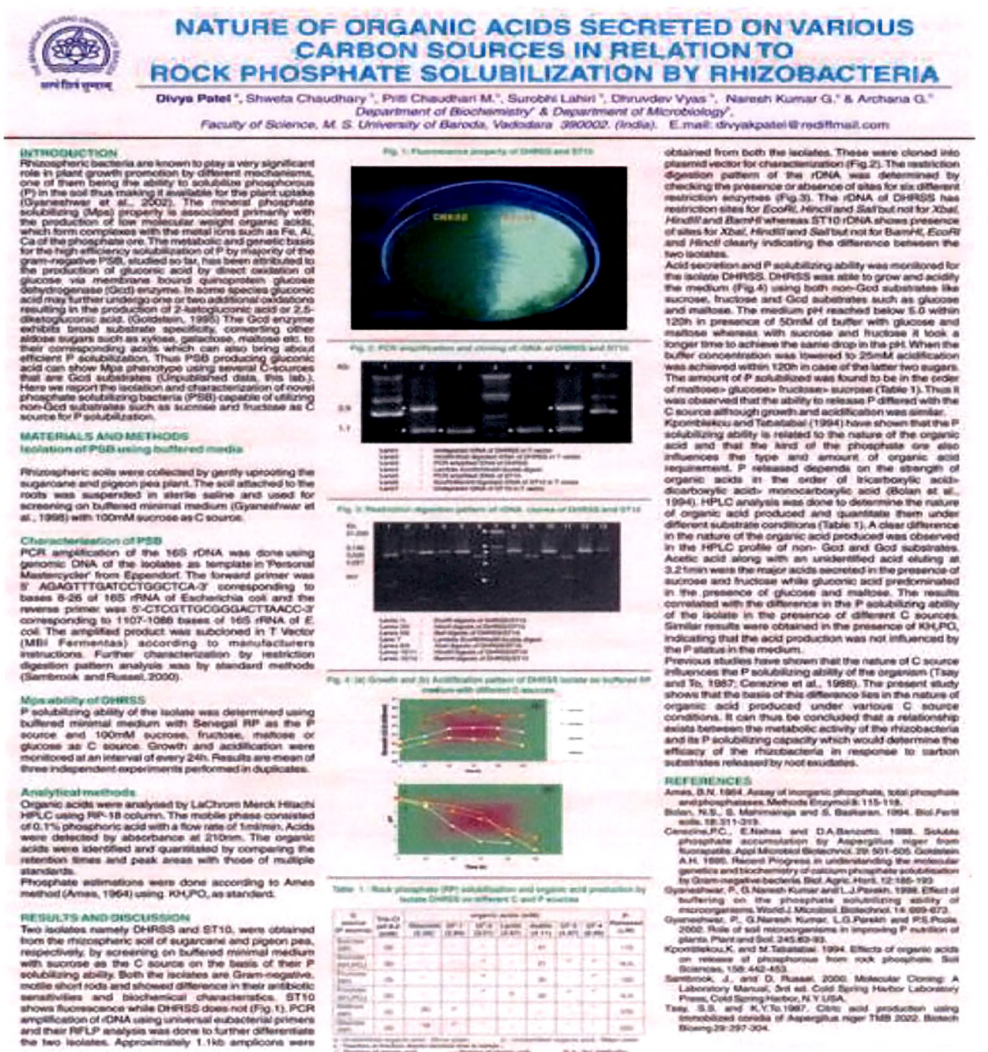
CHAPTER 2

Biochemical Characterization of Variation in the Nature of Organic Acid Secretion and Rock Phosphate Solubilization by Rhizobacteria, *Citrobacter* sp. DHRSS on Various Carbon Sources



NATURE OF ORGANIC ACIDS SECRETED ON VARIOUS CARBON SOURCES IN RELATION TO ROCK PHOSPHATE SOLUBILIZATION BY RHIZOBACTERIA

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INTRODUCTION

Rhizospheric bacteria are known to play a very significant role in plant growth promotion by different mechanisms, one of them being the ability to solubilize phosphorous (P) in the soil thus making it available for the plant uptake (Gyaneshwar et al., 2002). The mineral phosphate solubilizing (Mps) property is associated primarily with the production of low molecular weight organic acids, which form complexes with the metal ions such as Fe, Al, Ca of the phosphate ore. The metabolic and genetic basis for the high efficiency solubilization of P by majority of the gram-negative PSB, studied so far, has been attributed to the production of gluconic acid by direct oxidation of glucose via membrane bound quinoprotein glucose dehydrogenase (Gcd) enzyme. In some species gluconic acid may further undergo one or two additional oxidations resulting in the production of 2-ketogluconic acid or 2,5-diketogluconic acid. (Goldstein, 1995) The Gcd enzyme exhibits broad substrate specificity, converting other aldose sugars such as xylose, galactose, maltose etc. to their corresponding acids which can also bring about efficient P solubilization. Thus PSB producing gluconic acid can show Mps phenotype using several C-sources that are Gcd substrates (Unpublished data, this lab.). Here we report the isolation and characterization of novel phosphate solubilizing bacteria (PSB) capable of utilizing non-Gcd substrates such as sucrose and fructose as C source for P solubilization.

MATERIALS AND METHODS

Isolation of PSB using buffered media

Rhizospheric soils were collected by gently uprooting the sugarcane and pigeon pea plant. The soil attached to the roots was suspended in sterile saline and used for screening on buffered minimal medium (Gyaneshwar et al., 1998) with 100mM sucrose as C source.

