CHAPTER 4

BIOCHEMICAL CHARACTERIZATION OF PS ISOLATES

4.1 : INTRODUCTION

Phosphate solubilizing microorganisms are known to solubilize mineral phosphates by bringing a drop in pH of the media either by proton extrusion or secreting organic acids. The organic acids secreted are generally TCA cycle intermediates. However the biochemical and molecular basis for production of most of these acids is unknown.

Biochemistry of Gluconic Acid Production :

The PS mechanism characterized to certain extent is phosphate solubilization by gluconic acid secretion by certain gram negative organisms like *Erwinia herbicola* and *Pseudomonas cepacia* (Goldstein, 1993). Gram negative aerobic organisms have a direct oxidation pathway for glucose utilization in which glucose is converted to gluconate and then to ketogluconate in the periplasm. The first conversion is brought about by a quinoprotein glucose dehydrogenase (GDH) and the next conversion by gluconate dehydrogenase. The direct oxidative pathway can be the major pathway of glucose utilization under certain conditions (Lisse & Phibbs, 1984). GDH is dependent on the cofactor pyrroloquinolinequinone (PQQ). The enzyme is present on the outside of cell membrane. Two GDHs are present in *Acinetobacter calcoaceticus*. GDH-A is a monomer of 86.9 Kd and is localised in the plasma membrane whereas GDH-B is a homodimer of 50.2 Kd and is present on the outerside of periplamic membrane (Cleton-Jansen *et al.*, 1989). Both the enzymes can use glucose as a substrate. GDH-B can also act on disaccharides like maltose and lactose whereas 2-deoxyglucose is a specific substrate of GDH-A (Cleton-Jansen *et al.*, 1989). The GDH from *E. coli* (Cleton-Jansen *et al.*, 1990) and *Pseudomonas* is like GDH-A. There is not much homolgy of GDH-A with GDH-B. GDH from *P. aeruginosa* is 20% active with galactose as substrate as compared to GDH from *P. cepacia* which is as active with galactose as with glucose (Lisse & Phibbs, 1984).

The bacteria differ in their ability to synthesize PQQ, for e.g. species such as *Acinetobacter calcoaceticus* (Hauge, 1966), *Klebsiella aerogenes* (Neijssel *et al.*, 1983), *Pseudomonas cepacia* (Babukhan *et al.*, 1995) *Erwinia herbicola* (Liu *et al.*, 1992) and *Zymomonas* (Strohdeicher *et al.*, 1988) can synthesize PQQ whereas species like *E. coli, K. pneumoniae* and *A. lwoffi* can produce only the apo GDH and can not synthesize PQQ. These bacteria can oxidize glucose to gluconate only in the presence of externally added PQQ. However, *E. coli*, under forced circumstances can start producing PQQ endogeneously (Biville *et al.*, 1991). PQQ is also a chemotactic attractant for *E. coli* (deJonge *et al.*, 1996).

Genetics of Gluconic Acid Production :

PQQ synthesizing genes have been cloned from *A. calcoaceticus* (Goosen *et al.*, 1987) and *Erwinia herbicola* (Liu *et al.*,1992). The sequence of these genes has been determined and the genes have been expressed in *E. coli* (Goosen *et al.*, 1989; Liu *et al.*,1992).Two genes involved in PQQ biosynthesis have been cloned from *Methylobacterium extrogens* AM I. One of these belongs to a family of endopeptidase family (Springer *et al.*, 1996). In *Klebsiella pneumoniae*, six genes encoded by *pqq ABCDEF* are involved in PQQ synthesis and all six are nessecary for PQQ synthesis and secretion in *E. coli* (Velterop *et al.*, 1995). Mutants lacking *PqqB* or *PqqF* protein can synthesise PQQ at a slow rate and the PQQ synthesis probably requires oxygen as it is abolished under anaerobic conditions although the genes are expressed. Genes showing high homolgy to PQQ synthesis genes of *Klebsiella pneumoniae* have also been cloned from an antibiotic producing *Pseudomonas fluorescens* (Schnider *et al.*, 1995). Interestingly PQQ negative mutants also lacked GDHe. GDH genes have been cloned from *A. calcoaceticus* (Cleton- Jansen *et al.*, 1988) and *E. coli* (Cleton-Jansen *et al.*, 1990). In both these bacteria GDH is localised in the chromosome whereas in *Erwinia herbicola* ATCC 21998 GDH is localised on a 7.4 Kb extra chromosomal element (Koul *et al.*, 1995; 1997). There is also evidence of plasmid mediated glucose utilization in *Gluconobacter oxydans* (Qazi *et al.*, 1989; Verma *et al.*, 1994).

