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Full Length Research Paper

Isolation and molecular characterization of phosphate solubilizing *Enterobacter* and *Exiguobacterium* species from paddy fields of Eastern Uttar Pradesh, India

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Six phosphate solubilizing bacteria (PSB) were isolated from paddy fields of Eastern Uttar Pradesh, India harboring low available phosphorus. Taxonomic delineation employing morphological, biochemical, 16S rRNA gene sequences and phylogenetic affiliations suggests that they are members of *Enterobacter* and *Exiguobacterium* genera. Of the six isolates, *Enterobacter* sp. LCR1 and LCR2 exhibited high level (568 - 642 µg/ml) of phosphate solubilization in NBRIP liquid medium. *Exiguobacterium* sp. LCR4 and LCR5 showed increased phosphate solubilization efficiency under alkaline pH while *Enterobacter* sp. LCR3 remained unaffected. At high salt and temperature, *Enterobacter* sp. LCR1 and LCR2 produced 1.6 fold soluble phosphorus in comparison with earlier studies. Thus, these isolates may be useful for the development of potential bio-inoculants for soils having alkaline pH, high salt, temperature and insoluble phosphorus.

Key words: Phosphate solubilizing bacteria, phylogenetic analysis, *Enterobacter* spp., *Exiguobacterium* spp., 16S rRNA gene sequencing.

INTRODUCTION

Injudicious agricultural practices and canal irrigation have not only disturbed the soil nutritional balance but also caused a significant increase in soil salinity and pH. Of the 400 - 1200 mg/kg of soil phosphorus present in agricultural fields (Rodríguez and Fraga, 1999), a large fraction of this is locked in an insoluble form and only <10% enters the plant-animal cycle (Kucey et al., 1989). Phosphatic fertilizers being very expensive, Indian farmers are compelled to cut down its application (Sundara et al., 2002). Further, since phosphorus availability from the phosphate reserves under neutral and alkaline conditions is negligible, phosphate solubilizing microbes assume special significance for ensuring availability of locked phosphate reserves in the soils (Patil et al., 2002).

Several bacterial genera including *Pseudomonas, Bacillus, Enterobacter, Azotobacter, Agrobacterium, Achromobacter, Rhizobium, Burkholderia, Flavobacterium* and *Micrococcus* isolated from the temperate countries have been reported to solubilize phosphorous (Jeon et al., 2003; Rodríguez and Fraga, 1999; Son et al., 2006) but their establishment and performance is largely hampered by environmental variables like salinity, pH and temperature. Thus these phosphate solubilizers are highly unlikely to be successful in the Indian context. It is also known that the inocula developed from a particular soil fail to function as efficiently in soils having different properties (Rodríguez and Fraga, 1999). Notwithstanding, above the characterized phosphate solubilizing bacteria (PSBs) were identified only by one or two approaches, that is, biochemical characteristics and/or 16S rRNA gene sequencing (Pérez et al., 2007; Yi et al., 2008).

Some reports dealing with the isolation and characterization of phosphate solubilizing bacteria are also available from the Indian peninsula (Souza et al., 2000; Johri et al., 1999). However, these isolates were

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neither characterized at the molecular level nor their phylogenetic affiliations determined. Nevertheless, Indian isolates have been named after the name of the institute (National Botanical Research Institute, Lucknow, India) such as NBRI1, NBRI2 etc where the work had been conducted but the physicochemical properties of the soils have never been analyzed. Thus this restricts their use at the national and international levels.

While salinity affected area in India is estimated to be about 13 million hectare, the site selected for this study, Eastern Uttar Pradesh, is further affected by alkalinity (http://www.irri.org/cure/lowlandWG3.htm). In tropical countries including India, temperature during summer and rainy season ranges between 35 - 45°C, salt level in alkaline soil goes up to 2% and pH up to 10.5 unit (Nautiyal et al., 2000). Since rice is a staple crop in different parts of the world including India and its demand is likely to increase ~ 35% by 2030 (FAO, 2002), there is a pressing need to isolate efficient PSBs, capable of proliferation in saline, alkaline soil distressed with high temperature and characterize them by polyphasic approach, and complimented with physicochemical properties of the resident soils. This paper reconciles the above points and presents novel data on the isolation and polyphasic characterization of PSBs and *Enterobacter* capable of phosphate solubilization at high temperature, salt and alkaline pH.

MATERIALS AND METHODS

Soil sampling

Three soil samples were collected from paddy fields of Eastern Uttar Pradesh, India (82° 59' East, 25° 15' North and 82° 33' East, 25° 8' North). For each sample, three sub-samples (0 - 10 cm depth and 4 cm diameter) were collected and mixed thoroughly. These samples were stored in the laboratory at -70°C in a deep freezer (Krispcold, India).

Soil analysis

Soil samples were homogenously suspended in double distilled water in the ratio of 1:2 (wet w/v) and centrifuged at 5000 rpm for 5 min at 25°C. Electrical conductivity (EC) and pH were measured from clear supernatants using conductivity meter 306 and µpH SYSTEM 361, respectively, of Systronics, India.

Total phosphorus present in the air dried soil samples was extracted by strong acid (mixture of concentrated H_2SO_4:$HClO_4$; 4:1) digestion using V_2O_5 as a catalyst (López-Gutiérrez et al., 2004). Samples were digested by soil/acid mixture (1:30) in a fume hood on hot plate at 80°C. After digestion, the final volume was made up to 25 ml by adding double distilled warm water. The available form of phosphorus was extracted with addition of 200 mg activated charcoal at a soil/$NaHCO_3$ (0.5 mol/l, pH 8.5) ratio of 1:20 (Olsen et al., 1954). The samples were shaken at 100 rpm in a temperature controlled incubator shaker at 25°C for 1 h. Both types of extraction samples were filtered through Whatman No.1 filter paper and extract was acidified (for available phosphorus only) to pH 5.0 using concentrated H_2SO_4. The extracted phosphorus in the clear supernatant was determined by molybdophosphoric acid method (APHA, 1995).

Isolation of phosphate solubilizing bacteria

Bacterial isolation was carried out from each soil sample after homogenously suspending in sterile saline solution (0.85% sodium chloride). Aliquots of 10^6 diluted samples (serial dilutions) were spread on Pikovskaya's agar medium (Pikovskaya, 1948) and incubated in temperature controlled incubator shaker (Model-3597-ICOGMPR) at 30°C for 3 days. To avoid fungal contamination, 50 µg/ml cycloheximide was added to the medium before plating (Black et al., 2003). Eleven colonies producing clear halos were selected and purified on AT salt minimal medium (Johri et al., 1999). Six colonies displaying differential phosphate solubilization in NBRIP liquid medium (Nautiyal, 1999) were further characterized in detail.