Characterisation of PSB

PCR amplification of the 16S rDNA was done using genomic DNA of the isolates as template in 'Personal Mastercycler' from Eppendorf. The forward primer was 5' AGAGTTTGATCCTGGCTCA-3' corresponding to bases 8-26 of 16S rRNA of Escherichia coli and the reverse primer was 5'-CTCGTTGCGGGACTTAACC-3' corresponding to 1107-1088 bases of 16S rRNA of *E. coli*. The amplified product was subcloned in T Vector (MBI Fermentas) according to manufacturers instructions. Further characterization by restriction digestion pattern analysis was by standard methods (Sambrook and Russel, 2000).

Mps ability of DHRSS

P solubilizing ability of the isolate was determined using buffered minimal medium with Senegal RP as the P source and 100mM sucrose, fructose, maltose or glucose as C source. Growth and acidification were monitored at an interval of every 24h. Results are mean of three independent experiments performed in duplicates.

Analytical methods

Organic acids were analysed by LaChrom Merck Hitachi HPLC using RP-18 column. The mobile phase consisted of 0.1% phosphoric acid with a flow rate of 1ml/min. Acids were detected by absorbance at 210nm. The organic acids were identified and quantitated by comparing the retention times and peak areas with those of multiple standards.
Phosphate estimations were done according to Ames method (Ames, 1964) using KH_2PO_4 as standard.

RESULTS AND DISCUSSION

Two isolates namely DHRSS and ST10, were obtained from the rhizospheric soil of sugarcane and pigeon pea, respectively, by screening on buffered minimal medium with sucrose as the C source on the basis of their P solubilizing ability. Both the isolates are Gram-negative, motile short rods and showed difference in their antibiotic sensitivities and biochemical characteristics. ST10 shows fluorescence while DHRSS does not (Fig.1). PCR amplification of rDNA using universal eubacterial primers and their RFLP analysis was done to further differentiate the two isolates. Approximately 1.1kb amplicons were obtained from both the isolates. These were cloned into plasmid vector for characterization (Fig.2). The restriction digestion pattern of the rDNA was determined by checking the presence or absence of sites for six different restriction enzymes (Fig.3). The rDNA of DHRSS has restriction sites for *EcoRI*, *HincII* and *SalI* but not for *XbaI*, *HindIII* and *BamHI* whereas ST10 rDNA shows presence of sites for *XbaI*, *HindIII* and *SalI* but not for *BamHI*, *EcoRI* and *HincII* clearly indicating the difference between the two isolates.
Acid secretion and P solubilizing ability was monitored for the isolate DHRSS. DHRSS was able to grow and acidify the medium (Fig.4) using both non-Gcd substrates like sucrose, fructose and Gcd substrates such as glucose and maltose. The medium pH reached below 5.0 within 120h in presence of 50mM of buffer with glucose and maltose whereas with sucrose and fructose it took a longer time to achieve the same drop in the pH. When the buffer concentration was lowered to 25mM acidification was achieved within 120h in case of the latter two sugars. The amount of P solubilized was found to be in the order of maltose> glucose> fructose> sucrose (Table 1). Thus it was observed that the ability to release P differed with the C source although growth and acidification was similar.
Kpombekou and Tabatabai (1994) have shown that the P solubilizing ability is related to the nature of the organic acid and that the kind of the phosphate ore also influences the type and amount of organic acid requirement. P released depends on the strength of organic acids in the order of tricarboxylic acid> dicarbowylic acid> monocarbowylic acid (Bolan et al., 1994). HPLC analysis was done to determine the nature of organic acid produced and quantitate them under different substrate conditions (Table 1). A clear difference in the nature of the organic acid produced was observed in the HPLC profile of non- Gcd and Gcd substrates. Acetic acid along with an unidentified acid eluting at 3.2 min were the major acids secreted in the presence of sucrose and fructose while gluconic acid predominated in the presence of glucose and maltose. The results correlated with the difference in the P solubilizing ability of the isolate in the presence of different C sources. Similar results were obtained in the presence of KH_2PO_4 indicating that the acid production was not influenced by the P status in the medium.
Previous studies have shown that the nature of C source influences the P solubilizing ability of the organism (Tsay and To, 1987; Cerezine et al., 1988). The present study shows that the basis of this difference lies in the nature of organic acid produced under various C source conditions. It can thus be concluded that a relationship exists between the metabolic activity of the rhizobacteria and its P solubilizing capacity which would determine the efficacy of the rhizobacteria in response to carbon substrates released by root exudates.

REFERENCES

Ames, B.N. 1964. Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8: 115-118.
Bolan, N.S., S. Manimaraja and S. Balkaran. 1994. Biol.Fertil soils, 18:311-319.
Cerezine,P.C., E.Nahas and D.A.Banzotto. 1988. Soluble phosphate accumulation by Aspergillus niger from fluorapatite. Appl Microbiol Biotechnol. 29: 501-505. Goldstein A.H. 1995. Recent Progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilisation by Gram-negative bacteria. Biol. Agric. Hort. 12:185-193
Gyaneshwar, P., G.Naresh Kumar and L.J.Parekh. 1998. Effect of buffering on the phosphate solubilizing ability of microorganisms. World J. Microbiol. Biotechnol. 14: 669-673.
Gyaneshwar, P., G.Naresh Kumar, L.G.Parekh and P.S.Poole. 2002. Role of soil microorganisms in improving P nutrition of plants. Plant and Soil. 245:83-93.
Kpomblekou,K. and M.Tabatabai. 1994. Effects of organic acids on release of phosphorous from rock phosphate. Soil Sciences, 158: 442-453.
Sambrook, J., and D. Russel. 2000. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA.
Tsay, S.S. and K.Y.To. 1987. Citric acid production using immobilized conidia of Aspergillus niger TMB 2022. Biotech Bioeng.29: 297-304.

