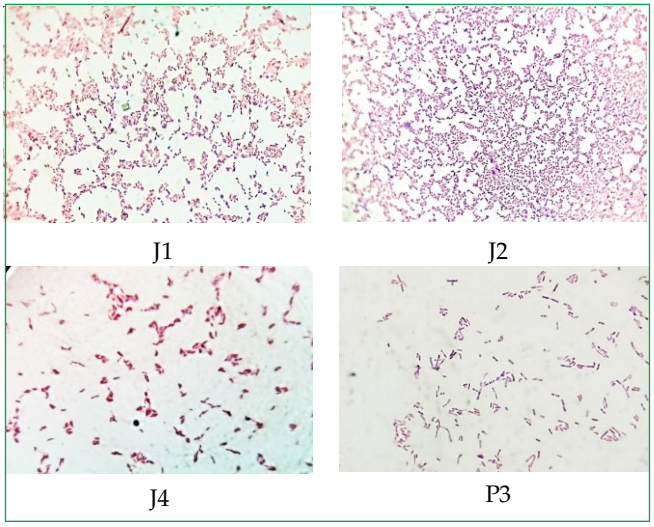


Table 2: Gram-stained PSB isolates under a laboratory microscope (1000x)

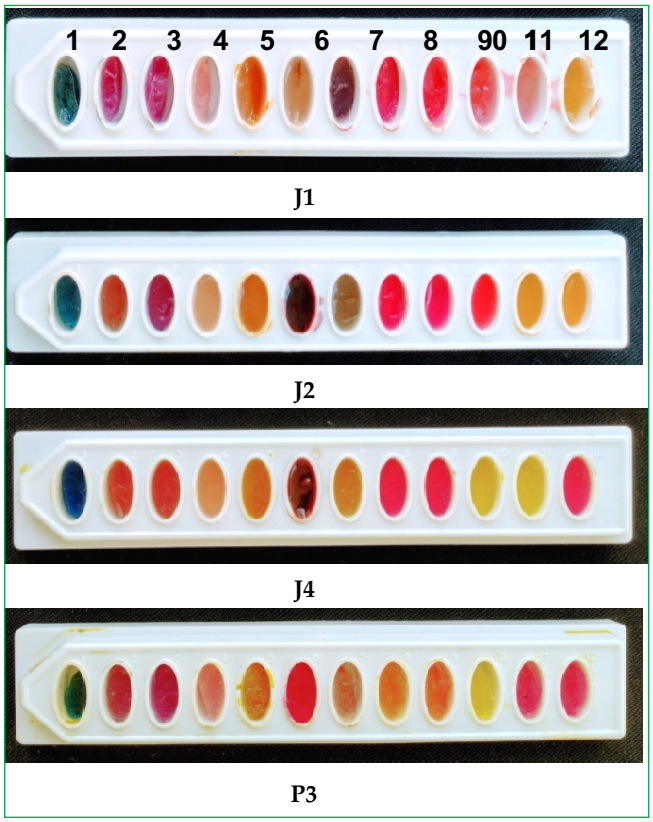


Table 3: Biochemical tests of four PSB isolates on HiMedia™ kit

1) citrate utilization, 2) lysine utilization, 3) ornithine utilization, 4) urease production, 5) phenylalanine deamination, 6) nitrate reduction, 7) H_2S production, 8) glucose utilization, 9) adonitol utilization, 10) lactose utilization, 11) arabinose utilization and 12) sorbitol utilization.

The citrate, lysine and ornithine tests were positive for all the isolates. Isolates J1 and J2 were positive for six, while J4 and P3 for seven and ten tests, respectively. In contrast, no isolate deaminated the phenylalanine and produced indole. The isolates J2, J4 and P4 reduced the nitrate in the medium while J1 was H_2S positive. Isolate P3 utilized glucose and adonitol, J4 and P3 used lactose, while J2 and P4 isolates metabolized sorbitol in the growth medium. P3 hydrolyzed starch and gelatin. The isolates were sequenced through 16s rRNA sequencing and obtained sequences were searched from NCBI website for homology. The isolates J1, J2, J4 and P3 were identified and named *Klebsiella aerogenes* LC515412, *Klebsiella pneumoniae* LC515413, *Kocuria flava* LC515414, *Enterobacter hormaechei* LC515415, respectively.

