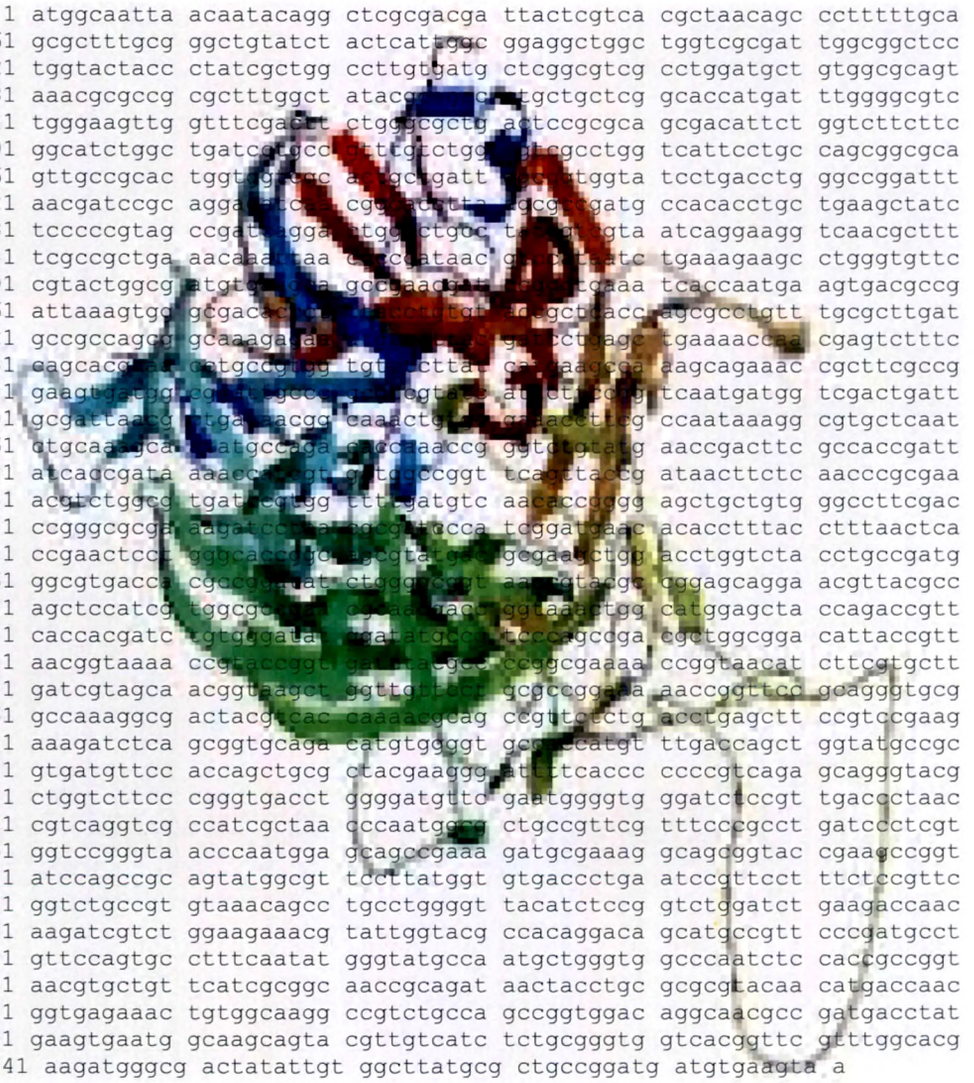
CHAPTER 2

Substrate specificity of Glucose dehydrogenase (GDH) of Enterobacter asburiae PSI3 and RP solubilisation with GDH substrates as C sources



2.1 INTRODUCTION

Rhizospheric microorganisms possessing the ability to solubilize mineral phosphates have been considered important for increasing P availability to the plants they associate with (Gyaneshwar et al., 2002; Khan et al., 2006). Most PSMs dissolve poorly soluble soil phosphates by the acidification of the environment through secretion of organic acids (Sperber, 1957; Rodríguez and Fraga, 1999; Khan et al., 2006). The organic acids lower down soil pH and more soluble form of P are released. Besides changing the pH, chelation of cations by organic acids, which bind phosphate anions also bring about release of phosphate in soil solution. Some metabolic end products like CO_2 and H_2S produced by organotrophs also play a role to a limited extent in solubilization of inorganic phosphates purely by chemical reaction (Yadav and Dadarwal, 1997). Among various PSMs studied, those secreting gluconic or 2-ketogluconic acids have been well-characterized, and acid secretion has been established as a basis of mineral phosphate solubilizing phenotype through gene cloning (Goldstein and Liu, 1987; Liu et al., 1992; Babu-Khan et al., 1995; Kim et al., 1997, 2003) as well as by mutational analysis (Gyaneshwar et al., 1999). Gluconic acid production in PSMs is mediated by oxidation of glucose to gluconic acid by a PQQ-dependent GDH (EC 1.1.5.2). Depending on the bacterial species, gluconic acid may undergo additional oxidation by gluconate dehydrogenase resulting in production of 2-ketogluconic acid (Goldstein, 1995). GDH from many bacterial sources has been shown to possess broad substrate specificity and can catalyze the oxidation of several aldosugars to their respective lactones (Goodwin and Anthony, 1998).