2.1: INTRODUCTION

Phosphorus (P) is the second major macronutrient required for plant growth (Theodorou and Plaxton, 1993; Vance, 2001). P is present in soil in abundance in both organic and inorganic forms, but the majority of it is immobilized and rendered unavailable for plant uptake as it complexes with cations of Ca, Fe, and Al, depending on the type of soils. Thus, only the phosphate in a soluble ionic form (Pi) is effective as a mineral nutrient (Kucey *et al.*, 1989; Ae *et al.*, 1991). Several attempts to overcome the P deficiency problem including the application of chemical P fertilizers have not been very effective due to the high refixing ability of phosphate in the soil (Goldstein *et al.*, 1993).

Rhizospheric bacteria are known to play a very significant role in plant growth promotion by different mechanisms, one of them being the ability to solubilize mineral phosphate in the rhizosphere, thus making it available for plant uptake (Kucey *et al.*, 1989; Gyaneshwar *et al.*, 2002). A variety of bacteria, belonging to diverse genera, and several groups of fungi have been reported to show mineral phosphate solubilizing (mps) ability (Cunningham and kuiack, 1992; Vassilev *et al.*, 1996; Yadav and Dadarwal, 1997; Sharma *et al.*, 2005). The mps property is due to a drop in pH, which has been associated with their ability to secrete low molecular weight organic acids such as gluconic, 2-ketogluconic, oxalic, citric, acetic, malic, and succinic, etc. (Goldstein *et al.*, 1993; Bolan *et al.*, 1994; Sharma *et al.*, 2005). *Aspergillus niger* and some *Penicillium* species have been shown to solubilize mineral phosphates by the secretion of organic acids such as gluconic, citric, and oxalic acid (Cerezine *et al.*, 1988; Cunningham and kuiack, 1992; Vassileva *et al.*, 1998; Reyes *et al.*, 1999a).

Solubilization of mineral phosphates mediated by gram-negative rhizobacteria has been extensively studied using glucose as the carbon source (Goldstein, 1995; Gyaneshwar *et al.*, 1999). Gluconic acid is one of the prominent organic acids responsible for P solubilization and is produced by direct oxidation of glucose via membrane bound quinoprotein GDH enzyme. The GDH enzyme is known to exhibit broad substrate specificity insome organisms (Sharma *et al.*, 2005) in that it can also convert other aldose sugars such as xylose, galactose, and maltose, in addition to glucose, to their corresponding aldonic acids, which can also bring about efficient P solubilization.

Root exudates are known to serve as a substantial source of reduced carbon compounds which are released in the rhizosphere (Katznelson and Bose, 1959; Lugtenberg *et al.*, 1999). Microbes in the rhizosphere utilize root exudates as their major nutrient source and this forms the basis for rhizosphere colonization. Apart from glucose, many different carbon sources are found in root exudates and sucrose is one of the common sugars (Jaeger *et al.*, 1999). Sucrose has been detected in large amounts in the soil near the root tip and large numbers of bacteria occur near the root area, with the highest sucrose and tryptophan exudation. Cowpea root exudates also contain arabinose, ribose, glucose, and sucrose as the main constituents (Odunfa and Werner, 1981). Glucose and fructose were the major components in all growth stages of stonewool-grown tomato (Kamilova *et al.*, 2006). Thus rhizobacteria demonstrating mps ability using sucrose and fructose as carbon sources for P solubilization could be very effective in field conditions. For such sugars which are not glucose dehydrogenase (GDH) substrates, the organic acid secreted is of interest since organic acids other than aldonic acids are expected. Here we report the isolation and characterization of bacteria from the sugarcane rhizosphere with mps ability on aldo- and keto-sugars.

2.2: MATERIALS AND METHODS

2.2.1: Screening Of Phosphate Solubilizing Bacterium (PSB) Using Buffered Media

Rhizospheric soil samples were collected by gently uprooting the sugarcane. The loosely adhering soil was removed by gentle shaking and the soil that remained attached to the roots was suspended in sterile saline. Serially diluted soil suspension was used for screening on buffered minimal medium with 100 mM sucrose as the sole carbon (C) source for isolation of potential PSB (Gyaneshwar *et al.*, 1998a). Commercial-grade Senegal rock phosphate (RP; 15.94% P; composition given in Ref. of Sharma *et al.*, 2005) at a concentration of 1.0 g/L was used as source of insoluble mineral phosphate and 1% methyl red as indicator dye for visualization of color change on organic acid production.

2.2.2: Identification Of PSB By 16S rRNA Gene Sequence Analysis

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was done using genomic DNA of the isolate as template in an Eppendorf Personal Mastercycler.

The forward primer was 5'-AGAGTTTGATCCTGGCTCA-3', corresponding to bases 8–26 of 16S rRNA of *Escherichia coli*, and the reverse primer was 5'-CTCGTTGCGGG ACTTAACC-3', corresponding to 1107–1088 bases of 16S rRNA of *E. coli* (Raul *et al.*, 2004). The PCR kit and primers were obtained from Bangalore Genie Pvt. Ltd, India. The amplified product was subcloned in pTZ57R vector (MBI Fermentas) according to the manufacturer's instructions. The nucleotide sequence was determined by the automated DNA sequencing service provided by Bangalore Genie Pvt. Ltd., using the M13 reverse primer. The partial 16S rRNA gene sequence has been deposited in GenBank (**Accession number DQ486057**). Molecular biology techniques were performed according to standard methods (Sambrook and Russel, 2001).