Table 3: Biochemical tests performed for PSB characterization

Biochemical test	Isolate code			
	J1	J2	J4	P3
Citrate utilization test	+	+	+	+
Lysine utilization	+	+	+	+
Ornithine utilization test	+	+	+	+
Urease detection	+	-	+	+
Phenylalanine deamination	-	-	-	-
Nitrate reduction	-	+	+	+
H_2S production	+	-	-	-
Glucose utilization	-	-	-	+
Adonitol utilization	-	-	-	+
Lactose utilization	-	-	+	+
Arabinose utilization	-	+	+	-
Sorbitol utilization	+	+	-	-
Starch hydrolysis	-	-	-	+
Gelatin hydrolysis	-	-	-	+
Indole production	-	-	-	-

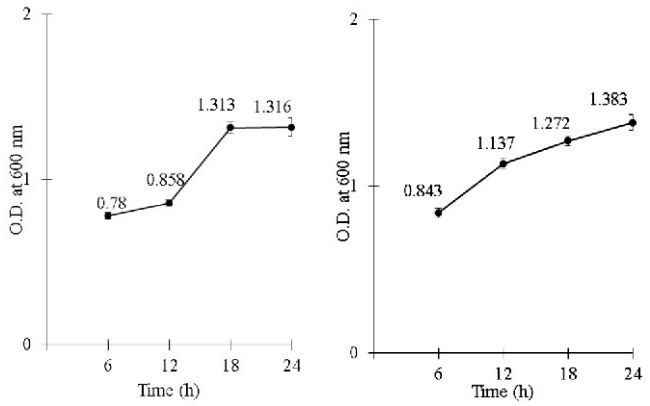
The sequences of the identified isolates were submitted to the DDBJ database and the accession numbers were obtained (Table 4).

Table 4: Identified PSB isolates with NCBI accession numbers

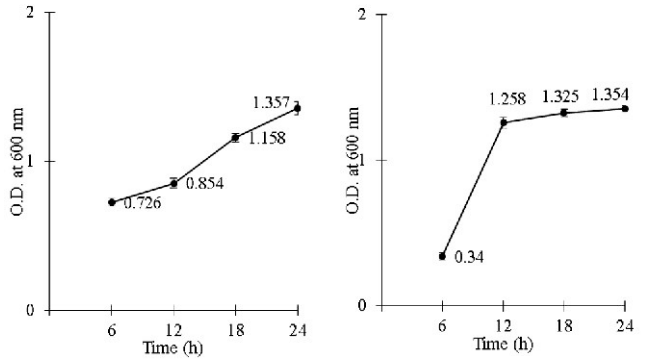
Isolate code	Isolate identification	NCBI accession no.
J1	*Klebsiella aerogenes*	LC515412
J2	*Klebsiella pneumoniae*	LC515413
J4	*Kocuria flava*	LC515414
P3	*Enterobacter hormaechei*	LC515415

Growth study of PSB Isolates

The growth of PSB isolates *viz. Klebsiella aerogenes* LC515412, *Klebsiella pneumoniae* LC515413, *Kocuria flava* LC515414 and *Enterobacter hormaechei* LC515415 was measured spectrophotometrically through the optical density (OD) of the inoculated nutrient broth flasks at 600 nm, every six hours for 24 h (Fig. 4). The exponential growth of *K. pneumoniae* LC515413 and *K. flava* LC515414 for 24 h was similar in pattern but different from *K. aerogenes* LC515412 and *E. hormaechei* LC515415. The OD based growth pattern of *E. hormaechei* LC515415 was dissimilar from all other isolates, which steeply increased during 6-12 h and became constant later. The linear OD increase was observed with *K. pneumoniae* LC515413. After 96 h of growth, the highest OD (1.383) was observed with *K. pneumoniae* LC515413 and lowest (1.316) with *K. aerogenes* LC515412.



a) Klebsiella aerogenes LC515412 *b) Klebsiella pneumoniae* LC515413



a) Kocuria flava LC515414 *b) Enterobacter hormaechei* LC515415

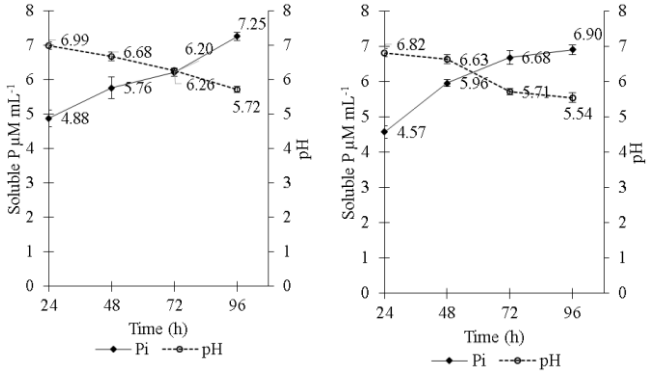
Fig. 4: The 24 h growth curve of the four PSB isolates[*α]

[*] Each value is a mean of three replicates.