2.1.1 Direct oxidation mediated mineralization of mineral phosphate by GDH

GDH is a member of the largest group of quinoproteins, those that require the cofactor 2,7,9-tricarboxyl-1H-pyrollo-(2,3)-quinoline-4,5-dione (PQQ). The enzyme is located on the outer face of cytoplasmic membrane and is involved in the first step of 'direct oxidation' pathway in conversion of glucose to gluconic acid (Goldstein et al., 1993). The biochemical complexity of aldose utilization is the result of the interactive and dynamic nature of cell growth. It is known that GDH mediated periplasmic oxidation of aldose sugars can contribute electrons directly to the respiratory electron transport pathway. The quinoprotein GDH controls the unique step in direct oxidation, where it transfers electrons from aldose sugars to electron

transport chain via two electrons, two proton oxidations mediated by the cofactor PQQ. In addition, protons generated from these oxidations can contribute directly to the transmembrane proton motive force. GDH is an enzyme with diverse functions, one of which is its role in mineral phosphate solubilization owing to its ability in the production of organic acid such gluconic and 2-ketogluconic by direct oxidation of a wide range of aldosugars.

2.1.2 Physiology and Isoforms of GDH

Two types of GDH enzymes have been identified to date: GDH A and GDH B, which differ in their subcellular localization and substrate specificity. GDH A, the more widespread isozyme, is membrane-bound and has been found in organisms such as *Acinetobacter calcoaceticus* (Cleton-Jansen et al., 1988), *Escherichia coli* (Ameyama et al., 1986), *Gluconobacter oxydans* (Ameyama et al., 1981), and *Pseudomonas fluorescens* (Matsushita et al., 1980). GDH type B enzyme is soluble in nature and has been demonstrated only in *A. calcoaceticus* (Cleton-Jansen et al., 1989). Sequences homologous to GDH B have been identified from the uncharacterized proteins in the genomes of other bacteria (Oubrie et al., 1999). GDH B is physiologically inactive and has more affinity for PQQ as compared to GDH A which is physiologically active. GDHs have been detected and characterized in wide variety of species, some of which synthesize the cofactor PQQ themselves (e.g. *A. calcoaceticus*) whereas others are dependent for GDH activity on the presence of PQQ in the growth medium (e.g. *E. coli*). The cloning of the gene coding for two different GDHs: GDH A and GDH B have been reported from *A. calcoaceticus*. GDH A is a protein of molecular weight 87,000 Da and shows 140 residue N-terminal regions with high hydrophobicity. GDH B is a homodimer of molecular weight 52,772 Da subunit. The amino acid sequence has no obvious homology between GDH A and GDH B and they also differ in substrate specificity. Disaccharides are specific GDH B substrates whereas 2-deoxyglucose is specific for GDH A.

2.1.3 Physiological implications

GDH is found in many bacteria as a membrane bound enzyme which could be implicated to different physiological importance in different bacteria (Anthony and Goodwin, 1998). *Pseudomonas putida* which cannot utilize xylose as a sole carbon source but could oxidize xylose show enhanced growth yield under limited carbon

source (glucose or lactose) (Hardy et al., 1993). Similarly improvement in growth due to energetic efficiency and N_2 fixation in *Gluconacetobacter diazotrophicus* is linked to the xylose oxidation via the periplasmic GDH (Luna et al., 2002). In *Acetinobacter diazotrophicus* GDH level increases three to four times under nitrogen fixing condition (Galar and Boiardi, 1995; Luna et al., 2000). In *Klebsiella pneumoniae* under low P condition the flux through the Glyceraldehyde 3-phosphate is low, direct oxidation through GDH provides the an alternate source of energy (Buurman et al., 1994). Additional role for direct oxidation of glucose in protecting the enteric bacteria against oxygen inactivation during transfer from aerobic to anaerobic fermentative growth is also been speculated (Anthony and Goodwin, 1998). Its role in protecting nitrogenase in conditions supporting microaerobic nitrogen fixation in *K. pneumoniae* is also been implicated (Smith et al., 1990; Hill et al., 1990; Juty et al., 1997).

2.1.4 Industrial application

NADP dependent GDH genes were cloned from *Bacillus megaterium* AS1.223 and AS1.151, to give approximately tenfold increase in GDH activity (58.7 U ml^{-1}), which could regenerate NADPH efficiently *in vivo* and *in vitro*. This coenzyme-regenerating system was coupled with a NADPH-dependent bioreduction for efficient synthesis of ethyl (R)-4-chloro-3-hydroxybutanoate from ethyl 4-chloro-3-oxobutanoate (Xu et al., 2007). *In vitro* conversion of acetophenone to its alcohol was achieved in a cell-free system with purified alcohol dehydrogenase (ADH) and GDH (Weckbecker and Hummel, 2005). GDH from *Sulfolobus solfataricus* utilizing D-idose, D-xylose and D-glucose, oxidise to generate NADPH which is cofactor of broad substrate specific alcohol dehydrogenase. This combination of protein overcomes many industrial problems such as cofactor concentration product inhibition of enzyme, toxicity to microorganism, separate work to separate the product. As these enzyme reactions could take place at the boiling point of ethanol, its synthesis and recovery by distillation were coupled (Blum, 2004; U.S. Patent 6737257).

Oxidation of glucose by *Thermoplasma acidophilum* GDH with concomitant oxidation of NADPH by *Pyrococcus furiosus* hydrogenase yields hydrogen at stoichiometric levels. This simple system may provide a method for the biological production of hydrogen from renewable sources with gluconic acid, is a high-value

chemical commodity (Woodward et al., 1996). GDH has also been exploited for continuous monitoring of blood glucose from diabetic patients (Bilen et al., 2007).