2.2.3: Characterization Of mps Ability Of The Isolate

The mps ability of the isolate was determined on minimal medium with Senegal RP as the sole P source and varying concentrations of Tris Cl, pH 8.0 (0, 25, 50, 75, and 100 mM). Detailed studies were done using the highest buffer concentration which allowed a drop in the pH of the growth medium. Thus, 25 mM Tris Cl, pH 8.0, buffer was used in the case of sucrose and fructose, whereas 50 mM Tris Cl, pH 8.0, was selected for maltose and glucose. All the C sources were used at a 100 mM concentration. Aliquots were withdrawn at 24-h intervals. Absorbance at 600 nm and pH drop were used as parameters for growth pattern and acidification, respectively. Culture supernatant when the pH dropped below 5.0 was used for identification of organic acids and for estimating soluble Pi. Results are Mean ± S.D. of three independent experiments performed in duplicate.

2.2.4: Measurement Of GDH Activity

Bacterial isolate was grown on RP-containing minimal medium with 100 mM either glucose or sucrose as the C source and 50 or 25 mM Tris Cl, pH 8.0, respectively. After the pH dropped below 5.0, cells were harvested (5000g for 10 min), washed with sterile saline, and resuspended in 50 mM Tris Cl, pH 8.75, and the whole-cell suspension was used as the source of enzyme in GDH assays done according to Matsushita and Ameyama (1982). To determine the substrate specificity of the GDH enzyme, glucose was replaced with a 100 mM concentration of other sugars such as maltose, xylose, arabinose, galactose, and mannose in the assay mixture. Units of activity of GDH are

defined as μmoles of 2,6-dichlorophenolindophenol (DPIP) reduced per minute. Specific activity is defined as units per milligram of protein.

2.2.5: Invertase Assay

The bacterial isolate was grown overnight in M9 minimal medium with sucrose or glucose as the carbon source. Cells were harvested by centrifugation at 5000g for 10 min, then washed with and resuspended in sterile saline. An aliquot of the cells was used for toluenization to permeabilize the cells. Whole cells as well as toluenized cells were used for enzyme assay. The assay system (2 ml) consisted of 0.01 M sucrose, 0.1 M buffer (acetate buffer, pH 5.0, phosphate buffer, pH 7.0, or Tris Cl buffer, pH 8.0), with an appropriate amount of cells as the source of enzyme. The reaction system was incubated at 37°C for 30 min and the reducing sugar produced was measured by the method of Miller (1959). One unit of activity is defined as the amount of enzyme that produced reducing sugar equivalent to 1 μmol of glucose per h. Specific activity is defined as units per milligram of protein.

2.2.6: Analytical Methods

Organic acids were analyzed by LaChrom Merck Hitachi high-performance liquid chromatography (HPLC). The culture supernatant was filtered through a 0.22 μm nylon filter. The organic acids were separated using an RP-18 column. The mobile phase consisted of 0.1% phosphoric acid at a flow rate of 1 ml/min. Acids were detected by absorbance at 210 nm. The organic acids were identified and quantitated by comparing the retention times and peak areas with those of standard acids.

Phosphate estimations were done according to the Ames method using KH2PO4 as standard (Ames, 1964). Total whole-cell protein was estimated using a modified Lowry's method (Peterson, 1979). Cell growth was estimated by optical density measurements at 600 nm. Dry cell mass was calculated considering that 1 unit of OD at 600 nm corresponds to a dry cell weight of 0.50 ± 0.01 g/L (Zheng *et al.*, 2005). C source consumed was determined by measuring the amount of residual sugars in the culture supernatant as reducing sugar by the method of Miller (1959). Sucrose was estimated by the same method after acid hydrolysis with 2 N HCl.

2.3: RESULTS

2.3.1: Isolation And Identification Of PSB Using Sucrose Containing Buffered Minimal Media

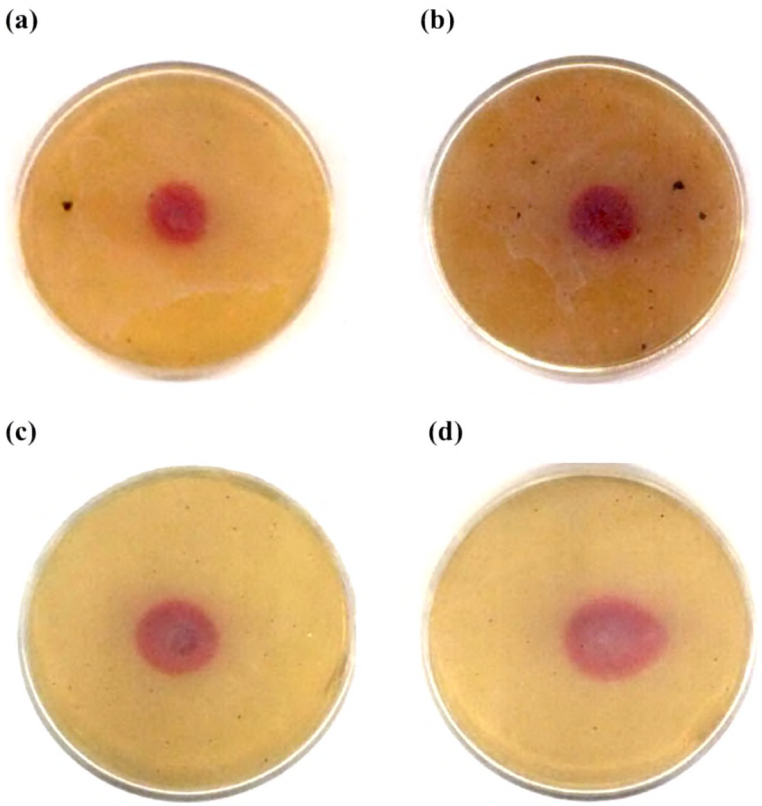
Sucrose was used as the preferred C source for the isolation of PSMs from the rhizosphere. Many isolates were obtained from the rhizospheres of different plants by screening in the presence of RP as the sole P source under buffered conditions. The isolates were characterized on the basis of their ability for growth and acidification on methyl red containing minimal medium plates.