Morphological and biochemical characterization

Morphology was studied by gram staining under compound microscope (KYOWA GETNER, OPTO-PLAN 2KT, Japan) having the facility of live image transfer to computer. Biochemical characterization of the isolated strains was carried out according to Brenner and Farmer (2005), Collee et al. (1996) and López-Cortés et al. (2006). Utilization of different carbon sources was studied following addition of 50 mg/l of tetrazolium chloride as colour indicator to the basal growth medium (Janisiewicz and Bors, 1995).

DNA isolation

DNA was isolated using the phenol/chloroform/isoamyl alcohol method (http://www.bio.vu.nl/geomicrob/protocols/) with slight modifications. Briefly, one ml of overnight grown culture was centrifuged at 8000 rpm for 5 min. The pellet was washed twice with TE buffer (Tris-HCl 10 mmol/l, EDTA 1 mmol/l, pH 8.0) and suspended in 567 µl TE buffer containing 2 mg/ml lysozyme. The suspension was incubated in a temperature controlled water bath at 37°C for 30 min. This was followed by addition of 3 µl proteinase K (20 mg/ml) and 30 µl of 10% SDS and incubated at 37°C for 1 h in a water bath. 100 µl of 5 mol/l NaCl was added and mixed thoroughly. To this, pre-warmed 80 µl of cetyl trimethyl ammonium bromide (CTAB, 10%) was added and then incubated at 65°C for 10 min. Now samples were allowed to cool down to room temperature and equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and vortexed gently. This was subjected to centrifugation at 12000 rpm for 5 min at 4°C and the upper aqueous phase was aspirated out.

Equal volume of chloroform/isoamyl alcohol (24:1) was now added to the aqueous phase, mixed by gentle vortexing and centrifuged at 12000 rpm for 5 min at 4°C. The clear aqueous phase was precipitated using double volume of chilled ethanol and one tenth volume of 3 mol/l sodium acetate (pH 5.2). Samples were left overnight at -20°C (Vestfrost deep freezer, Blue Star, India) to allow DNA precipitation. Precipitated DNA pellet was collected by centrifugation at 12000 rpm for 10 min at 4°C. Pellet was washed with 70% ethanol and air dried followed by suspension in 100 µl of TE buffer (Tris-HCl 10 mmol/l, EDTA 1 mmol/l, pH 8.0). The DNA sample so prepared was qualitatively checked on 0.8% agarose gel and stored at -20°C for further work.

PCR amplification and sequencing of 16S rRNA gene

The PCR amplification of the partial genes encoding 16S rRNA was carried out in an Icycler (Bio-Rad, USA). Primers 8F: AGAGTTTGATCCTGGCTCAG and 518R: ATTACCGCGGTGCTGG (Benlloch et al., 2002) were commercially synthesized from Sigma Chemical Co., USA. 25 µl of PCR reaction mixture was prepared as reported earlier (Srivastava et al., 2007).

Table 1. Physicochemical characteristics of the soil samples used for isolation of phosphorus solubilizing bacteria.

Experimental site	pH	Electrical conductivity (ds/m)	Total P (µg/g dry soil)	Available P (µg/g dry soil)	Isolated strains
Agriculture farm I (B.H.U.)	7.92 ± 0.05	1.00 ± 0.10	265.35 ± 17.70	32.20 ± 1.10	LCR1 and LCR2
Agriculture farm II (B.H.U.)	8.03 ± 0.14	2.10 ± 0.20	295.50 ± 31.20	54.69 ± 3.70	LCR3
Parashurampur (Mirzapur)	7.15 ± 0.09	1.40 ± 0.00	210.56 ± 29.60	21.62 ± 1.30	LCR4, LCR5 and LCR6

Thermal cycler profile was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of incubation each consisting of 1 min denaturation at 94°C, 1.5 min annealing at 58°C, 2 min extension at 72°C and a final extension of 10 min at 72°C. Amplified PCR products were resolved on 1.2% agarose gel and stained with ethidium bromide (1 µg/ml). PCR products (500 ng) were lyophilized in a speed vac concentrator, Model- SPD111V (Thermo Electron Corporation, USA). Lyophilized PCR products were subjected to purification and sequencing on commercial basis (Macrogen Inc., Korea). Partial 16S rRNA gene sequences of the isolated strains were submitted in GenBank database with accession numbers EU304794 to EU304799.

Phylogenetic analysis

The obtained sequences were manually corrected by deleting the unambiguous nucleotides from 3' and 5' ends and subjected to BLAST homology search in the NCBI database. Reference 16S rRNA gene sequences of type strains were retrieved from ribosomal database project (http://rdp.cme.msu.edu/hierarchy/hb_intro.jsp). Multiple sequence alignment and Neighbour-Joining tree construction were carried out using CLUSTAL_X version 1.83 software (Thompson et al., 1997) with 1000 bootstrap replicates.

Determination of rate and phosphate solubilizing efficiency of isolated strains

Phosphate solubilization efficiency of isolated strains was determined on NBRIP medium (Nautiyal, 1999) containing glucose (10.0), $Ca_3(PO_4)_2$ (5.0), $MgCl_2.6H_2O$ (5.0), $MgSO_4.7H_2O$ (0.25), KCl (0.2), $(NH_4)_2SO_4$ (0.1) g/l and initial pH 7.0. The inoculum was prepared by preculturing the bacteria in NBRIP medium at 30°C in a temperature controlled incubator shaker at 100 rpm. 100 ml (250 ml capacity flask) of medium was inoculated with exponentially growing 142 ± 12 x 10^4 colony forming units. Samples (5.0 ml) from each flask were withdrawn aseptically at different time intervals and used for monitoring of growth rate, pH drop and soluble phosphorus level in the supernatant (APHA, 1995). Phosphate solubilization rate (increase in soluble phosphorus concentration/h) was measured during the exponential phase of the culture. Growth was measured after diluting the samples with 1.0 mol/l HCl (1:1) to dissolve the left over $Ca_3(PO_4)_2$ (Pérez et al., 2007).

Experimental conditions for testing the effect of pH, salt and temperature

To study the phosphate solubilization activity under alkaline conditions, the initial pH of the NBRIP medium was varied from 7.0 - 10.0 using 0.1 mol/l NaOH. Effect of salt was studied by changing sodium chloride concentration from 0.5 - 2.0% in NBRIP medium. Likewise temperature effect was studied by incubating the flasks at 30, 40 and 50°C for 24 h. Wherever not specified, all experiments were carried in original NBRIP medium (pH 7.0) at 30°C for 24 h.