Gluconic Acid Producing Genes from PS Bacteria :

There has been an interest to clone the gene involved in Mineral Phosphate Solubilization (*mps*) to use them in renewable P fertilizer technology (Goldstein, 1986; Goldstein & Liu, 1987; Liu *et al.,* 1992). A phenotypic screening procedure for isolation of *mps* genes has been developed based on the inability of *E. coli* to synthesize PQQ and thus its inability to produce gluconic acid (Goldstein & Liu, 1987). This allows the selection of *mps*$^{+}$ phenotype based on the solubilization of acid soluble Ca-P complex like hydroxyapatite (Liu *et al.*, 1989; Babukhan *et al.*,1995) thus allows the cloning of gene(s) required for PQQ biosynthesis.

Using this screening procedure, PQQ synthase gene has been cloned from *Erwinia herbicola* in *E. coli* (Goldstein & Liu, 1987). The *E. coli* containing this gene could solubilize HAP only under P starvation conditions. Another *mps* gene was cloned from *Pseudomonas cepacia* which could code for either protein involved in membrane transport, probably of PQQ or a regulatory protein involved in the regulation of PQQ biosynthesis (Babukhan *et al.*, 1995). However the GDH from these gluconate producers has not been identified.

In this chapter, the biochemical basis for such high levels of gluconic acid production by the PS *Rhizobium* sp. isolated using buffered media conditions has been studied. Mutants in GDH activity and mineral phosphate solubilization (*mps-*) have been isolated and characterized.

4.2 : MATERIALS AND METHODS

Bacterial Strains :

The PS bacteria described in the preceding chapter were maintained on buffered rock phosphate plates and were inoculated in defined media for GDH assay as described below. *E. coli* containing pRK 2013 and pSUP 202-1 were maintained on Luria agar plates containing 20 μg/ml kanamycin and 100 μg/ml ampicillin, respectively. These were provided by Dr. P. S. Poole, University of Reading, U.K. *Pseudomonas fluorescens* ATCC 13525 and *Rhizobium leguminosarum* ATCC 10004 were obtained from MTCC, Chandigarh and were maintained on media as described by MTCC.

Induction of GDH and Alkaline Phosphatase (AP) of Soil Isolates :

The isolates were inoculated in minimal medium containing 100mM glucose, 10mM ammonium chloride, 10mM KH_2PO_4 and the micronutrients as given in Chapter 2. The media was buffered with 100mM Tris-HCl pH 8.0. The cells were harvested at 0.4 O.D and washed twice with normal saline. The cells were then resuspended in same medium with 100μM P. To monitor the effect of protein synthesis inhibitor on the induction of GDH, chloramphenicol was added at 170μg/ml. The cells were harvested at different time intervals, washed thrice with saline and resuspended in equal volume of 50mM Tris-HCl pH 8.0 and were used for GDH and AP assay. To study the levels of P concentration that was sufficient for GDH induction, different concentrations of K_2HPO_4 were added. The cells were harvested after 6 hours, washed and used for GDH assay.

Effect of C Source on GDH of Soil Isolates :

The isolates were inoculated from the maintenance media into liquid media containing 100mM glucose, 10mM NH_4Cl, 10mM K_2HPO_4 and trace elements as described in previous chapter and were allowed to grow on a rotary shaker overnight. Glucose was replaced with other carbon sources at 100mM concentration for determining on GDH.

These cells were harvested by centrifugation in a table top centrifuge at 5,000 rpm, washed twice with 0.9 % saline and distributed in the above described media either with 10 mM KH_2PO_4 for P sufficient or with 100 μM for P deficient conditions. The cells were further allowed to grow for 6 hours and then harvested by centrifugation at 5,000 rpm. The cells were washed twice with 0.9% saline and resuspended in equal volume of 50 mM Tris-HCl pH 8.0 and were used for enzyme assays.