About 13 PSMs were obtained from the rhizospheres of tuver and chili plants, an almost-equal number was obtained from the rhizospheres of cotton, and a few from sugarcane and jowar. These PSMs were further characterized based on the time taken to grow and acidify the plates and the extent and intensity of the zone of clearance. An isolate, namely, DHRSS, obtained from the rhizospheric soil of sugarcane, was selected for further characterization on the basis of its higher efficiency to show a red zone of acid secretion and, thereby, P solubilization **(Fig. 2.1a).**

Approximately 1.1 kb of the hypervariable region of the 16S rRNA gene was amplified from the genomic DNA of DHRSS by PCR using primers corresponding to the highly conserved region of the *E. coli* 16S rRNA gene sequence **(Fig. 2.2).** About 400 bp of the rDNA was sequenced using the M13 reverse primers from the plasmid backbone **(Fig. 2.3 a).** The sequence information obtained was subjected to sequence alignment in the online homology search programe, Ribosomal Database Project (RDP) II. The highest homology was detected with *Citrobacter freundii* (0.988%), indicating that the isolate belonged to the genus *Citrobacter* of family *Enterobacteriaceae* **(Fig. 2.3 b).** The GenBank accession number obtained for this partial 16S rDNA sequence is **DQ486057** and the accession profile is given in **Fig. 2.3 c**.

Preliminary biochemical characterization of DHRSS was performed according to Bergey's manual of determinative bacteriology and it was shown to be Gram-negative, motile short rods belonging to the *Enterobacteriaceae* family.

Fig. 2.1: Acidification on RP minimal medium containing methyl red by *Citrobacter* sp. DHRSS



(a) sucrose and 25mM Tris Cl (pH 8.0); (b) fructose and 25mM Tris Cl (pH 8.0); (c) glucose and 50mM Tris Cl (pH 8.0) and (d) maltose and 50mM Tris Cl (pH 8.0)

Fig. 2.2: PCR amplification and cloning of 16S rDNA of *Citrobacter* sp. DHRSS

Lane1: Undigested rDNA of DHRSS in T vector
Lane2: *Hind*III/*Xba*I digested rDNA of DHRSS in T vector
Lane3: 1.1kb PCR amplified rDNA of DHRSS
Lane4: Lambda *Eco*RI/*Hind*III double digest

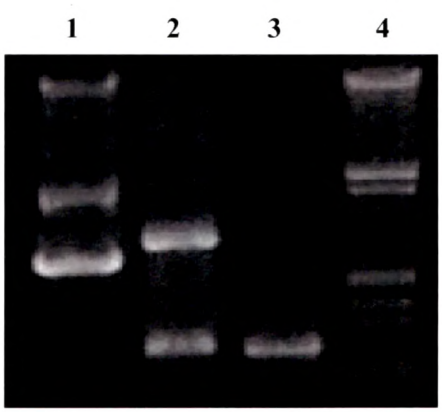


Fig. 2.3 (a): Partial 16S rDNA sequence of *Citrobacter* sp. DHRSS

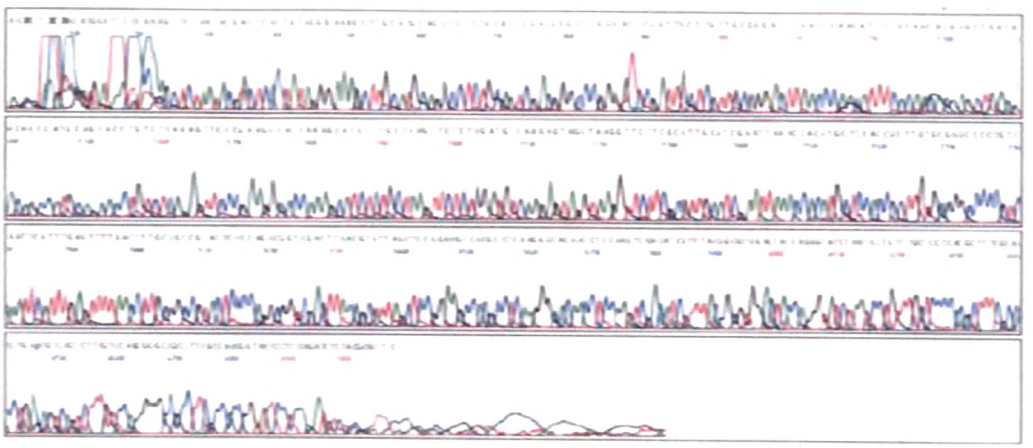


Fig. 2.3 (b): Homology search result of partial 16S rDNA of *Citrobacter* sp. DHRSS using RDP II

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class Gammaproteobacteria (0/20/13993)
order Enterobacteriales (0/20/2305)
family Enterobacteriaceae (0/20/2305)
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genus Enterobacter (0/16/238)
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S000139838 0.988 1409 unidentified bacterium; GLB-2; AY345576
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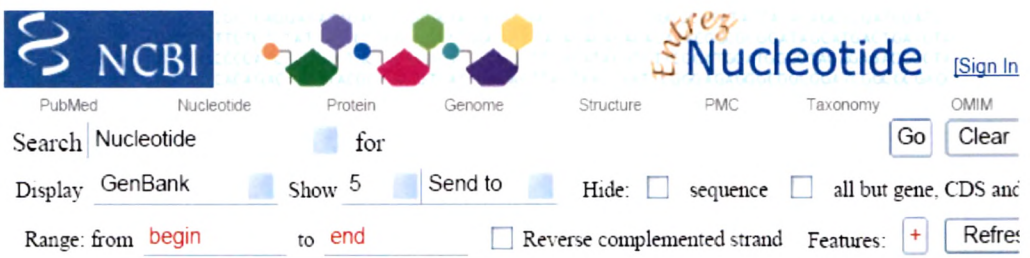
Fig. 2.3 (c): Genbank accession result of partial 16S rDNA sequence of *Citrobacter* sp. DHRSS