Assessment of phosphate solubilization in pot experiment

Pot experiments were carried out in earthenwares measuring 12 cm diameter x 14 cm height containing 300 gram of sterile as well as unsterile soils by inoculating with all the six isolates having ~10^3 colony forming units (cfu) per g soil. Both control and experimental pots supplemented with 5.0 g/kg (wet soil) tricalcium phosphate as insoluble phosphate were incubated at 30°C and watered daily to keep the moisture level at saturation. After six days of incubation, pots were withdrawn, the available phosphorus was extracted (Olsen et al., 1954) and quantified by molybdophosphoric acid method (APHA, 1995).

Statistical analysis

All the experiments were performed in triplicate and the average values with ±SD were reported in figures and tables. Separate statistical analysis (ANOVA) was done for each organism and different sets of experiments using Duncan's multiple range test (SPSS version 10.0) at 5% probability level ($P \leq 0.05$).

RESULTS

Soil characteristics and isolation of phosphate solubilizing bacteria

Table 1 shows the physicochemical properties of the three soil samples where pH ranged between 7.15 (Parashurampur) to 8.03 (agriculture farm II), and the electrical conductivity between 1.0 (agriculture farm I) to 2.1 (agriculture farm II) representing slightly alkaline nature of samples. Furthermore, the presence of 7 - 9 fold unavailable than the available phosphorus in the soil samples, suggests the preponderance of the unavailable form. The soil sample from the agricultural farm II contained the highest amount of both unavailable and available phosphorous.

Of the six phosphate solubilizing bacteria characterized, *Enterobacte*r sp. LCR1 and LCR2 were from the soil of agricultural farm I, *Enterobacter* sp. LCR3 from agricultural farm II and *Exiguobacterium* sp. LCR4, LCR5 and LCR6 from Parashurampur (Table 1).

Table 2. Biochemical characteristics of isolated strains.

Biochemical parameters	Isolated strains					
	***Enterobacter* sp. LCR1**	***Enterobacter* sp. LCR2**	***Enterobacter* sp. LCR3**	***Exiguobacterium* sp. LCR4**	***Exiguobacterium* sp. LCR5**	***Exiguobacterium* sp. LCR6**
Urease test	+	+	+	-	-	-
Methyl Red test	-	-	-	+	-	+
Oxidase test	-	-	-	+	-	+
Hugh-Leiffson reaction	O+/ F+	O+/ F+	O+/ F+	O+/ F-	O+/ F-	O+/ F-
Oxidative utilization of sorbitol and acid production	+, acid production	+, acid production	+, acid production	w, acid production	w, no acid production	w, acid production
Fermentation of sorbitol and acid production	+, acid production	+, acid production	+, acid production	w, acid production	-	w, acid production
Oxidative utilization of glycerol and acid production	+, acid production	+, acid production	+, acid production	+, acid production	+, no acid production	+, acid production
Fermentation of glycerol and acid production	+, acid production	+, acid production	w, w	w, w	+, no acid production	+, w
Phenylalanine deaminase	-	-	w	-	-	w
Orinithine decarboxylase	+	+	+	w	-	-
Lysine decarboxylase	+	+	w	-	-	-
Utilization of Adonitol	-	-	-	+	+	+
Xylose	w	w	-	+	-	+
Dulcitol	+	+	w	+	+	+
Raffinose	w	w	-	+	+	+
Cellobiose	-	-	w	+	+	+
Manitol	-	-	-	+	-	+
Fructose	-	-	w	w	-	+
Sucrose	-	-	+	w	w	+
Malonate	+	+	+	+	-	+
Citrate	+	-	-	-	w	-

+, tested positive/utilized as substrate; -, tested negative/not utilized as substrate; w, tested weakly positive/weakly utilized substrate/weak acid producer, O: oxidation; F: fermentation.

Morphological and biochemical characterization of bacteria

Strains LCR1, LCR2 and LCR3 were gram negative, positive for urease test, Hugh-Leiffson reaction, orinithine decarboxylase, lysine decarboxylase and negative for methyl red, oxidase, utilized dulcitol and malonate carbon sources. Further, oxidative and fermentative utilization of sorbitol and glycerol as well as acid production were observed. Likewise, LCR4, LCR5 and LCR6 were gram variable, negative for urease, phenylalanine deaminase, orinithine decarboxylase, lysine decarboxylase test and positive for oxidative Hugh-Leiffson reaction, utilization of adonitol, xylose, dulcitol, raffinose, cellobiose, manitol and malonate (Table 2).