Enzyme Assays :

The assay mixture for GDH consisted of 1 ml of 50mM Tris-HCl buffer pH 8.75, 0.1 ml of 6.7 mM dichlorophenol indophenol (DCIP), 0.1 ml 20 mM, phenazine methosulfate (PMS), 0.1 ml of 1M glucose and 0.1 ml of cells in a final vloume of 3 ml. GDH activity was determined by measuring the decrease in absorbance at 600 nm of DCIP mediated with PMS (Mutscushita & Ameyama, 1982). The reaction was started by addition of substrate. Different substrates were used at same concentration as glucose in the assay mixture. For AP assay 0.1 ml of the cells were added in the assay system consisting of 1 mg/ml pNPP in 30 mM Tris-HCl pH 9.0. The activity was determined by estimating p-Nitrophenol liberated from p-Nitrophenylphosphate (Smart *et al.,* 1994) and comparing it to absorbance of standard paranitrophenol at 420 nm. Protein estimations were done by the dye binding method (Bradford, 1976). Unit defined as nanomoles of DCIP reduced per minute for GDH and nanomoles of paranitrophenol liberated per minute for AP.

Transposon Mutagenesis of IS 3 :

Transposon Tn5 encoding kanamycin resistance was mobilized with the help of helper plasmid pRK 2013. Tri-parental matings were carried out as described by Simon (Simon *et al.,* 1983). In the first mating logarithmically growing *E. coli* containing pSUP 2021 and pRK 2013 were allowed to mate for 30 minutes on filter membrane (0.2 μ). The next mating was started by addition of approximately 10^8 cells of isolate 3. The cells were allowed to mate for 12 hours, resuspended in distilled water and plated on selective media. As the *E. coli* strains used in the conjugation are auxotrophs, the transconjugants were selected on minimal media containing 100mM Glucose, 10mM Ammonium chloride, 100mM Tris-HCl pH 8.0. and DCP as sole P source. Methyl red was added at 50 mg% to monitor pH drop. Kanamycin was added at a concentration of 100 μg/ml. *mps*$^-$ mutants were selected based on their inability to show zone of clearance and pH reduction on the above media.

Characterization of *mps-* Transposon Mutants :

The various mutants were assayed for their ability to show zone of clearance and pH reduction on DCP plates in the presence and absence of PQQ in solid media of the above mentioned media buffered with either 50 or 100 mM Tris-HCl pH 8.0. The putative mutants were grown on minimal media in P sufficient or P starvation conditions and were used for GDH and AP assays as described above. For determining the effect of PQQ on the GDH activity, PQQ was added to the assay mixture at a concentration of 1 μM.

RP Solubilization by *mps*$^-$ Mutants :

The *mps-* mutants were inoculated in media containing 100 mM glucose, 10 mM ammonium chloride and other micronutrients as described in previous chapters. RP was provided as sole P source. Growth, pH and P release was monitored after different time intervals as indicated. The pH of the media was adjusted to 8.0 by NaOH in case of unbuffered media and 100 mM Tris-HCl pH 8.0 was added in the buffered media.

P Solubilization from Alkaline Soil by *mps*⁻ Mutants :

The *mps-* mutants were inoculated in media containing 100 mM glucose, 10 mM ammonium chloride, 1 g/ml of alkaline soil was added as P source. The growth of the bacteria was monitored by viable counts at different time intervals. Culture was collected and centrifuged in a microfuge at 10,000 rpm for 5 mins. The supernatant was used for pH and P estimations.