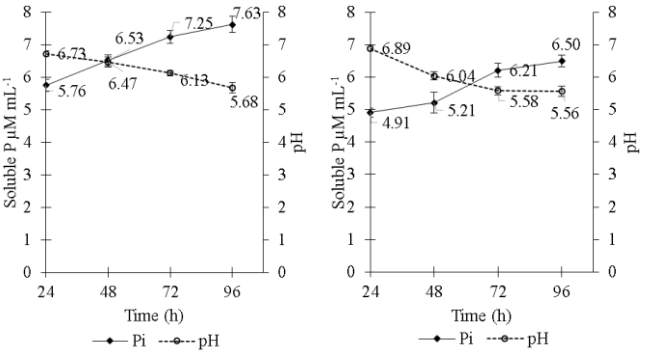
[α] Vertical bars represent SEm

Determination of Pi Solubilizing Potential of PSB Isolates

Fig. 5 shows the P dissolved by PSB isolates grown in Pikovskaya's broth with pH change in the growth medium. Overall, the P solubilization patterns of all four isolates were similar. After 96 h of growth the isolates, namely, *K. aerogenes* LC515412, *K. pneumoniae* LC515413, *K. flava* LC515414 and *E. hormaechei* LC515415 solubilized 7.25, 6.90, 7.63 and 6.5 µM mL[-1] P with a pH drop to 5.72, 5.54, 5.68 and 5.5, respectively in the growth medium. *K. flava LC515414* had comparatively higher P solubilization values throughout the observation period. In contrast, *K. pneumoniae* LC515413 and *K. flava* LC515414 had uniform P solubilization values. The nutrient broth pH decreased temporally, possibly due to the bacterial secretion of organic acids (Liu et al., 2015).



a) Klebsiella aerogenes LC515412 *b) Klebsiella pneumoniae* LC515413



a) Kocuria flava LC515414 *b) Enterobacter hormaechei* LC515415

Fig.5: Released Pi and pH change in Pikovskaya's broth containing four PSB isolates[*α]

[*] Each value is a mean of three replicates

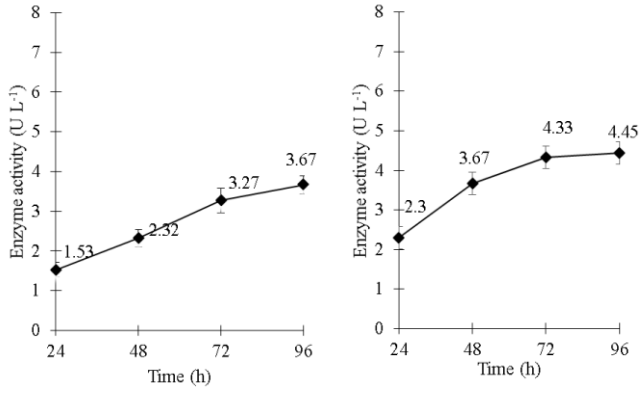
[α] Vertical bars represent SEm

Pi-Inorganic phosphorous

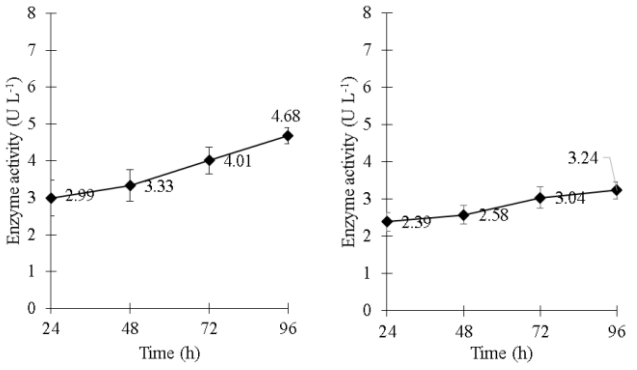
Determination of acid and alkaline phosphatase activity of PSB isolates