Cells of all the taxa were rod shaped, non-spore

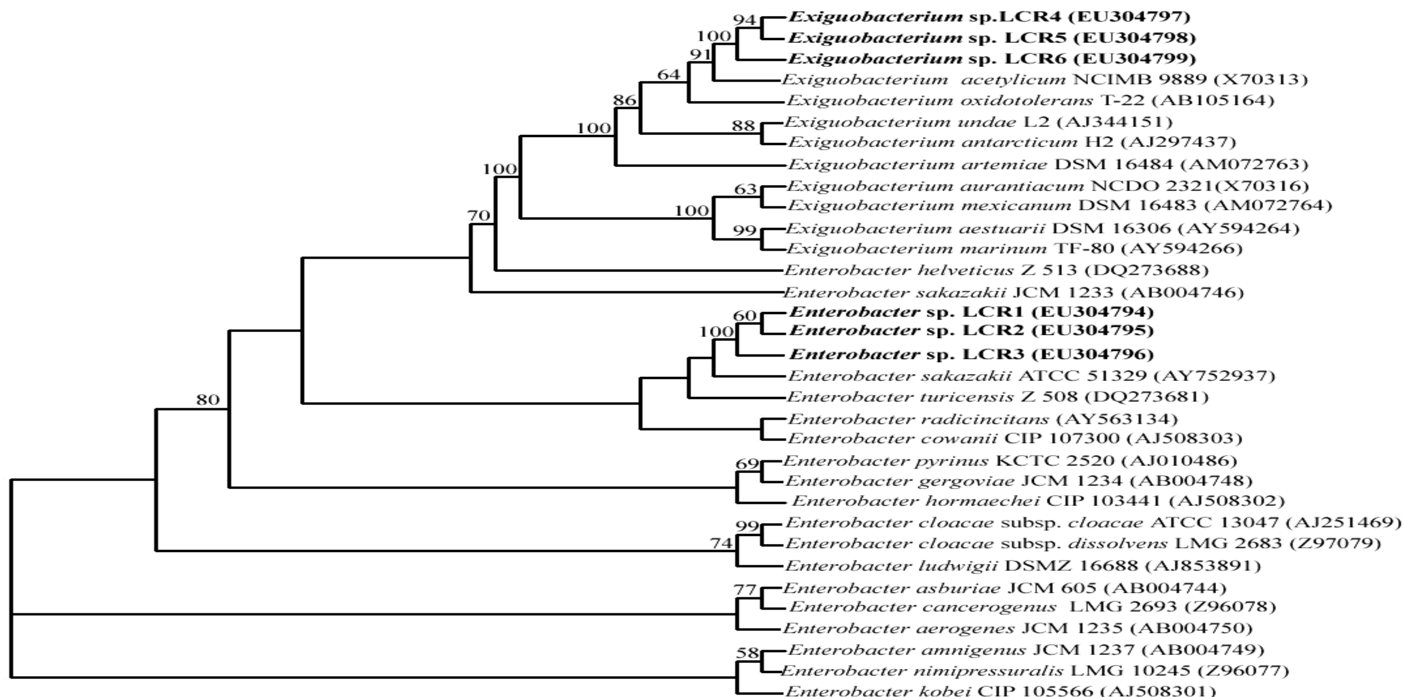

Figure 1. Unrooted Neighbour-Joining phylogenetic dendrogram of 16S rRNA gene sequences showing the positions of *Enterobacter* type strains, *Exiguobacterium* type strains, *Enterobacter* sp. LCR1, LCR2, LCR3 and *Exiguobacterium* sp. LCR4, LCR5 and LCR6. More than 50% bootstrap values are shown at branching point.

forming, negative for Voges-Proskauer test, gelatin hydrolysis and positive for catalase test.

Identification and phylogenetic analysis of isolates

16S rRNA gene sequences comparison with available data in GenBank using BLAST homology search were used to identify the isolates at the generic level. LCR1, LCR2 and LCR3 displayed close homology to *Enterobacter* species while LCR4, LCR5 and LCR6 to *Exiguobacterium* species. As shown in the phylogenetic dendrogram (Figure 1), *Enterobacter* sp. LCR1, LCR2 and LCR3 clustered together in the *Enterobacter* species clade. These strains showed respectively, 96.9, 97.4 and 97.4% gene sequence similarities and grouped most closely with *Enterobacter sakazakii* ATCC 51329 (AY752937). However, the 16S rRNA gene sequence of strain LCR1 was 98.9 and 99.1% similar to LCR2 and LCR3, respectively. Gene sequence similarity between LCR2 and LCR3 was 99.4%. Likewise *Enterobacter* isolates LCR4, LCR5 and LCR6 clustered with *Exiguobacterium* (Figure 1). 16S rRNA gene sequence of *Exiguobacterium* sp. LCR4 showed 99.2 and 98.6% similarity with *Exiguobacterium* sp. LCR5 and LCR6, respectively, and 98.8% similarity was found between *Exiguobacterium* sp. LCR5 and LCR6. LCR4, LCR5 and LCR6 showed maximum relatedness to *Exiguobacterium acetylicum* NCIMB 9889 (X70313) with 96.8, 96.8 and 96.9% similarity, respectively.

Characterization of phosphate solubilizing bacteria

Phosphate solubilization by all the six strains was studied over a time period of 72 h by monitoring pH drop and available phosphorus in the culture medium. All the strains except LCR3 (which required 6 - 8 h), entered in the exponential phase after 4 h of culturing (data not shown). Maximum phosphate solubilization, that is, 568.0, 642.0, 24.4, 4.5, 4.9 and 5.0 µg/ml was detected after 24 h growth for LCR1, LCR2, LCR3, LCR4, LCR5 and LCR6, respectively, along with a significant pH decrease (Figure 2B). LCR1, LCR2, LCR3, LCR4, LCR5 and LCR6 displayed 41.0, 37.0, 3.0, 0.4, 0.3, 0.45 µg/ml/h phosphate solubilization rate, respectively. Linear increase in phosphate solubilization efficiency was observed for strains LCR1 and LCR2, while LCR3, LCR4, LCR5 and LCR6 showed decreased phosphate solubilization efficiency (Figure 2A).

Tables 3A and B and Figure 3 show the effect of initial pH (7.0 - 10.0) on phosphate solubilization potential and growth rate of different PSBs. *Enterobacter* sp. LCR1 and LCR2 exhibited maximum solubilization at pH 7.0. *Enterobacter* sp. LCR3 showed insignificant change in solubilization efficiency with increase of initial pH from 7.0 - 9.0. Interestingly, *Exiguobacterium* sp. LCR4 and LCR5 registered an increase in phosphate solubilization

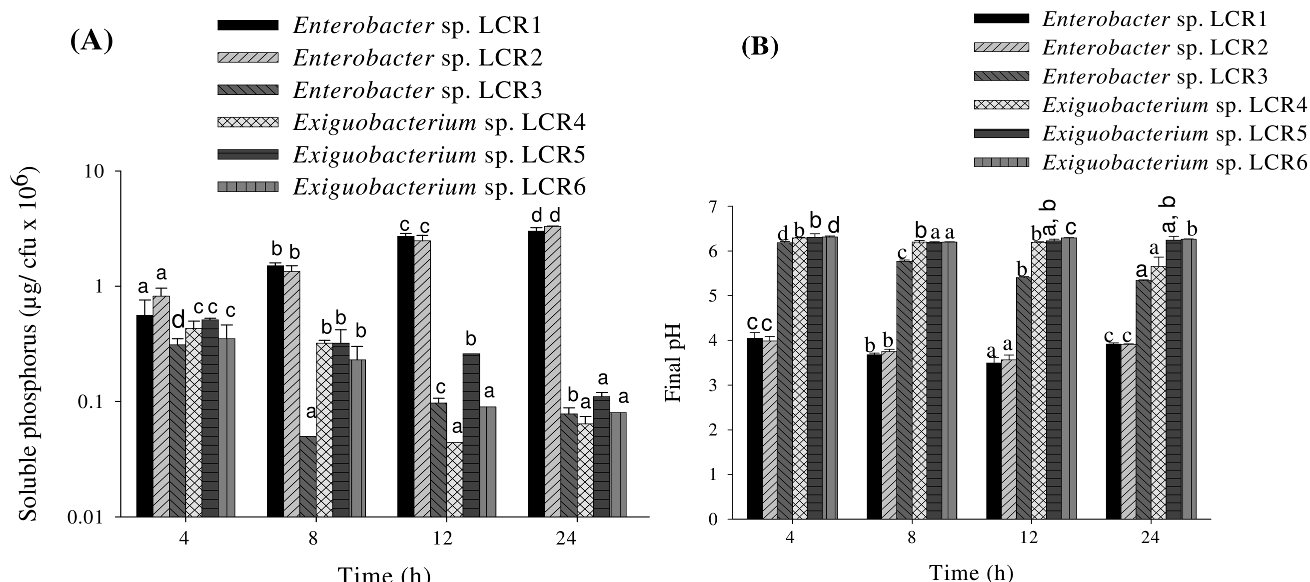

Figure 2. (A) Phosphorus solubilization efficiency of isolates during growth on NBRIP medium at 30°C. (B) Final pH of the medium at different time periods of growth in NBRIP medium at 30°C. Separate statistical analysis (Duncan's multiple range test) has been performed for each isolate and bars having different letters show significantly different ($P \leq 0.05$) values at different time periods.

following upshift of the pH from 7.0 to 9.0.