4.3 : RESULTS

Induction of GDH and AP :

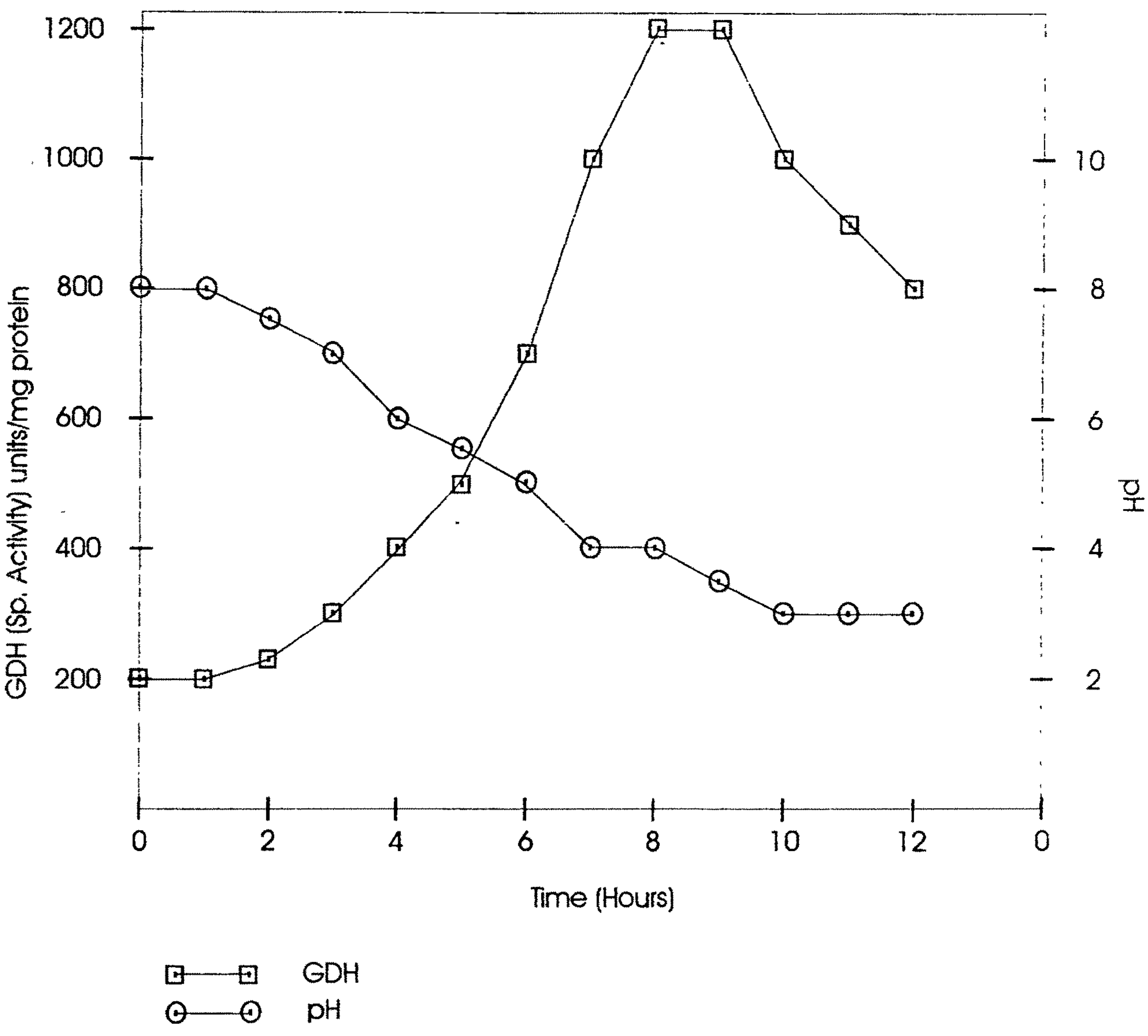
Basal level of GDH activity was about 250 U/mg protein which was increased to 5 fold in 6 h by P starvation in all the isolates (Fig. 4.1-4.3). The increase in GDH activity correlated with the decrease of media pH by the isolates. Maximum induction of GDH in all isolates was found at 0.1 mM P concentration (Table 4.1). That the cells were induced for P starvation was determined by measuring the activity of alkaline phosphatase (AP) which is known to be induced upon P starvation. GDH of these isolates was induced only upon P starvation and was independent of the carbon source used for the growth of the cells (Table 4. 2). The increase in activity upon P starvation required *de novo* protein synthesis as addition of chloramphenicol abolished the induction of GDH (Table 4.3).