Acid phosphatase activity

After 96 h of growth the isolates namely, *K. aerogenes* LC515412, *K. pneumoniae* LC515413, *K. flava* LC515414 and *E. hormaechei* LC515415 had acid phosphatase activity of 3.67, 4.45, 4.68, 3.24 U L[-1], respectively. The isolate *K. flava* LC515414 expressed the highest acid phosphatase activity and *E. hormaechei* LC515415 the lowest (Fig. 6). The initial 24 h old PSB acid phosphatase activity of isolates ranged between 1.53-2.99 U L[-1] that spanned between 3.24-4.68 U L[-1] in the final observations. Overall, the consistently lower but constant acid phosphatase activity was observed with *E. hormaechei* LC515415 throughout the experiment (2.39-3.24 U L[-1]). The enzyme activity of *E. hormaechei* LC515415 remained constant with only 35.6 % increase from the initial value, the lowest among all the four isolates. The acid and alkaline phosphatase



a) Klebsiella aerogenes LC515412 *b) Klebsiella pneumoniae* LC515413



a) Kocuria flava LC515414 *b) Enterobacter hormaechei* LC515415

Fig. 6: Acid phosphatase activity of four PSB isolates*α

* Each value is a mean of three replicates

α Vertical bars represent SEm

activity stabilized after 72 h of growth, possibly due to a drop in enzyme activity, similar to the observation of Xiao *et al.* (2009). The reduction in activity at the later stages could be attributed to the accumulation of soluble phosphorus, which can inhibit the activity of phosphatase. Some PSBs with high phosphatase can release high phosphorus in the growth medium due to substrate-specific phosphatase secretion (Deepa et al., 2010).

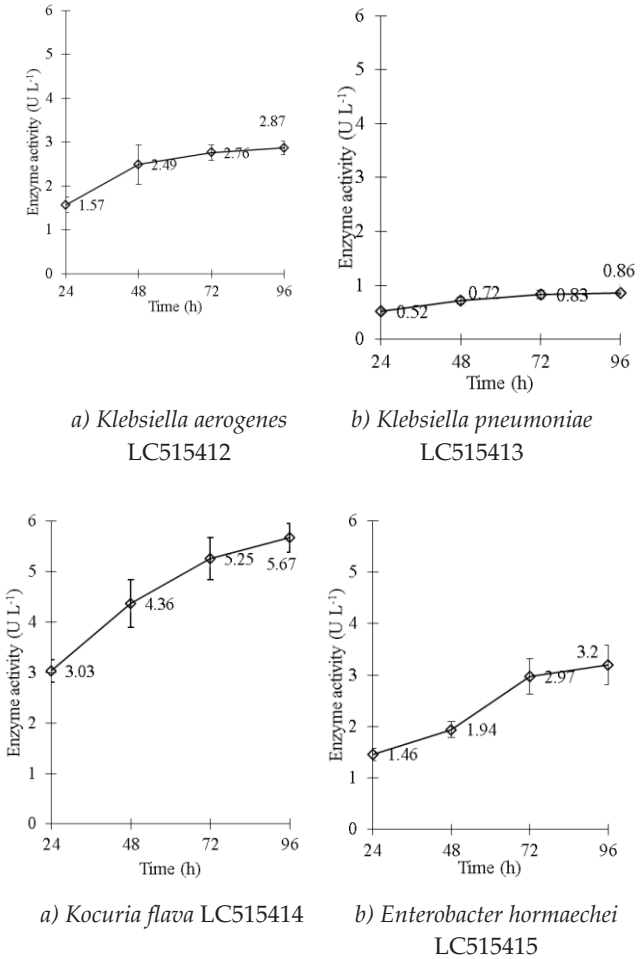
Bacterial acid phosphatases are a group of enzymes secreted as soluble periplasmic proteins or retained as membrane-bound lipoproteins. Phosphatase dephosphorylates organic phosphodiesters of nucleotides, sugar phosphates and phytic acid to acquire inorganic P and organic byproducts. Phosphatases are a diverse enzyme group to hydrolyze phosphodiester bonds in various substrates under different conditions (Vincent et al., 1992). The acid phosphatase activity of PSB is common in nature and has been recorded in several strains of *Bacillus, Citrobacter, Enterobacter, Klebsiella, Proteus, Pseudomonas, Rhizobium* and *Serratia* genera (Jha and Saraf 2015). However, phosphatase does not act directly on inorganic P. It lowers the pH of the culture medium through dephosphorylation and produces organic acids (Achal et al., 2007). The dephosphorylation reactions are involved in the hydrolysis of phosphodiester or phosphoanhydride bonds. The phosphohydrolases remain clustered in acid or alkaline conditions.