Table 3A and B shows NaCl concentration dependent decrease in phosphate solubilization efficiency for all the isolated strains except *Enterobacter* sp. LCR3. In fact, undetectable level of soluble phosphorus was observed above 1.0% salt for *Exiguobacterium* sp. LCR4, LCR5 and LCR6 (Table 3B). *Enterobacter* sp. LCR3 appeared to be salt tolerant as phosphate solubilization increased at 0.5% salt concentration and a further increase in salt concentration up to 2.0% did not produce any significant decrease in phosphate solubilization. Notwithstanding, above increase in temperature caused a significant decline in the phosphate solubilzation efficiency of all the isolated PSBs except *Enterobacter* sp. LCR3 (Table 3 A and B).

Phosphate solubilization in pot experiment

Pot experiments demonstrated that *Enterobacter* sp. LCR1, LCR2 and LCR3 solubilized phosphate significantly (32.21 - 8.49 µg/g dry soil) in sterile and non sterile conditions while *Exiguobacterium* strains failed to do so as the amount of soluble phosphorus was not significantly different from control (Figure 4).

DISCUSSION

This study presents a combination of morphological, biochemical including phosphate solubilization potential and 16S rRNA gene sequences approaches for the identification of PSBs, which were further validated at phylogenetic level. The gram negative rod shaped morphology, fermentative metabolism, acid and gas production, negative for oxidase test and gelatin liquifaction attributes of LCR1, LCR2 and LCR3 qualifies them to be the members of Enterobacteriaceae (Brenner and Farmer, 2005; López-Cortés et al., 2006; Stephan et al., 2007). Similarly, gram variable, rod shaped morphology, positive for oxidase and catalase as well as for cellobiose utilization attributes of LCR4, LCR5 and LCR6 denominated them to be the members of Bacillaceae (Kim et al., 2005). In addition to above, other biochemical characteristics (Table 2) were closely related to *Enterobacter* and *Exiguobacterium* (Brenner and Farmer, 2005; López-Cortés et al., 2006; Stephan et al., 2007). Furthermore, the clustering of *Enterobacter* sp. LCR1, LCR2, LCR3 and LCR4, LCR5 and LCR6 was supported by 100, 60, 94 and 100% bootstrap values, respectively. The similarity level between the different *Exiguobacterium* species lied between 92.09 (*Exiguobacterium artemiae* and *Exiguobacterium aurantiacum*) and 98.9% (*Exiguobacterium artemiae* and *Exiguobacterium undae*) and that of *Enterobacter* species between 95.15 (*Enterobacter sakazakii* JCM 1233 and *Enterobacter turicensis* Z 508) to 99.02% (*Enterobacter asburiae* JCM 6051 and *Enterobacter cancerogenus* LMG 2693).

All isolates displayed highest phosphate solubilization after 24 h. This may be attributed to maximum growth and pH drop, and supported by the earlier studies (Babenko et al., 1984; Gen-Fu and Xue-Ping., 2005) where a linear relationship between soluble phosphorus level in the supernatant and growth of culture was

Table 3A. Effect of initial pH, salt and temperature on phosphate solubilization efficiency and pH drop after 24 h of growth.

Parameter		*Enterobacter* sp. LCR1		*Enterobacter* sp. LCR2		*Enterobacter* sp. LCR3	
		Soluble phosphorus (µg/cfu x 10^6)	Final pH	Soluble phosphorus (µg/cfu x 10^6)	Final pH	Soluble phosphorus (µg/cfu x 10^6)	Final pH
Initial pH	7.0	3.00 ± 0.23^{c}	3.91 ± 0.03^{a}	3.31 ± 0.02^{d}	3.91 ± 0.01^{a}	0.08 ± 0.01^{b}	5.34 ± 0.01^{a}
	8.0	2.68 ± 0.50^{b}	3.93 ± 0.04^{a}	2.69 ± 0.17^{c}	3.92 ± 0.07^{a}	0.08 ± 0.01^{b}	4.96 ± 0.47^{a}
	9.0	0.28 ± 0.08^{a}	4.84 ± 0.14^{b}	1.18 ± 0.10^{b}	4.21 ± 0.03^{b}	0.07 ± 0.00^{b}	5.40 ± 0.02^{a}
	10.0	0.12 ± 0.00^{a}	5.60 ± 0.03^{c}	0.12 ± 0.00^{a}	5.22 ± 0.02^{c}	0.01 ± 0.00^{a}	6.95 ± 0.07^{b}
Salt (%)	0.5	2.80 ± 0.37^{b}	3.61 ± 0.02^{a}	2.73 ± 0.11^{b}	3.60 ± 0.01^{a}	0.11 ± 0.00^{b}	5.64 ± 0.2^{a}
	1.0	2.61 ± 0.01^{b}	3.69 ± 0.00^{b}	2.71 ± 0.02^{b}	3.67 ± 0.00^{a,b}	0.10 ± 0.00^{a}	5.78 ± 0.04^{a}
	1.5	2.42 ± 0.37^{a,b}	3.75 ± 0.02^{c}	2.18 ± 0.20^{a}	3.76 ± 0.07^{b,c}	0.10 ± 0.00^{a}	5.78 ± 0.01^{a}
	2.0	2.20 ± 0.02^{a}	3.75 ± 0.00^{c}	2.15 ± 0.12^{a}	3.79 ± 0.04^{c}	0.10 ± 0.00^{a}	5.80 ± 0.08^{a}
Temperature (°C)	30	3.00 ± 0.23^{c}	3.91 ± 0.03^{a}	3.31 ± 0.02^{c}	3.91 ± 0.01^{a}	0.08 ± 0.01^{a}	5.34 ± 0.01^{b}
	40	2.81 ± 0.01^{b}	3.87 ± 0.01^{a}	2.78 ± 0.07^{b}	3.86 ± 0.00^{a}	0.12 ± 0.00^{b}	4.85 ± 0.02^{a}
	50	2.34 ± 0.16^{a}	4.22 ± 0.03^{b}	2.52 ± 0.15^{a}	4.31 ± 0.05^{b}	0.09 ± 0.00^{a}	5.55 ± 0.05^{c}

All values are mean ±SD of three replicates. Separate analysis (Duncan's multiple range test) has been done for each isolate and each experiment, different letters in superscript show significant differences ($P \leq 0.05$).