2.1.5 P-solubilisation and biochemical Characteristics *E. asburiae* PSI3

PSMs proficient at solubilizing mineral phosphates in laboratory media could not liberate P from alkaline vertisols (Gyaneshwar et al., 1998). The high buffering capacity of alkaline vertisols and low amounts of organic acid secretion were attributed for their inability to release P from soils. Incorporation of 100mM Tris-Cl buffer of pH 8.0 in the screening medium resulted in the isolation of *Enterobacter asburiae* strains, which secreted high amounts of gluconic acid and released P from alkaline Indian soils (Gyaneshwar et al., 1999). *E. asburiae* PSI3, isolated from the rhizosphere of pigeon pea (*Cajanus cajan*), secreted up to 55mM of gluconic acid when grown in the presence of 100mM glucose. *gdh* mutants confirmed that P solubilisation was due to gluconic acid secretion mediated by PQQ dependent GDH enzymes as the mutants appeared to be defective in biosynthesis of GDH apoprotein as well as PQQ (Gyaneshwar et al., 1999).

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and culture conditions

E. asburiae PSI3, isolated from the rhizosphere of pigeon pea (Gyaneshwar et al., 1999), was used throughout these studies. *E. asburiae* PSI3 was grown on minimal medium for growth, acidification and P-solubilisation pattern, (Gyaneshwar et al., 1998) with RP as the sole P source in the presence of 100mM Tris-Cl, pH 8.0, and glucose, xylose, arabinose, galactose, mannose, maltose, or cellobiose as sole C sources at 75mM and 50mM concentrations each. These 7 sugars were taken in mixtures at concentrations of 15 and 10mM each. Senegal RP used in the study contains (%) silica 5-6, P_2O_5 36-37, CaO 49-51, fluorine 3-4, insoluble acid 5-6, CO_2 1-2, Fe_2O_3 0.6-0.8, sulphate 0.3-0.6, and Al_2O_3 0.6-0.8. For organic acid analysis, *E. asburiae* PSI3 was grown on M9 minimal medium containing (for 1 L) Na_2HPO_4 30 g, KH_2PO_4 15 g, NH_4Cl 5 g, NaCl 2.5 g, and $CaCl_2$ 15 mg, supplemented with micronutrients consisting of (for 1 L) $FeSO_4 \cdot 7H_2O$ 3.5 mg, $ZnSO_4 \cdot 7H_2O$ 0.16 mg, $CuSO_4 \cdot 5H_2O$ 0.08 mg, H_3BO_3 0.5 mg, $CaCl_2 \cdot 2H_2O$ 0.03 mg, and $MnSO_4 \cdot 4H_2O$ 0.4 mg.

2.2.2 Mineral Phosphate Solubilization by *E. asburiae* PSI3

2.2.2.1 Inoculum preparation

E. asburiae PSI3 was grown overnight at 37°C at 200 rpm. Cells were subjected to centrifugation at 9200 x g for 3 min. and then washed thrice with physiological saline, finally suspending in 0.5 ml of saline. Aliqouts of the cells were inoculated to maintain the initial $O.D._{600}$ less than 0.03.

2.2.2.2 Plate assay

Center point inoculations of *E. asburiae* PSI3 were done on Tris minimal solid medium of above mentioned composition with RP as sole P source and requisite sugar concentration. The P solubilization by acid production was determined by zone of pH reduction (change in colour from yellow to red) around the colony.

2.2.2.3 Kinetics of growth, acidification and P solubilization in liquid medium

The culture was grown at 37°C at 200 rpm in the Tris minimal liquid medium each containing 75mM and 50mM of different sugars individually and 15mM and 10mM in mixture of 7 sugars. RP was used as the P source and 100mM Tris-Cl (pH 8.0) as buffer, aliquots were drawn at 12h intervals until the pH was below 5.0. Absorbance at 600 nm and pH drop were used as parameters for growth pattern and acidification, respectively. Cells were centrifuged at 9200 x *g* for 5 min. when the pH dropped below 5.0 and culture supernatant was used for P estimation by ascorbate method (Ames, 1964).

2.2.3 Measurement of GDH activity of *E. asburiae* PSI3

E. asburiae PSI3 was grown for 30h on RP containing minimal media with requisite concentration of the C- source. After growth, cells were harvested at 9200 x *g* for 3 min., washed several times with physiological saline, and resuspended in 50mM Tris-Cl, pH 8.75. Whole-cell suspensions were used as the source of enzyme in GDH assays carried out according to Matsushita and Ameyama (1982). The reaction mixture consists of 50mM Tris-Cl pH 8.75, 0.1ml of 6.7 mM DCIP, 0.1ml of 20mM PMS, 0.1ml of 2M Glucose and 0.2 ml of whole cells as the enzyme source. The reaction volume was made up to 3ml with distilled water. The reaction was initiated by addition of glucose. Enzyme activity was measured as the initial reduction

of 2,6-Dichlorophenol-indo-phenol (DCIP). For determining the substrate specificity, glucose was replaced with different sugars in the assay mixture. One unit of GDH enzyme was defined as the amount of enzyme that brings about a decrease in 0.1 $O.D._{600nm}$ min^{-1}.