Behera *et al.* (2017) isolated several PSB from the soil, performed acid phosphatase activity by p-nitrophenyl phosphate method, and observed maximum acid phosphatase by *Serratia* sp. (76.8 U) mL^{-1}) after 48 h of incubation. However, P solubilization is not restricted to soil bacteria. Several enteric bacteria also harbor the P solubilizing trait (Mihara et al. 2001; Torriani 1960), which they might have acquired the trait through horizontal gene transfer. Several researchers have reported a significant amount of acid phosphatase activity from several soil bacteria (Gomes et al. 2004; Prasanna et al. 2012; Walpola and Yoon 2013a).

Alkaline Phosphatase Activity

After 96 h of growth, the isolate *K. aerogenes* LC515412, *K. pneumoniae* LC515413, *K. flava* LC515414 and *E. hormaechei* LC515415 had alkaline phosphatase activity of 2.87, 0.86, 5.67 and 3.2 UL^{-1}, respectively (Fig. 7). The slow temporal increase in enzyme activity was observed in *K. aerogenes* LC515412 and *K. pneumoniae* LC515413. The temporal alkaline phosphatase activity increase was highest in *K. flava* LC515414. *K. pneumoniae* LC515413, on the other hand, displayed the lowest but constant enzyme activity throughout the observation period.

Overall, a wide variation was observed in the alkaline phosphatase activity of isolates. As illustrated in the scientific literature, alkaline phosphatase activity increases with increased pH (Dick et al. 2000). The pH change of the medium occurs due to the accumulation of secondary metabolites that causes variability in alkaline phosphatase production. The alkaline and acid phosphatase activity of microorganisms remains similar in the soil except that the former has an operational pH range of 8-9. Alkaline phosphatase is a homodimer of 86 kDa. Bacterial alkaline phosphatase catalyzes nonspecific removal of phosphomonoester bonds. The alkaline phosphatase is a heat-stable enzyme with maximum activity at higher pH ranges (Ross *et al.*, 1951). Gram-negative bacterial alkaline phosphatase remains present in the periplasmic spaces and requires specific methods to be released from the cell. Bacterial alkaline phosphatase removes 3' and 5' phosphates from DNA and RNA and remains active till 65°C.



a) Klebsiella aerogenes LC515412 *b) Klebsiella pneumoniae* LC515413

a) Kocuria flava LC515414 *b) Enterobacter hormaechei* LC515415

Fig. 7: Alkaline phosphatase activity of four PSB isolates*α

* Each value is a mean of three replicates

α Vertical bars represent SEm

CONCLUSIONS

In the present study, 33 PSB were screened from eight locations of North Gujarat, India. Four morphologically and biochemically characterized isolates, namely, J1, J2, J4 and P3 with PSI ≥ 1.55 were identified through 16S rRNA sequencing as *Klebsiella aerogenes* LC515412, *Klebsiella pneumoniae* LC515413, *Kocuria flava* LC515414 and *Enterobacter hormaechei* LC515415. The isolates had varied acid and alkaline phosphatase activity that parallels to bacterial growth observations for similar durations (Margalef et al. 2017; Ponmurugan and Gopi 2006). After 96 h of growth *K. flava* LC515414 had the highest acid and alkaline phosphatase activity (4.68 U L^{-1} and 5.67 U $L^{-1,}$ respectively). Also, *K. flava* LC515414 solubilized the highest P (7.63 µm P mL^{-1}) from Pikovaskays broth after 96 h. The alkaline phosphatase activity of *K. flava* LC515414 was moderately higher than the acid phosphatase activity, implying the dominant nature of alkaline phosphatase in the isolate. The study highlights the in vitro P solubilizing potential of four PSB isolates and suggests their trial as biofertilizer in plant-based experiments. The use of potent PSB with higher P solubilizing capability can decrease the usage of chemical fertilizers and pesticides to make our agricultural practices greener.

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