Table 3B. Effect of initial pH, salt and temperature on phosphate solubilization efficiency and pH drop after 24 h of growth.

Parameter		*Exiguobacterium* sp. LCR4		*Exiguobacterium* sp. LCR5		*Exiguobacterium* sp. LCR6	
		Soluble phosphorus (µg/cfu x 10^6)	Final pH	Soluble phosphorus (µg/cfu x 10^6)	Final pH	Soluble phosphorus (µg/cfu x 10^6)	Final pH
Initial pH	7.0	0.06 ± 0.01^{a}	5.65 ± 0.20^{c}	0.11 ± 0.00^{a}	6.24 ± 0.08^{c}	0.07 ± 0.00^{c}	6.26 ± 0.00^{a}
	8.0	0.29 ± 0.00^{d}	4.90 ± 0.00^{a}	0.37 ± 0.03^{c}	4.91 ± 0.02^{a}	0.03 ± 0.00^{b}	5.95 ± 0.21^{a}
	9.0	0.27 ± 0.00^{c}	4.94 ± 0.02^{a}	0.24 ± 0.00^{b}	4.97 ± 0.02^{a}	0.02 ± 0.00^{b}	6.14 ± 0.38^{a}
	10.0	0.16 ± 0.01^{b}	5.33 ± 0.07^{b}	0.08 ± 0.01^{a}	5.36 ± 0.16^{b}	0.01 ± 0.00^{a}	6.19 ± 0.01^{a}
Salt (%)	0.5	0.04 ± 0.01^{b}	5.90 ± 0.20^{a}	0.02 ± 0.00^{b}	5.98 ± 0.15^{a}	0.04 ± 0.01^{b}	5.90 ± 0.14^{a}
	1.0	0.01 ± 0.00^{a}	6.15 ± 0.01^{ab}	0.01 ± 0.00^{a}	5.93 ± 0.05^{a}	0.01 ± 0.00^{a}	6.18 ± 0.07^{b}
	1.5	Nd	6.23 ± 0.02^{a,b}	Nd	6.14 ± 0.04^{a,b}	Nd	6.18 ± 0.03^{b}
	2.0	Nd	6.30 ± 0.14^{b}	Nd	6.27 ± 0.00^{b}	Nd	6.21 ± 0.02^{b}
Temperature (°C)	30	0.06 ± 0.01^{b}	5.65 ± 0.2^{a}	0.11 ± 0.00^{b}	6.24 ± 0.08^{b}	0.07 ± 0.00^{b}	6.26 ± 0.00^{c}
	40	0.03 ± 0.01^{a}	5.79 ± 0.09^{a,b}	0.01 ± 0.00^{a}	5.97 ± 0.00^{a}	0.02 ± 0.00^{a}	5.92 ± 0.03^{a}
	50	Nd	6.17 ± 0.00^{b}	Nd	6.13 ± 0.02^{a,b}	Nd	6.09 ± 0.06^{b}

All values are mean ±SD of three replicates. Separate analysis (Duncan's multiple range test) has been done for each organism and each experiment and different letters in superscript show significant differences ($P \leq 0.05$). Nd = not detectable.