2.2.4 Determining the *E. asburiae* PSI3 GDH activity grown on different sugars

To determine the GDH production on different sugars, *E. asburiae* PSI3 was grown on various sugars individually at 100mM concentration and 15mM in case of mixture of 7 sugars as sole C sources. Cells were harvested by subjecting the cells to centrifugation at 9200 x *g* for 2min., after 30h, washed several times with physiological saline, and used for GDH assay. GDH activity was measured using 100mM glucose as substrate in the assay mixture.

2.2.5 Determining the substrate specificity of *E. asburiae* PSI3 GDH

To determine the substrate specificity of the GDH enzyme, cells grown on glucose for 30 h were used for the enzyme assay with different sugars at 100mM concentration as substrates. These experiments were repeated 3 times in duplicates. Results are expressed as Mean ± SD.

2.2.6 Analytical methods

Total protein was estimated using modified Lowry's method (Peterson, 1979). Culture supernatant was used for pH and P estimation as described. P estimation was done by the ascorbate method (Ames, 1964).

2.2.7 Organic acid analysis

E. asburiae PSI3 was grown on M9 minimal medium with different sugars at 100mM concentration as sole C sources. The organism was grown until the pH of the medium was reduced to 5.0. The culture supernatant was filtered using nylon-66 membrane filters of 0.22 µm pore size and subjected to HPLC with the following column specifications: HPLC LaChrom, Merck, Darmstadt, Germany), reverse phase, ion pairing column (RP-18) column. The mobile phase consisted of 0.1% phosphoric acid at a flow rate of 1 ml min^{-1}. Detection was performed by a UV/VIS detector at 210 nm. The HPLC profile of the culture supernatants was analyzed by comparison with the elution profile of pure organic acids.

2.3 RESULTS

2.3.1 Determining the substrate specificity of GDH of *E. asburiae* PSI3

Substrate specificity and activity of GDH on different C sources GDH activity of glucose-grown *E. asburiae* PSI3 was monitored with several sugars as substrates to determine its specificity (**Table 2.1**). GDH activity with galactose (110%) and xylose (94%) was comparable to activity with glucose (100%), whereas it was slightly low with maltose (75%), mannose (72%), and arabinose (66%). Cellobiose and 2-deoxyglucose showed 37% and 14% activity, respectively. When a mixture of 7 sugars, each at 15mM, was used as the substrate significant enzyme activity (46%) was found.

Table 2.1: GDH activity of *E. asburiae* PSI3

Sugar	GDH activity (U mg^{-1} protein) using different sugars as	
	Substrates[a]	C-source[b]
D-Glucose	0.83 ± 0.01 (100%)	0.38 ± 0.02 (100%)
L-Arabinose	0.55 ± 0.02 (66%)	0.18 ± 0.02 (47%)
D-Xylose	0.78 ± 0.01 (94%)	0.37 ± 0.01 (97%)
D-Galactose	0.91 ± 0.03 (110%)	0.31 ± 0.02 (82%)
D-Maltose	0.62 ± 0.01 (75%)	0.28 ± 0.02 (74%)
D-Mannose	0.60 ± 0.01 (72%)	0.26 ± 0.01 (68%)
D-Cellobiose	0.31 ± 0.03 (37%)	0.22 ± 0.02 (58%)
Mixture	0.38 ± 0.01 (46%)	0.36 ± 0.01 (95%)

One U of enzyme is defined as the amount that oxidizes 1µmol of D-glucose (or other sugars) or reduces 1µmol of DCIP min^{-1} at 25°C under specified conditions. Mixture indicates 15mM of each of the seven sugars independently tested. Results are expressed as Mean ± S.D. of three independent experiments in duplicates. a: Cells were grown on glucose as sole C source and different sugars were used as substrates in the GDH assay to measure their rates of direct oxidation. b: Cells were grown on the various sugars as C-sources and glucose was used as a substrate to measure GDH activity.

2.3.2 Determining the GDH production of *E. asburiae* PSI3 on different sugars

GDH activity of *E. asburiae* PSI3 was also monitored after growth on different sugars (**Table 2.1**) using 100mM glucose as the substrate. GDH activity on xylose (97%) and on galactose (82%) was similar to that on glucose (100%). GDH activity was lower on other sugars, such as maltose (74%), mannose (68%), cellobiose (58%), and arabinose (47%). When *E. asburiae* PSI3 was grown on a mixture of 7 sugars at 15mM each, GDH activity was found to be 95% of that when grown on 100 mM glucose.

2.3.3 Solubilization of RP by *E. asburiae* PSI3 on different sugars

2.3.3.1 Plate assay

E. asburiae PSI3 when grown on RP as a sole P source with 100mM Tris-Cl buffer, pH 8.0 showed red zone with 75mM glucose was used as sole carbon source (**Fig 2.1 A**). Similar results were obtained when glucose was replaced with with xylose, arabinose, galactose, mannose, maltose, or cellobiose as individual C sources at a 75mM concentration. The red colour on the methyl red plate was also observed when all the sugars were used as a mixture at a 15mM concentration (**Fig 2.1 C**). However, no acid production or red zone was observed when the above sugars were used at 50mM concentration individually and 10mM in mixture, although growth appeared normal (**Fig 2.1 B and D**).