As compared to PS isolates, *P. fluorescens* showed similar basal level activity of GDH but there was no induction upon P starvation even when PQQ supplemented in the assay (Table 4.4). *Rhizobium leguminosarum* showed low activity of GDH which was induced by 2 fold upon P starvation. In both *P. fluorescens* and *R. leguminosarum*, higher GDH activity was detected when gluconate was used as 'C' source (Table 4.4)

Substrate Specificity of GDH of Soil Isolates :

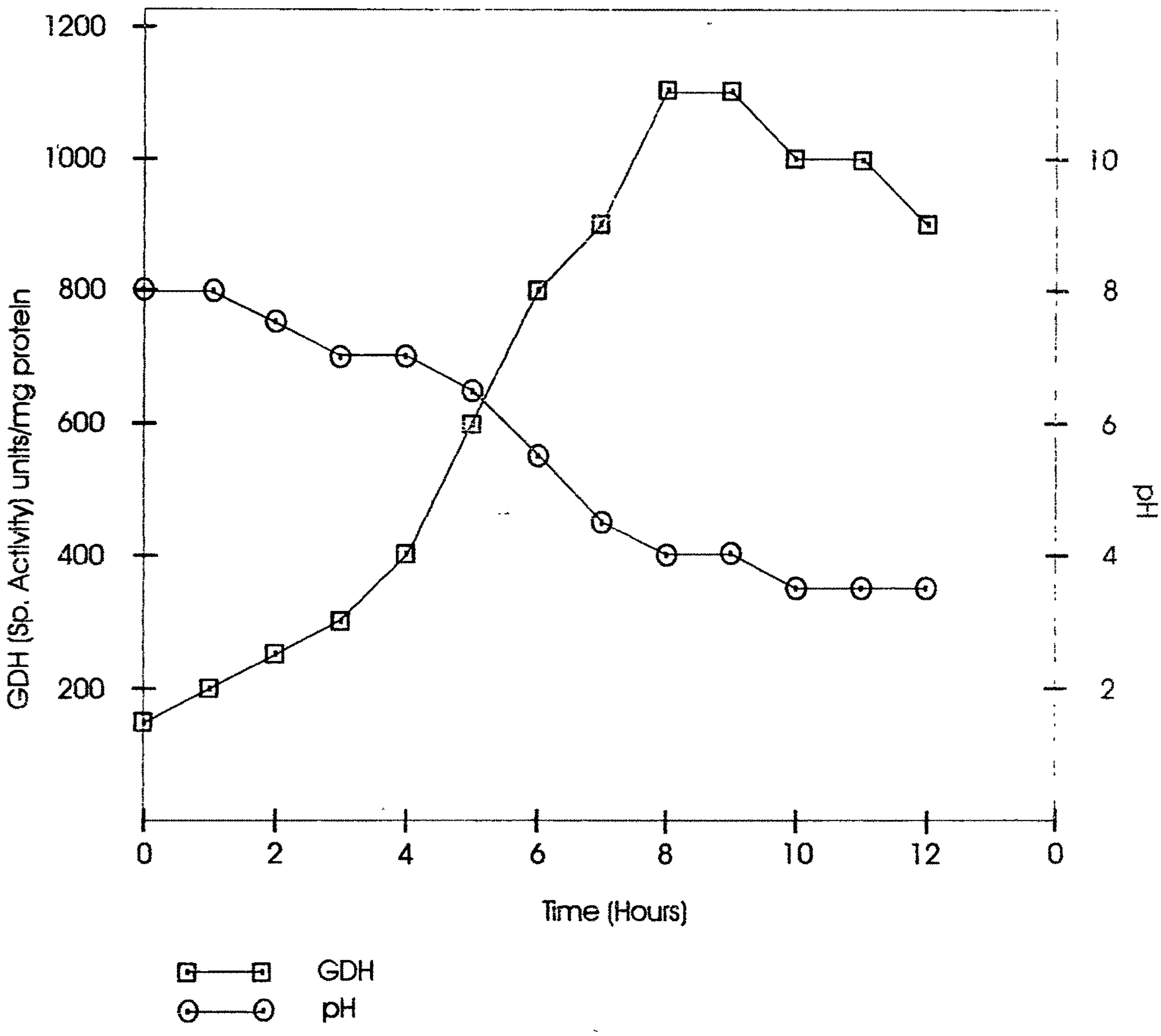
In order to determine whether the GDH of these isolates was of GDH-A or GDH-B type the GDH activity was assayed with different substrate. The whole cells of all the three soil isolates grown in P-sufficient and P-deficient conditions could show activity with maltose and galactose as substrates but could not use 2-deoxyglucose as a substrate (Table 4.5).