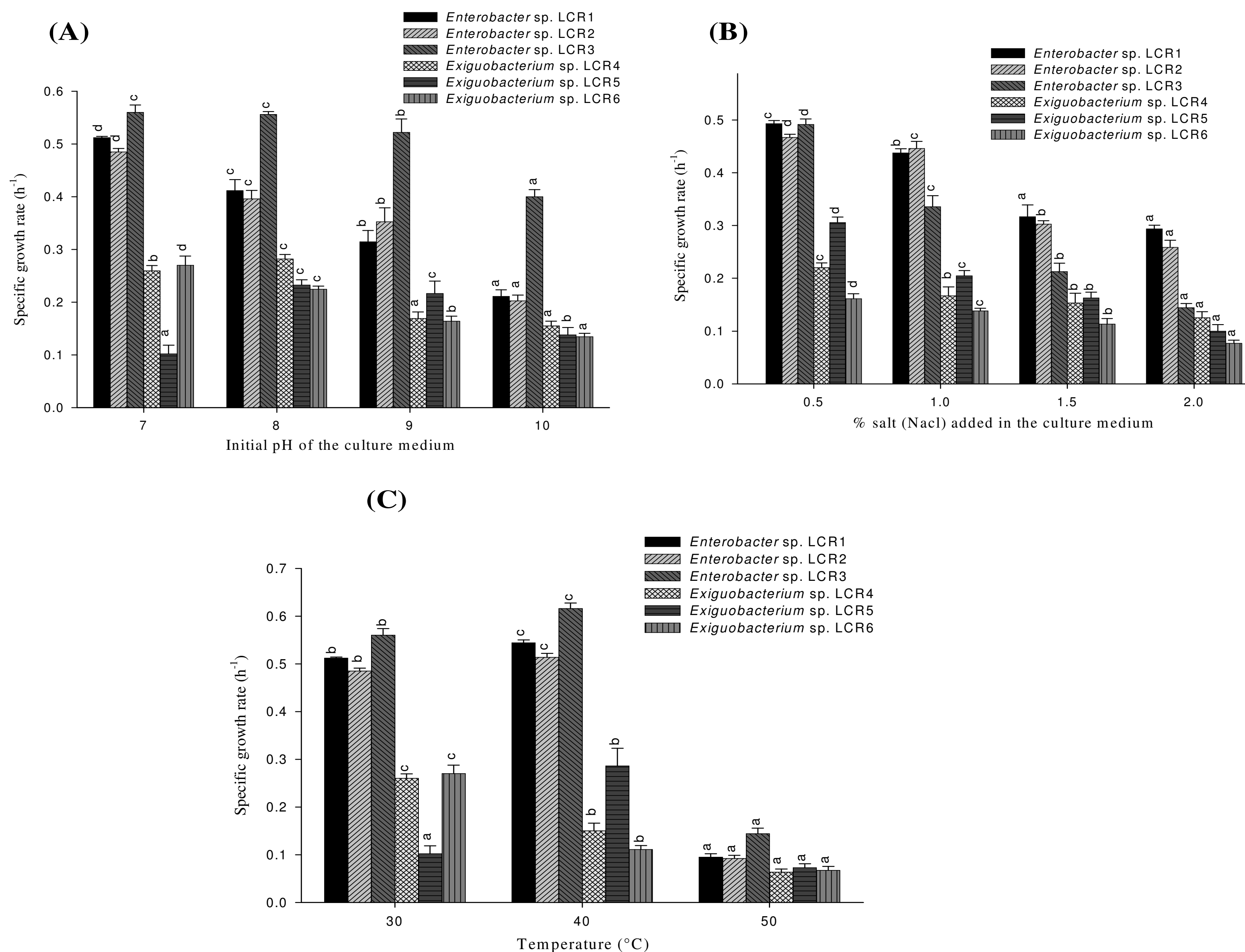

Figure 3. (A) Growth rates of isolates at different initial pH values in NBRIP medium at 30°C. (B) Effect of salt (NaCl) on growth rate of isolates in NBRIP medium at 30°C and initial pH 7.0. Except varying the salt concentration, all other ingredients remained same as in the original NBRIP medium. (C) Growth rates of isolates at different temperatures in NBRIP medium at pH 7.0. Separate statistical analysis has been done for each isolate and bars having different letters show significantly different ($P \leq 0.05$) values.

observed. The *Enterobacter* isolates of this study appear promising in comparison to other isolates as reflected by a high (2.2 - 4.5 fold) and quick phosphate solubilization potential (Chung et al., 2005; Nautiyal et al., 2000; Malboobi et al., 2009). The high phosphate solubilization rate of LCR1 and LCR2 appears justified due to their high solubilization efficiency, while LCR3, LCR4, LCR5 and LCR6 are less efficient solubilizers (Figure 2A). While phosphate solubilizing efficiency and cell density increase for LCR1 and LCR2 goes hand in hand, LCR3, LCR4, LCR5 and LCR6 failed to do so (Figure 2A). This might be due to the co-precipitation of soluble phosphorus following increased secretion of organic metabolite (Illmer and Schinner, 1992).

To test the performance of isolated strains under varying environmental conditions, their phosphate solubilization efficiency was assessed at high pH, salt and temperature. The species of *Enterobacter* LCR1 and LCR2 and *Exiguobacterium* LCR4, LCR5 and LCR6 displayed sensitivity to high salt and temperature as reflected by decreased phosphate solubilization efficiency and reduced growth rate under stressed environment (Table 3 and Figures 3B, C). At high temperature (40°C), growth rate of LCR1, LCR2, LCR3 and LCR5 was

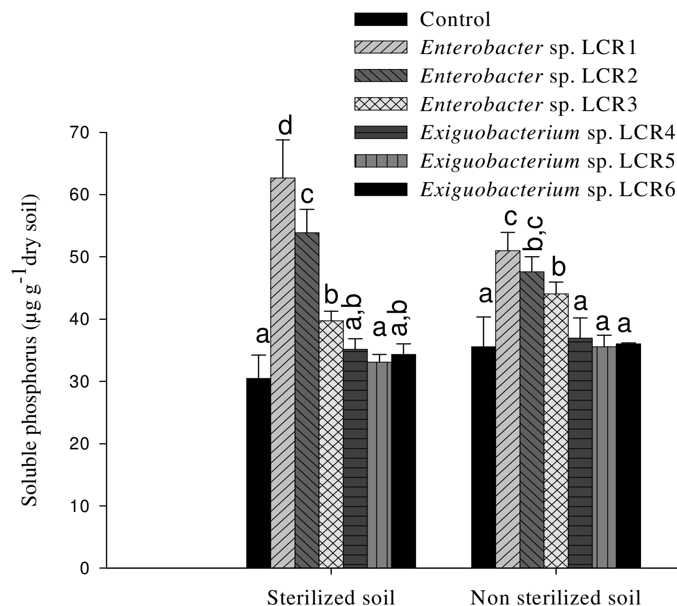

Figure 4. Soluble phosphorus produced by different isolates after six days of inoculation in pot soils. Separate statistical analysis has been done for sterilized and non sterilized samples and bars having different letters show significantly different (P ≤ 0.05) values.

increased while the growth after 24 h was less as compared to 30°C. In contrast to above, *Enterobacter* sp. LCR3 showed tolerance to salt and temperature as evident from phosphate solubilization efficiency (Table 3A). The enhanced phosphate solubilization by LCR3 at low salt concentration finds support from Johri et al. (1999). Under stressful environment, organisms grow slowly which results in decreased solubilization of phosphorus. In spite of reduced growth rate and phosphate solubilization efficiency, *Enterobacter* species isolated from the agriculture farm I, still solubilized 1.6 –2.5 fold phosphate compared to earlier studies (Nautiyal et al., 2000; Malboobi et al., 2009) under given stresses.

Interesting results were also obtained when the effect of increase in pH on the phosphorous solubilization efficiency of the isolated strains was studied. While *Enterobacter* exhibited reduced phosphorous solubilization, *Exiguobacterium* registered increased phosphate solubilization. In contrast to this, phosphate solubilization of *Enterobacter* sp. LCR3 remained unaffected at high pH. Similar trend was also observed by Nautiyal et al. (2000). A reduced phosphate solubilization by *Enterobacter* sp. LCR1 and LCR2 (Figure 3A) may be attributed to the reduced growth, while increased phosphate solubilization by LCR3 and *Exiguobacterium* may be due to the availability of optimum pH required for growth. The results of pH drop also support the data of phosphate solubilization, which is associated with acidification of the culture medium, but the extent of phosphate solubilization and pH drop are not proportionally co-related (Rodríguez and Fraga, 1999).

Pot experiments demonstrated the potential of isolates to serve as biofertilizers. *Enterobacter* strains solubilized phosphate significantly (8.49 - 32.21 µg g^{-1} dry soil) in sterile and non-sterile soils thereby indicating its competence to be established in the natural field. A low amount of soluble phosphorus produced by isolates in compareson to *in vitro* studies could be due to the limitation of nutrients, competition with indigenous microbes as well as precipitation of soluble phosphorus with cations present in the soil (Rodríguez and Fraga, 1999).

Thus *Enterobacter* spp. in general emerged a very efficient phosphate solubilizer under a wide range of pH, salt and temperature as reflected by their high phosphate solubilization efficiency. These isolates grow well at elevated salt level, pH and temperature, thus may be apt for the development of potential bio-inoculants for soils containing insoluble phosphorus. However, a field based trial shall be necessary for understanding their potential in agroecosystem.