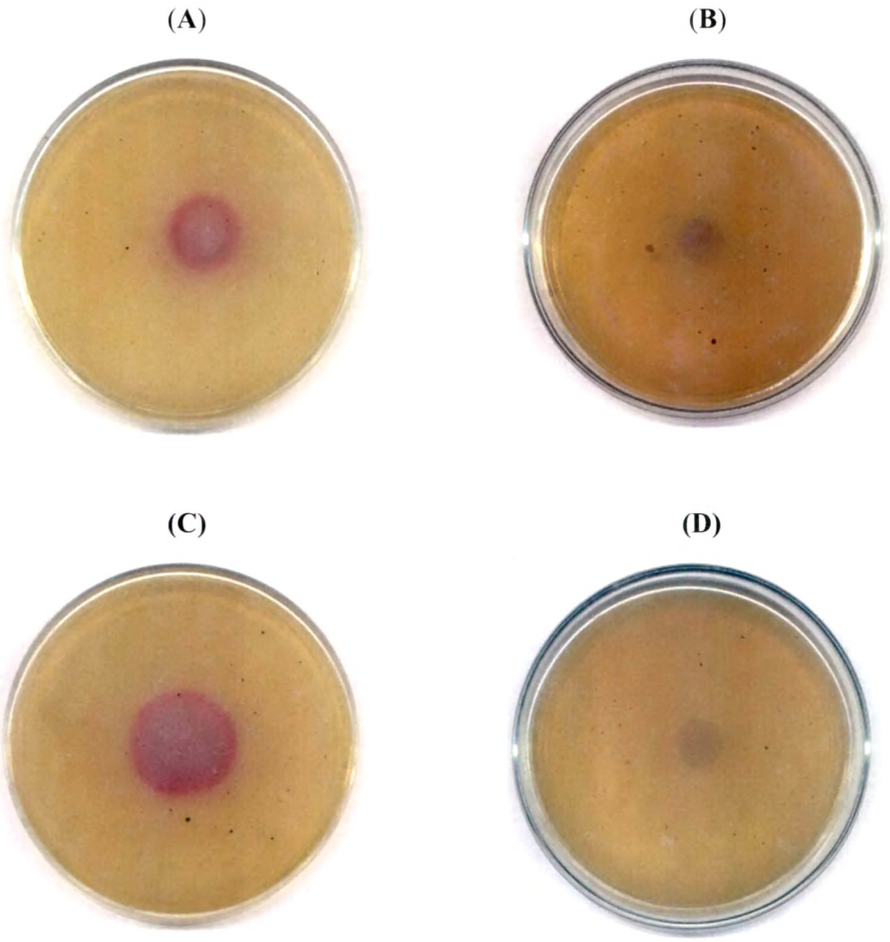
2.3.3.2 Kinetics of RP solubilization in liquid media with different sugars

E. asburiae PSI3, when grown on RP as the sole P source in the presence of 100mM Tris-Cl buffer, pH 8.0, acidified the medium to pH less than 5.0 with glucose (**Fig 2.2 B**), xylose, arabinose, galactose, mannose, maltose, or cellobiose as individual C sources at a 75mM concentration (**Table 2.2**). These sugars when used at 50mM concentration did not show acidification (**Fig 2.2 B**).

On sucrose and fructose, *E. asburiae* PSI3 did not show RP solubilization, although these sugars could be used as C sources for growth in the presence of soluble P (data not shown). The time required for acidification of the medium varied from 30h in the case of cellobiose to 96h for arabinose. Growth ranged from 0.22 $O.D._{600}$ in the case of glucose (**Fig 2.2 A**) to 0.78 $O.D._{600}$ on mannose. The amount of soluble P

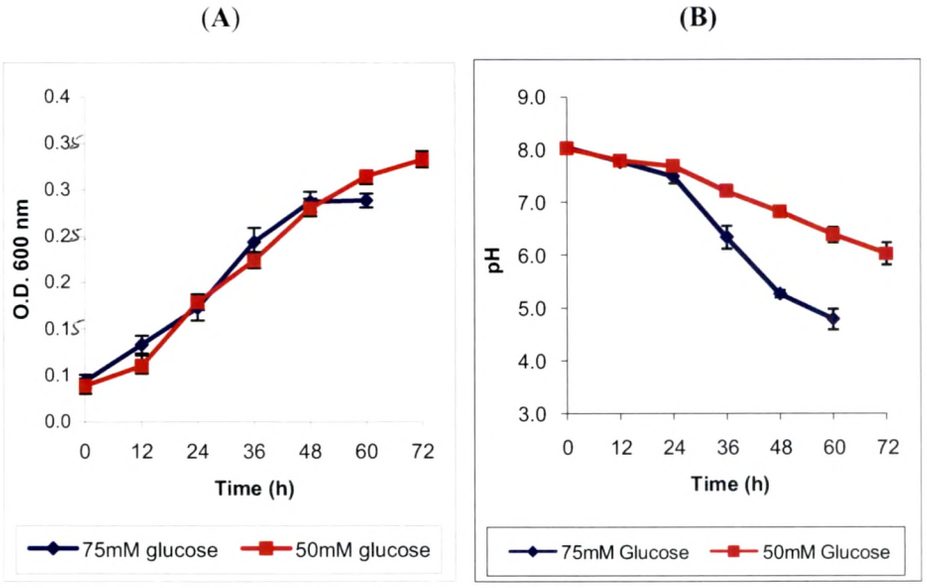
varied from 450µM for arabinose to 890µM for glucose (**Table 2.2**). A mixture of all 7 sugars, at 15mM each, in medium buffered with 100mM Tris-Cl, pH 8.0, resulted in the acidification of medium to pH less than 5.0 within 96h (**Fig. 2.3 B**), and the amount of P solubilized was 410µM. A mixture of 10mM of each sugar could bring about acidification of the medium to pH 6.23 in 96h (**Fig. 2.3 B**), and P released in the supernatant was found to be 100µM. The growth at 15mM and 10mM mixture was 0.43 $O.D._{600}$ and 0.70 $O.D._{600}$ respectively (**Fig. 2.3 A**).