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            Phosphate Solubilization by Citrobacter sp. DHRSS in the Presence
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  JOURNAL   Curr. Microbiol. 56 (2), 168-174 (2008)
   PUBMED   17965911
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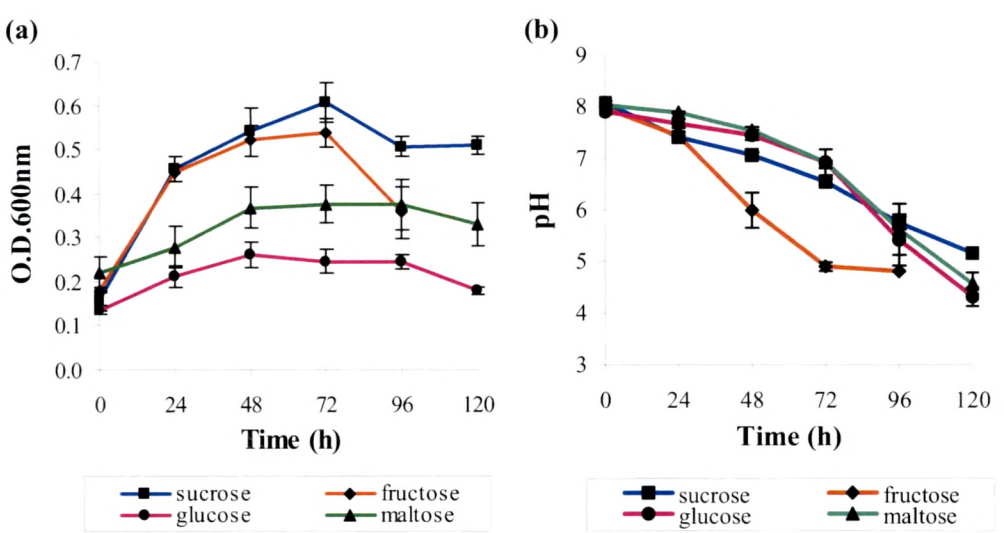
2.3.2: Characterization Of The mps Ability Of *Citrobacter* sp. DHRSS

In addition to sucrose, *Citrobacter* sp. DHRSS showed a red zone of acidification on buffered RP minimal medium plates containing methyl red and fructose, glucose, or maltose as C source **(Fig. 1b–d).** The level of buffering that allowed a drop in the medium pH was found to vary with the C source used. On sucrose and fructose, *Citrobacter* sp. DHRSS was unable to decrease the medium pH beyond 25 mM Tris Cl (pH 8.0) buffering, whereas with glucose and maltose as C sources, the concentration to which *Citrobacter* sp. DHRSS was able to grow and acidify the medium was 50 mM. Medium was acidified to pH<5.0 by 72 h on fructose and 120 h on sucrose, glucose, and maltose (**Fig. 2.4a and b).** A maximum of 570 µM (54.7 mg/L) P was released in the presence of maltose, followed by glucose 520 µM (50 mg/L), amounting to about 30%–35% of the total P. The specific RP solubilizing ability was also found to be highest with these C sources **(Table 2.1).** Thus, the amount of RP solubilized was found to depend on the nature of the sugar in the order of maltose>glucose>sucrose>fructose.

Fig. 2.4: (a) Growth and (b) acidification pattern of *Citrobacter* Sp. DHRSS on buffered RP medium with different C sources



25 mM Tris Cl (pH 8.0) medium was used for fructose and sucrose; 50 mM Tris Cl (pH 8.0) medium was used for glucose and maltose; Values given are Mean ± S.D. of three independent experiments performed in duplicates.

Table 2.1: Rock phosphate (RP) solubilization and organic acid production by *Citrobacter* sp. DHRSS on different C sources

		Sucrose	Fructose	Glucose	Maltose
C source consumed mM (g/L)		52.1 ± 1.4 (17.8 ± 0.5)	34.4 ± 1.1 (6.2 ± 0.2)	73.9 ± 2.9 (28.8 ± 0.2)	80 ± 0.6 (13.3 ± 0.5)
Dry cell mass [a] (g/L)		0.255 ± 0.01	0.178 ± 0.03	0.1 ± 0.004	0.165 ± 0.02
Organic acids mM (g/L)	Gluconic (2.36) [b]	ND	ND	19 ± 2.5 (4.1 ± 0.5)	20 ± 5.7 (4.4 ± 1.2)
	Pyruvic (3.21) [b]	ND	4.28 ± 0.8 (0.4 ±0.1)	ND	ND
	Acetic (4.11) [b]	49 ± 6.4 (2.9 ± 0.4)	35 ± 4.5 (2.1± 0.3)	ND	ND
Organic acid yields [c] (g/g)		0.16 ± 0.03	0.41 ± 0.02	0.31 ± 0.02	0.15 ± 0.01
Organic acid productivity [d] (g/g)		11.5	14.2	41.4	26.4
P released (µM)		170 ± 2.8	100 ± 4.2	520 ± 31.1	570 ± 28.2
Phosphate released (mg/L)		16.3 ± 0.3	9.6 ± 0.3	50 ± 0.5	54.7 ± 0.6
Sp. RP solubilization [e] (mg /g)		64 ± 4	54 ± 3	500 ± 3.1	331 ± 7.5
Soluble P/total P (%)		10.24	6.02	31.36	34.33