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REFERENCES

American Public Health Association (1995). Standard methods for examination of water and waste water. In Eaton AD, Clesceri LS, Greenberg AE (eds), 19th Ed., Washington, D.C.

Babenko YS, Tyrygina G, Grigoryev EF, Dolgikh LM, Borisova TI (1984). Biological activity and physiologo-biochemical properties of bacteria dissolving phosphates. Mikrobiology, 53: 533-539.

Benlloch S, López-López A, Casamayor EO, Øvreås L, Goddard V, Daae FL, Smerdon G, Massana R, Joint I, Thingstad F, Pedrós-Alió C, Rodríguez-Valera F (2002). Prokaryotic genetic diversity throughout the salinity gradient of a coastal solar saltern. Environ. Microbiol., 4: 349-360.

Black JIH, Parekh NR, Chaplow JS, Monson F, Watkins J, Creamer R, Potter ED, Poskitt JM, Rowland P, Ainsworth G, Hornung M (2003). Assesing soil biodiversity across Great Britain: national trends in the occurrence of heterotrophic bacteria and invertebrates in soil. J. Environ. Manage., 67: 225-266.

Brenner JD, Farmer III JJ (2005) Order XIII, Enterobacteriales. In Brenner DJ, Krieg NR, Staley JT (Eds) Bergey's, Springer, New York. Man. Syst. Bacteriol., 2: 587-850.

Chung H, Park M, Madhaiyan M, Seshadri S, Song J, Cho H, Sa T (2005). Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biol. Biochem., 37: 1970-1974.

Collee JC, Miles RS, Watt B (1996). Tests for the identification of bacteria. In Collee JG, Marmion BP, Fraser AG, Simmons A (Eds), Churchill Livingstone Inc, New York, pp. 131-149.

Souza MJBDD, Nair S, Chandramohan D (2000). Phosphate solubilizing bacteria around Indian peninsula. Indian J. Mar. Sci., 29: 48-51.

FAO (2002). World Agriculture: Towards 2015/2030 Summary Report. FAO, Rome, Italy.

Gen-Fu W, Xue-Ping Z (2005). Characterization of phosphorus-releasing bacteria in a small eutrophic shallow lake, Eastern China. Water Res. 39: 4623-4632.

Illmer P, Schinner F (1992). Solubilization of inorganic phosphates by microorganisms isolated from forest soils. Soil Biol. Biochem., 24: 389-395.

Janisiewicz WJ, Bors B (1995). Development of a microbial community of bacterial and yeast antagonist to control wound-invading postharvest pathogens of fruits. Appl. Environ. Microbiol., 61: 3261-3267.

Jeon JS, Lee SS, Kim HY, Ahn TS, Song HG (2003). Plant growth promotion in soil by some inoculated microorganisms. J. Microbiol., 41: 271-276.

Johri JK, Surange S, Nautiyal CS (1999). Occurrence of salt, pH, and temperature-tolerant, phosphate solubilizing bacteria in alkaline soils. Curr. Microbiol., 39: 89-93.

Kim IG, Lee MH, Jung SY, Song JJ, Oh TK, Yoon JH (2005). *Exiguobacterium aestuarii* sp. nov. and *Exiguobacterium marinum* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. Int. J. Syst. Evol. Microbiol., 55: 885-889.

Kucey RMN, Janzen HH, Leggett ME (1989). Microbially mediated increases in plant available phosphorus. Adv. Agron., 42: 199-225.

López-Cortés A, Schumann P, Pukall R, Stackebrandt E (2006). *Exiguobacterium mexicanum* sp. nov. and *Exiguobacterium artemiae* sp. nov., isolated from the brine shrimp *Artemia franciscana.* Syst. Appl. Microbiol., 29: 183-190.

López-Gutiérrez JC, Toro M, López-Hernández D (2004). Seasonality of organic phosphorus mineralization in the rhizosphere of the native savanna grass, *Trachypogon plumosus.* Soil Biol. Biochem., 36: 1675-1684.

Malboobi MA, Owlia P, Behbahani M, Sarokhani E, Moradi S, Yakhchali B, Deljou A, Heravi KM (2009). Solubilization of organic and inorganic phosphates by three highly efficient soil bacterial isolates. World J. Microbiol. Biotechnol., 25: 1471-1477.

Nautiyal CS (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol. Lett., 170: 265-270.

Nautiyal CS, Bhadauria S, Kumar P, Lal H, Mondal R, Verma D (2000). Stress induced phosphate solubilization in bacteria isolated from alkaline soils. FEMS Microbiol. Lett., 182: 291-296

Olsen SR, Cole CV, Watanabe FS, Dean LA (1954). Estimation of available phosphorus in soils by extraction with sodium bicarbonate, US Government Printing Office, USDA Washington, D.C. Circ. p. 939.

Patil MG, Sayyed RZ, Chaudhari AB, Chincholkar SB (2002). Phosphate solubilizing microbes: a potential bioinoculant for efficient use of phosphate fertilizers. In Proceedings of National Symposium: Bioinoculants for Sustainable Agric. for.

Pérez E, Sulbarán M, Ball MM, Yarzábal LA (2007). Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. Soil Biol. Biochem., 39: 2905-2914.

Pikovskaya RI (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Mikrobiology, 17: 362-370.

Rodríguez H, Fraga R (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol. Advan., 17: 319-339.

Son HJ, Park GT, Cha MS, Heo MS (2006). Solubilization of insoluble inorganic phosphates by a novel salt- and pH-teolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere. Biores. Technol., 97: 204-210.

Srivastava AK, Ara A, Bhargava P, Mishra Y, Rai SP, Rai LC (2007). A rapid and cost-effective method of genomic DNA isolation from cyanobacterial culture, mat and soil suitable for genomic fingerprinting and community analysis. J. Appl. Phycol., 19: 373-82.

Stephan R, Trapen SV, Cleenwerck I, Vancanneyt M, Vos PD, Lehner A (2007). *Enterobacter turicensis* sp. nov. and *Enterobacter helviticus* sp. nov., isolated from fruit powder. Int. J. Syst. Evol. Microbiol., 57: 820-826.

Sundara B, Natarajan V, Hari K (2002). Influence of phosphorus solubilizing bacteria on the changes in soil available phosphorus and sugarcane and sugar yields. Field Crops Res., 77: 43-49.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acid. Res., 25: 4876-4882.

Yi Y, Huang W, Ge Y (2008). Exopolysaccharide: a novel important factor in the microbial dissolution of tricalcium phosphate. World J. Microbiol. Biotechnol., 24: 1059-1065.