Fig. 4.1 : Induction of GDH of soil isolate 1



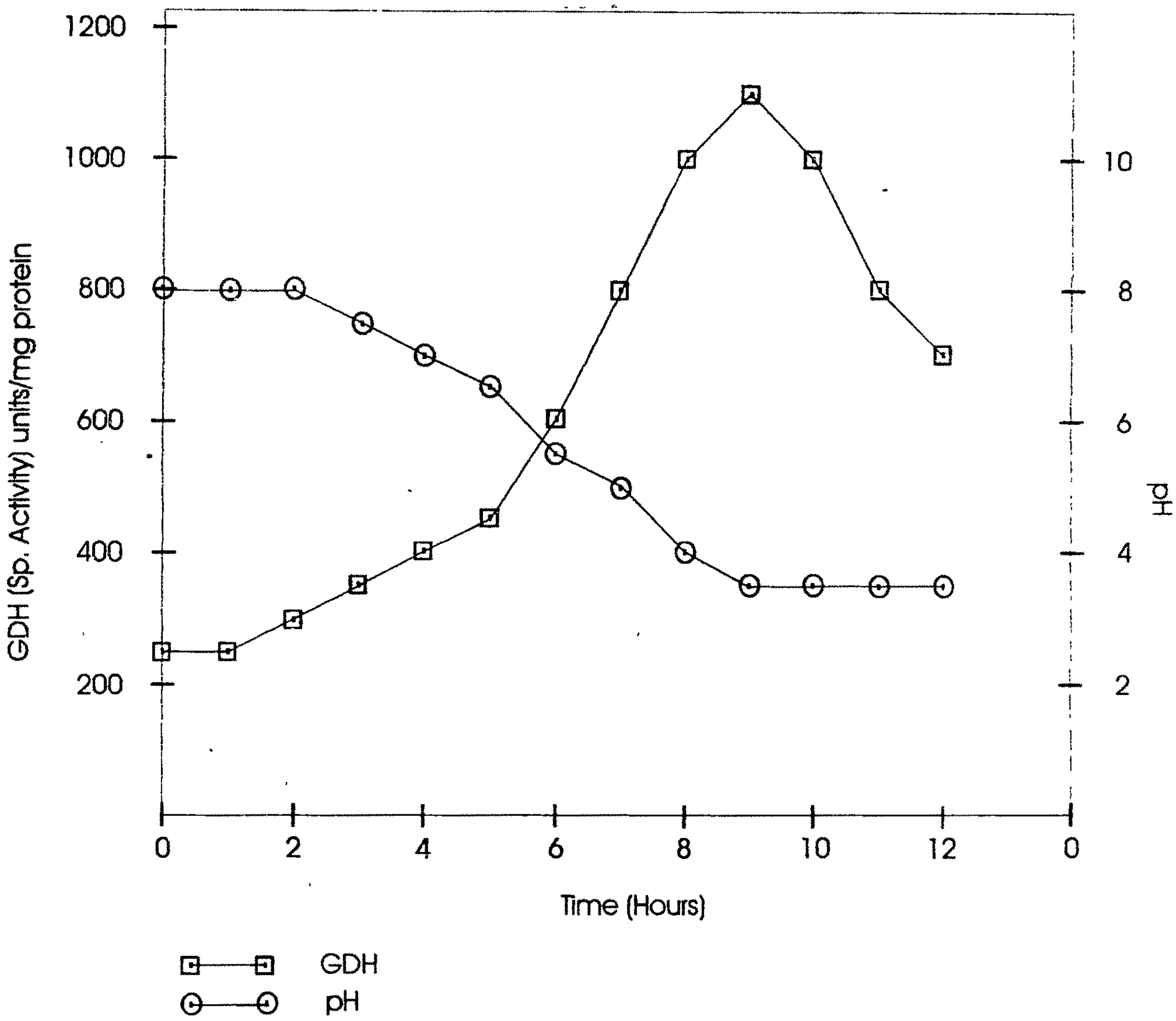
Specific activity is as defined in the text.