Fig. 2.1: RP solubilisation by *E. asburiae* PSI3 on buffered methyl red plates



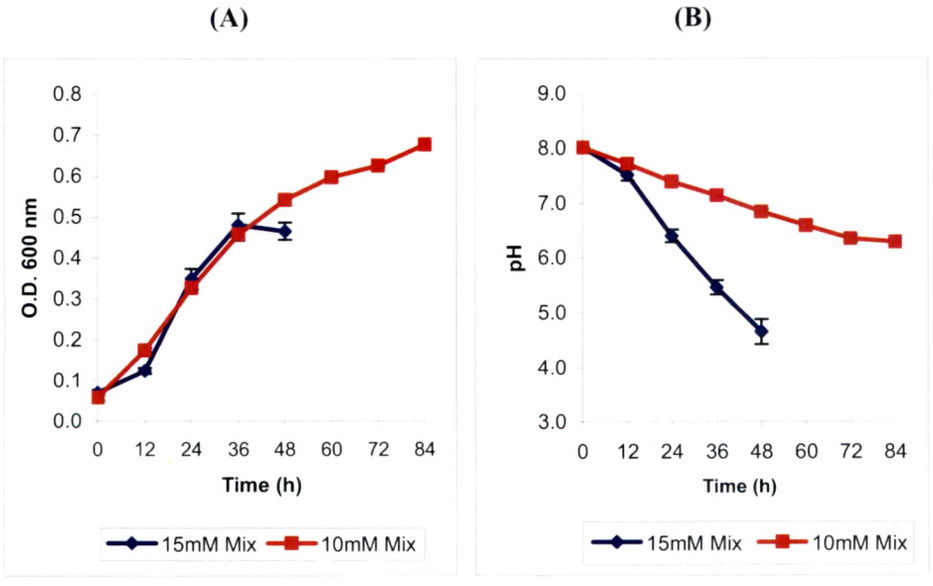
(A) Methyl red plates showing red zone with 75mM glucose, (B) No zone with 50mM glucose, (C) Methyl red plates showing red zone with 15mM mixture, (D) No zone with 10mM mixture.

Fig. 2.2: Growth profile and acidification pattern of *E. asburiae* PSI3 on Tris RP minimal medium with 75mM and 50mM glucose concentration respectively.



(A) Growth profile (B) Acidification pattern

Fig. 2.3: Growth profile and acidification pattern of *E. asburiae* PSI3 on Tris RP minimal medium with 15mM and 10mM sugar mixture respectively.



(A) Growth profile (B) Acidification pattern

Table 2.2: Solubilisation of RP by *E. asburiae* PSI3 on different carbon sources.

C source (mM)	Time taken for pH drop (h)	pH	O.D.$_{600}$[a]	P released (mM)
D-Glucose (75)	60 ± 0	4.42 ± 0.10	0.24 ± 0.06	0.89 ± 0.06 (100%)
D-Cellobiose (75)	30 ± 0	4.88 ± 0.11	0.54 ± 0.05	0.78 ± 0.03 (88%)
D-Xylose (75)	68 ± 6	4.64 ± 0.20	0.24 ± 0.04	0.75 ± 0.02 (84%)
D-Mannose (75)	46 ± 3	4.65 ± 0.21	0.78 ± 0.08	0.68 ± 0.06 (76%)
D-Galactose (75)	50 ± 3	4.90 ± 0.23	0.73 ± 0.03	0.60 ± 0.10 (67%)
D-Maltose (75)	40 ± 6	4.35 ± 0.04	0.66 ± 0.06	0.47 ± 0.15 (53%)
L-Arabinose (75)	96 ± 0	4.86 ± 0.09	0.24 ± 0.00	0.45 ± 0.03 (50%)
Mixture (15 each)	40 ± 4	4.68 ± 0.25	0.43 ± 0.07	0.41 ± 0.15 (46%)
Mixture (10 each)	96 ± 5	6.23 ± 0.21	0.70 ± 0.07	0.10 ± 0.13 (11%)

Results are expressed as Mean ± S.D. of 6 independent experiments; a: The initial O.D.$_{600}$ was approximately 0.05.