[a] Dry cell mass is calculated considering1.0 O.D. = 0.5 ± 0.01 g/L dry cell mass; [b] Numbers in brackets denote retention time in minutes for standard organic acids as determined by HPLC analysis; [c] The organic acid yields are calculated as g of organic acid produced per g of C source consumed; [d] The organic acid productivity is calculated as g of organic acid produced per g dry cell mass; [e] Sp. RP solubilization is calculated as mg of phosphate released per g of dry cell mass; Values given are Mean ± S.D. of three independent experiments performed in duplicates; ND - Not Detected

2.3.3: Determination Of The Nature Of Organic Acids

HPLC analysis of the medium supernatant was done to determine the nature and amount of organic acids secreted by *Citrobacter* sp. DHRSS. Approximately 49 mM (2.94 g/ L) and 35 mM (2.1 g/L) acetic acid was secreted when sucrose and fructose were used as C source, respectively. A low level (~ 4 mM; 0.44 g/L) of pyruvic acid was also detected on fructose. On the contrary, approximately 20 mM (4.36 g/L) gluconic acid alone was secreted on glucose and maltose **(Table 2.1).** However, no gluconic acid secretion was detected in the presence of sucrose or fructose. Acid production was not found to be growth related. The acid accumulation was detected only in the late log phase when the pH of the medium fell below 5.0. The maximum yields of organic acids per gram of C source utilized were obtained on fructose and glucose, whereas the productivity of the organic acids (expressed per unit biomass) was found to be maximum in the presence of glucose and maltose.

In order to account for the nature of organic acids produced in the presence of different C sources, invertase and GDH activity of *Citrobacter* sp. DHRSS was monitored. *Citrobacter* sp. DHRSS was grown on sucrose minimal medium and invertase assay was done using toluenized cells as well as whole cells. No activity was observed in the case of whole cells. In the case of toluenized cells invertase activity was 94 ± 13 and 62 ± 6 units at pH 7.0 and 8.0, respectively, but no activity was found at pH 5.0. Invertase activity was also not found on glucose-grown cells, indicating that the intracellular invertase is induced in the presence of sucrose.

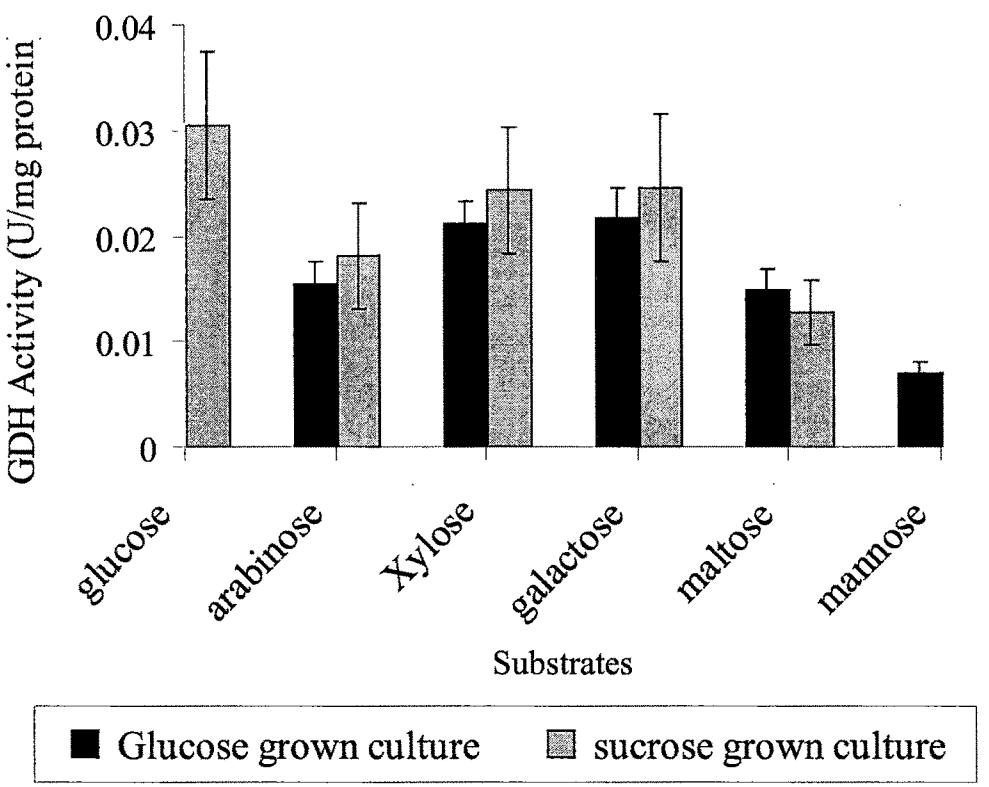
GDH activity was measured after growing the culture on buffered RP minimal medium with either glucose or sucrose as C source. The isolate showed low levels of GDH activity but exhibited a broad substrate range **(Fig. 2.5).** The enzyme was able to utilize xylose, arabinose, and galactose in addition to glucose and maltose as substrates.

2.4: DISCUSSION

Citrobacter sp. DHRSS, a facultative anaerobe belonging to the *Enterobacteriaceae* family, was isolated from the rhizosphere of sugarcane using stringent buffered conditions with sucrose as the C source and RP as the P source, with

the intention of obtaining a PSB that could exploit the sugar sucrose, which is present in abundance in the rhizosphere of a variety of plants (Jaeger *et al.*, 1999), and show P solubilization. This isolate also showed P-solubilizing ability when provided with fructose as the C source. In both cases the isolate *Citrobacter* sp. DHRSS showed similar growth, acidification, and P solubilization properties. However, the time taken to achieve P solubilization was faster in the presence of fructose than on sucrose.