Fig. 4.3 : Induction of GDH of soil isolate 3



Specific activity is as defined in the text.

Fig. 4.2 : Induction of GDH of soil isolate 2



Specific activity is as defined in the text.

Table 4.1

Induction of GDH and AP activity in the soil isolates

	P (mM)				
	0.05	**0.1**	**1.0**	**5.0**	**10.0**
Isolate 1					
GDH	1300 ± 100	1200 ± 80	800 ± 25	600 ± 30	200 ± 10
AP	55 ± 7	50 ± 5	10 ± 2	UD	UD
Isolate 2					
GDH	1500 ± 125	1400 ± 100	900 ± 30	800 ± 20	300 ± 10
AP	40 ± 4	35 ± 2.5	5 ± 1	UD	UD
Isolate 3					
GDH	1300 ± 140	1100 ± 120	700 ± 20	400 ± 15	200 ± 10
AP	40 ± 4	40 ± 8	10 ± 3	UD	UD

UD : Undetectable.

Activities are given as Units/mg protein.

Values are expressed as mean ± S.D. of three independent observations.

Table 4.2

GDH activity of soil isolates with various C sources

C Source	+ P	-P
Glucose		
Isolate 1	262 ± 4.1	1390 ± 149
Isolate 2	265 ± 7.5	1737 ± 125
Isolate 3	285 ± 8.5	1500 ± 55
Glycerol		
Isolate 1	242 ± 7.5	1303 ± 100
Isolate 2	274 ± 10	1600 ± 60
Isolate 3	236 ± 5.1	1418 ± 125
Mannitol		
Isolate 1	225 ± 6.0	1193 ± 130
Isolate 2	250 ± 6.5	1395 ± 75
Isolate 3	240 ± 5.0	1158 ± 100
Gluconate		
Isolate 1	200 ± 10.0	1000 ± 100
Isolate 2	220 ± 5.5	900 ± 65
Isolate 3	250 ± 5.0	1200 ± 120

Values are expressed as mean ± S.D. of three independent observations.

Table 4.3

Effect of chloramphenicol on induction of GDH in the soil isolates

	GDH (Units/mg protein)	
	Chl+	Chl-
Isolate 1	300 ± 55	1000 ± 100
Isolate 2	250 ± 40	1200 ± 125
Isolate 3	200 ± 50	900 ± 75

Values are expressed as mean ± S.D. of three independent experiments.

Table 4.4

Effect of P starvation on GDH of other PGPR

C-Source	P	GDH (Specific Activity) Units/mg Protein	
		-PQQ	+PQQ
P. fluorescens :			
Glucose	+	250 ± 25	275 ± 50
	-	225 ± 50	200 ± 55
Gluconate	+	400 ± 50	450 ± 60
	-	450 ± 35	400 ± 25
R. leguminosarum			
Glucose	+	100 ± 5	90 ± 10
	-	250 ± 10	275 ± 15
Gluconate	+	250 ± 20	240 ± 25
	-	600 ± 50	650 ± 30

Values are expressed as mean ± S.D. of three independent observations.

Table 4.5

Substrate specificity of GDH of soil isolates

Substrate	**GDH Activity (Units/mg protein)**					
	Isolate 1		**Isolate 2**		**Isolate 3**	
	+P	**-P**	**+P**	**-P**	**+P**	**-P**
Maltose	100 ± 5	600 ± 25	150 ± 10	700 ± 20	150 ± 15	750 ± 30
Galactose	50 ± 5	200 ± 10	50 ± 5	150 ± 5	60 ± 5	250 ± 20
Lactose	UD	UD	UD	UD	UD	UD
2-Deoxyglucose	UD	UD	UD	UD	UD	UD

UD Undetectable.

Values are expressed as mean ± S.D. of three independent observations.

The Extra Chromosomal Elements of the PS Isolates :

The isolates did not show the presence of extrachromosomal elements by alkaline SDS and boiling preparation methods (Sambrook *et al.*, 1989).