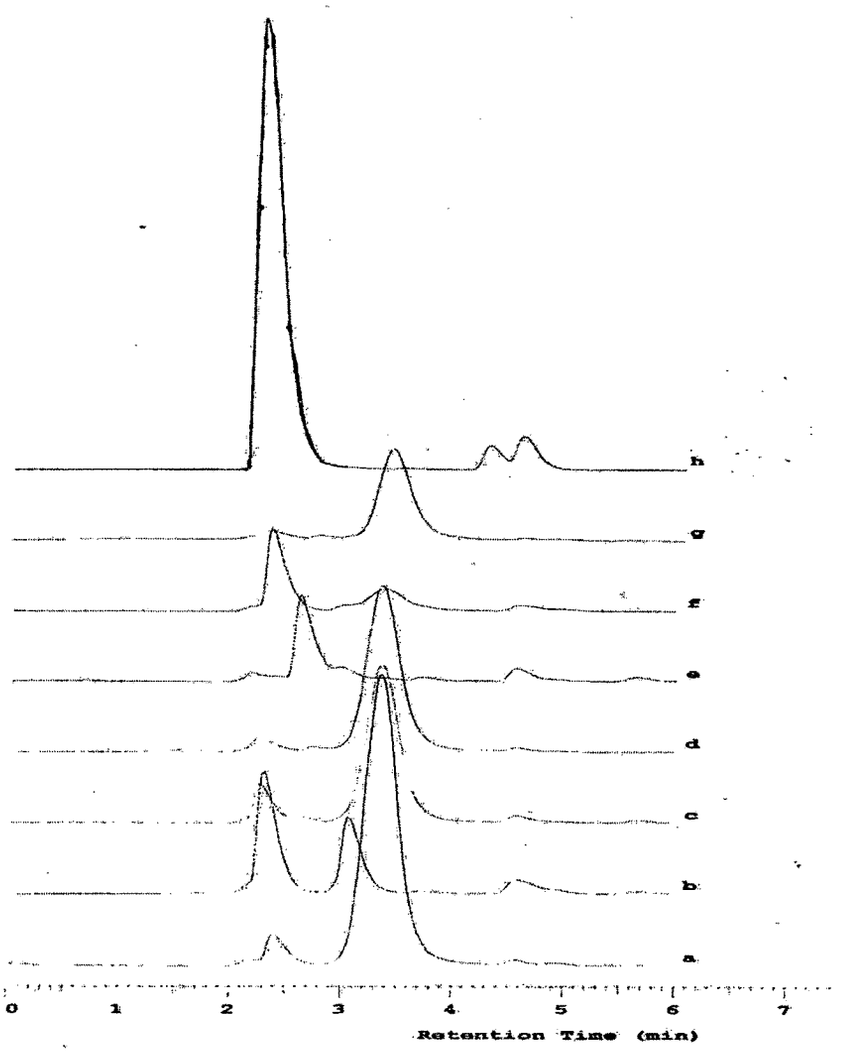
2.3.4 Organic acid analysis

To determine the nature of organic acids secreted by *E. asburiae* PSI3 while growing on different sugars, the culture supernatants were collected after the pH drop and analyzed by HPLC. Culture supernatants showed major peaks at the following retention times (in minutes) with different sugars as C sources: 2.40 on glucose, 2.36 and 3.10 on arabinose, 3.40 on galactose, 3.40 on mannose, 3.35 on xylose, 3.44 on maltose, and 2.66 on cellobiose. Of these, the peak obtained on glucose-containing medium with a retention time of 2.40 min corresponded to pure gluconic acid. None of the other peaks matched with the following standard acids, whose retention times were (in minutes) 2.26 for oxalic acid, 4.27 for lactic acid, and 4.54 for acetic acid **(Fig. 2.4)**.

2.3.5 Comparison of the substrate specificity of GDH enzymes from various bacteria.

E. asburiae PSI3 GDH has broad substrate range for mono as well as disaccharides whereas various bacteria such as *E. coli, P. fluorescens, G. oxydans* and *A. calcoaceticus* showed high substrate specificity for only mono-saccharides. *A. calcoaceticus* GDH B can act on disaccharides but not on mono-saccharides **Table 2.3.**

Fig. 2.4: Organic acid analysis of *E. asburiae* PSI3 on different GDH substrates



(a) D-Xylose, (b) L-Arabinose, (c) D-Mannose, (d) D-Galactose, (e) D-Cellobiose, (f) D-Glucose, (g) D-Maltose and (h) Standard organic acids.

Table 2.3: Comparison of the substrate specificity of GDH enzymes from various bacteria

Substrates	*E. coli* GDH A (Ameyama et al., 1986)	*A. calcoaceticus*		*P. fluorescens* GDH A (Matsushita et al., 1980)	*G. oxydans* GDH A (Ameyama et al., 1981)	*E. asburiae* PSI3 This work
		GDH A (Cleton-Jansen et al., 1988)	GDH B (Cleton-Jansen et al., 1989)			
D-Glucose	100%	100%	100%	100%	100%	100%
D-Allose	n.t. (++)[a]	n.t.	66%	n.t.	n.t.	n.t.
L-Arabinose	n.t. (++)[a]	95%	35%	3%	n.t.	66%
2-Deoxyglucose	n.t. (++)[a]	100%	4%	n.t.	n.t.	14%
D-Fucose	91%	n.t.	n.t.	n.t.	n.t.	n.t.
D-Galactose	41%	90%	18%	7%	0%	110%
D-Mannose	38%	n.t.	4%	9%	0%	72%
L-Rhamnose	20%	0%	n.t.	8%	0%	n.t.
D-Ribose	0% (+)[a]	80%	1%	0%	0%	n.t.
D-Xylose	54%	100%	20%	13%	0%	94%
Cellobiose	n.t.	n.t.	70%	n.t.	n.t.	37%
Lactose	n.t.	0%	65%	n.t.	n.t.	n.t.
Maltose	10%	0%	90%	3%	5%	75%
Melibiose	n.t. (++)[a]	n.t.	10%	n.t.	n.t.	n.t.

n.t: Not tested; a: Symbols in parenthesis denote the results from Cozier et al., (1999). (+): V_{max} approx. <300 µmol/min per mg; (++): V_{max} approx. >400 µmol/min per mg.