Fig. 2.5: Substrate specificity of glucose dehydrogenase activity of *Citrobacter* Sp. DHRSS using different sugars as substrates



Units of activity of GDH are defined as µmol of DPIP reduced per minute; Specific activity is defined as units per mg protein; Values given are Mean ± S.D. of three independent experiments performed in duplicates.

In addition to sucrose and fructose, *Citrobacter* sp. DHRSS also showed acid production and P-releasing ability on glucose and maltose in buffered medium. Several fungi have been reported to solubilize P in the presence of sugars such as sucrose, fructose, maltose, and glucose (Tsay and To, 1987; Cerezine *et al.*, 1988; Narsian *et al.*, 1995; Reyes *et al.*, 1999a). Among bacteria, only *Azotobacter chroococcum* isolate from the wheat rhizosphere showed P solubilization on sucrose as the C source with tricalcium phosphate (TCP) and Mussoorie RP (MRP) as insoluble inorganic P sources (Kumar and Narula, 1999).

P solubilization by *Citrobacter* sp. DHRSS was found to be much higher in the presence of maltose and glucose compared to sucrose and fructose. Narsian *et al.* (1995) have also reported the use of sucrose, fructose, maltose, and glucose for P solubilizing activity of *Aspergillus aculeatus* and found it to be best on glucose. Although growth and acidification were similar on all the C sources, a clear difference in the nature and amount of organic acid profile was observed in the case of different sugars, which was also reflected in the amount of RP solubilized by *Citrobacter* sp. DHRSS. The P solubilization ability of *Citrobacter* sp. DHRSS, on sucrose and fructose was brought about predominantly by the secretion of acetic acid in the medium, whereas GDH-mediated gluconic acid production was the main mechanism of P solubilization in the presence of glucose and maltose. It has been shown using pure organic acids that the P solubilizing ability is related to the nature of the organic acid and that the kind of mineral P also influences the type and amount of organic acid requirement (Bolan *et al.*, 1994; Kpomblekou and Tabatabai, 1994). However, gluconic acid was not included in these studies. In another report using alkaline vertisol as the source of mineral P, the efficacy of organic acids in P solubilization was in the order citric = oxalic>gluconic>succinate>acetate (Gyaneshwar *et al.*, 1998a). In gram-negative bacteria, gluconic and 2-ketogluconic acids are the main acids implicated in P solubilization (Goldstein, 1995). In agreement with these findings, it was observed that although *Citrobacter* sp. DHRSS produced higher molar concentrations of organic acids on sucrose and fructose, the P released was higher on glucose and maltose, sugars which supported gluconic acid production. In contrast, the P-solubilizing fungus *P. rugulosum* has been shown to secrete gluconic and citric acids on sucrose (Reyes *et al.*, 1999b). In terms of the amount of P released as a fraction of the total P and specific RP solubilizing abilities, fungal species are better than *Citrobacter* sp. DHRSS, possibly due to secretion of strong acids such as citric and oxalic in addition to gluconic acid (Cerezine *et al.*,

1988; Cunningham and kuiack, 1992; Vassilev *et al.*, 1996; Vassileva *et al.*, 1998; Wakelin *et al.*, 2004).

Citrobacter sp. DHRSS is distinct from *Citrobacter koseri* in its ability to produce gluconic acid on glucose; the former instead produces 0.1 mM oxalic, 0.8 mM succinic, and 1.2 mM citric acid on glucose (Gyaneshwar *et al.*, 1998a). Gluconic acid secretion being an important mechanism of P solubilzation (Goldstein,1995), *Citrobacter* sp. DHRSS is an efficient PSM compared to *C. koseri*, which did not show P solubilization under buffered conditions (Gyaneshwar *et al.*, 1998a). *Enterobacter asburiae* PSI3 has been shown to secrete a higher amount of gluconic acid (55 mM; 11.99 g/L) on glucose and can liberate a higher amount of soluble P (800 lM) than *Citrobacter* sp. DHRSS (Gyaneshwar *et al.*, 1999). However, on sucrose and fructose, *E. asburiae* PSI3 did not show RP solubilization (Sharma *et al.*, 2005), whereas *Citrobacter* sp. DHRSS was proficient at using these two C sources for P solubilization, indicating it to be more versatile than *E. asburiae* PSI3. In addition, *Citrobacter* sp. DHRSS showed significant GDH activity using a variety of aldose sugars as substrates, converting them to their corresponding aldonic acid extracellularly, which may contribute to the P-solubilizing ability on GDH substrates (Sharma *et al.*, 2005). This is the first report of a bacterial isolate to show P solubilizing ability on both GDH substrates, such as glucose and maltose, and non-GDH substrates, such as sucrose and fructose. On both types of C sources the mechanism of P solubilization with respect to the organic acids involved is different. Another unique property of *Citrobacter* sp. DHRSS is its ability to solubilize P on these sugars under buffered medium conditions, which mimic alkaline vertisol soil conditions (Gyaneshwar *et al.*, 1998a).

Citrobacter sp. DHRSS could be an effective P solubilizer in the rhizosphere, as variation exists in the nature and amount of sugars in the root exudates of different plants (Odunfa and Werner, 1981). Glucose and fructose have been shown to be the most frequent and abundant sugars detected in plant root exudates (Rovira, 1965). The major exudates in tomato root were glucose, fructose and maltose, along with small amounts of sucrose (Lugtenberg *et al.*, 1999). Thus, the ability of *Citrobacter* sp. DHRSS to solubilize RP in the presence of a variety of sugars could be of importance regarding the rhizosphere colonization, and further work is needed to test its efficacy under field conditions, especially alkaline vertisols.