2.4 DISCUSSION

Gluconic acid production as a result of the activity of periplasmic or membrane-bound GDH is one of the best studied mechanisms by which PSMs liberate P from poorly soluble mineral phosphates (Goldstein, 1995). Although GDH enzymes from various bacteria are well-characterized in terms of kinetic properties and mechanisms (Goodwin and Anthony, 1998; Elias et al., 2004), substrate range characteristics of GDH from P-solubilizing isolates have not been previously reported. The substrate specificity of GDH from P-solubilizing *E. asburiae* PSI3 presented in this paper is compared with that of reported GDH enzymes in **Table 2.3**. The GDH enzyme of *E. asburiae* PSI3 shows broad substrate range, including hexoses, pentoses, as well as disaccharides. The results are in agreement with the general opinion that the enzymatic characteristics of bacterial GDHs vary depending on the specific bacterial sources (Yoshida et al., 1999). *E asburiae* PSI3 GDH enzyme differs from other GDH enzymes by the ability to act on both mono and disaccharides.

The GDH activity of *E. asburiae* PSI3 varied, depending on the nature of the sugars present in the medium, the greatest activity being present on glucose and xylose. The GDH enzyme of this organism is present in cells grown on several sugars as C sources (including those that are not GDH substrates, such as sucrose and fructose, which could be used by *E. asburiae* PSI3 as C sources for growth but not for RP solubilization, data not shown). This indicates the constitutive nature of the enzyme and is in contrast to the *E. coli* enzyme, whose levels are negatively regulated by glucose through cAMP (Yamada et al., 1993; Izu et al., 2002). Bacteria are known to utilize sugars that serve as GDH substrates by different pathways. High GDH activity was observed when *E. asburiae* PSI3 was grown on glucose or xylose. These sugars are utilized mainly through glycolysis and pentose phosphate pathways, respectively. Mannose, utilized by the glycolytic pathway, showed low GDH activity. Thus, levels of GDH of *E. asburiae* PSI3, when grown on a particular sugar, do not seem to show a relation with the pathway of sugar metabolism.

A novel aspect of this study is the correlation between the broad substrate specificity of the GDH enzyme and the manifestation of the P-solubilization phenotype on several C sources. Previous studies from this and other laboratories on gluconic-acid-producing PSMs were primarily concerned with the P- solubilization

phenotype under conditions in which glucose was the sole C source. When *E. asburiae* PSI3 was grown with 75mM of each of the other GDH substrates as the sole C source, pH drop was observed under strongly buffered conditions, and Pi was liberated. Substitution of KH_2PO_4 as the P source in place of RP allowed enhanced growth (data not shown), indicating that growth on the buffered RP medium was probably strongly dependent on P solubilization. Upon use of 50mM of individual sugars as C sources under similar conditions, the pH did not drop to below 5, and P solubilization was insignificant up to 96 h (data not shown). HPLC analysis revealed the absence of secreted fermentation products, such as acetate and lactate, and the acidification of the medium was possibly due to the conversion of different sugars to their respective aldonic acids by GDH-mediated direct oxidation. *E. asburiae* PSI3 grown on sucrose and fructose did not solublize RP on plates as well as in the liquid medium. The results suggest that GDH-mediated acidification is the main mechanism of P solubilization by *E. asburiae* PSI3, and aldonic acids generated by the action of GDH on various sugars can bring about P solubilization. The role of GDH *E. asburiae* PSI3 in P solubilization has been demonstrated by the isolation of GDH mutants with a defective P-solubilization phenotype (Gyaneshwar et al., 1999).

Reports that demonstrate the use of various C sources for determining optimum conditions for P solubilization generally use each sugar individually at high concentrations (Cerezine et al., 1988). An important aspect of this work is the demonstration of P solubilization with a mixture of sugars, each at a concentration much lower than that generally used and too low to support P solubilization independently. When all the 7 GDH substrates were supplied as a mixture at 15mM each (the combined sugar concentration was 105mM), substantial pH drop and P solubilization were observed. Even when individual sugars in the mixture were at a concentration of 10mM each, 100μM P was released. This indicates the concurrent action of GDH on different sugars to produce sufficient acid to bring about P release.

The availability of adequate and appropriate C sources in the soil is known to be one of the major limiting factors for the survival, growth, and functioning of microorganisms in soils. Using the *Pfic* reporter gene system in *P. fluorescens*, C source has been demonstrated to be limited in soils (Koch et al., 2001). However, rhizospheric soil is richer in C source since plants secrete nearly 5%–21% of

photosynthetically fixed carbon through root exudates (Walker et al., 2003). With the use of *inaZ* reporter under *scrY* promoter, sucrose has been shown to be present at 100-fold higher concentration in the rhizosphere than in the bulk soil (Jaeger III et al., 1999). Generally, root exudates contain several sugars at low concentrations (Matsushita and Ameyama, 1982). It has been suggested that an organism that is capable of using a variety of C sources for its bioenergetics could have an advantage over competitors that use limited C sources in natural environments (Goodwin and Anthony, 1998). The versatile use of various C sources for P solubilization by *E. asburiae* PSI3 could thus be significant from the perspective of field performance.