Isolation of *mps*$^-$ Mutants of Isolate 3 with Tn-5 :

Kanamycin resistant transposon mutants were obtained at the frequency of 10^{-5} as reported earlier (Simon *et al.,* 1983). Approximately 4,000 colonies were screened for the *mps*- phenotype. 10 putatives were isolated which could not show zone of P solubilization on DCP plates at a frequency expected of a single hit mutation (Fig. 4.3). Based on their *mps*- phenotype, the various mutants could be catagorised broadly into 3 different types : (1) Mutants which do not show zone with PQQ but regain the *mps* phenotype in the presence of PQQ, (2) Mutant which show zone of pH reduction in the presence of 50 mM Tris-HCl pH 8.0 but not in media buffered with 100mM Tris pH 8.0 in the absence of PQQ but show *mps* phenotype on 100mM buffered plates in the presence of PQQ, (3) Mutants which do not show zone either in presence or absence of PQQ even in 50mM Tris buffered plates.

Biochemical Characterization of *mps*$^-$ Transposon Mutants :

mps-1 would not show GDH activity both in presence and absence of free P but showed GDH activity when PQQ was supplemented in the assay mixture. The *mps*-2 mutant showed GDH activity which could only increase 2 fold upon P starvation. However the activity increased further in the presence of PQQ in the assay mixture (Table 4.6). Both the mutants were not affected in AP activity.

P Solubilization by *mps*- Mutants :

In order to find out the RP solubilization by the *mps*- mutants which were isolated in solid media, the mutants were grown in minimal media with RP as sole P source. All

Fig.4.3 : *mps*- phenotype of the transposon mutants.

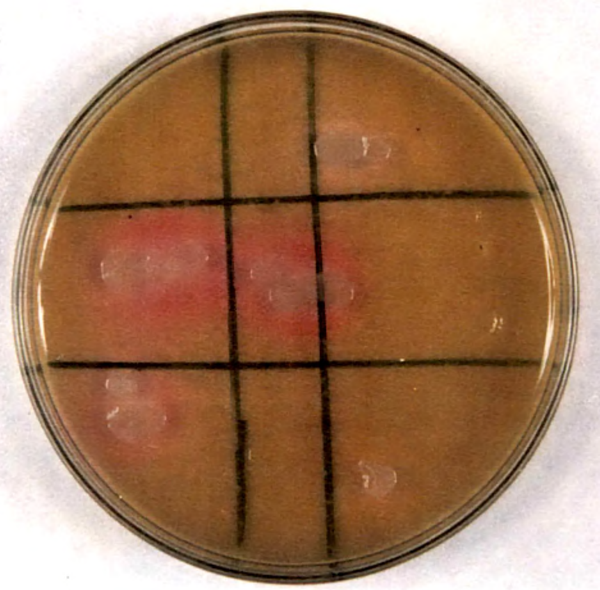


Table 4.6

GDH and AP activities of *mps-* mutants of isolate 3

Mutant No.	+P		-P	
	-PQQ	+PQQ	-PQQ	+PQQ
GDH (Units/mg protein)				
1.	UD	150 ± 8	UD	900 ± 40
2.	150 ± 10	200 ± 15	450 ± 10	1000 ± 45
3.	UD	UD	UD	UD
AP (Units/mg protein)				
1.	UD		35 ± 2	
2.	UD		30 ± 5	
3.	UD		40 ± 6	

UD : Undetectable.

Values are expressed as mean ± S.D. of three independent observations.

three mps- mutants could solubilize RP under unbuffered conditions, but mutants 1 and 3 could not solubilize RP in the presence of buffer in the medium. However, mutant number 2 (regulatory) could solubilize P to 0.1 mM level (Table 4.7).

The three *mps*- mutants could grow well when alkaline soil was provided as a sole P source. However, none of them could either drop the soil pH or release P from soil (Table 4.8).

Table 4.7

RP solubilization by *mps⁻* mutants with ammonium chloride as N source

Mutant	Buffer	Growth ($O.D_{600\ nm}$)	pH	Soluble P (mM)
1	-	0.10 ± 0.04	4.00 ± 0.20	1.00 ± 0.10
	+	0.10 ± 0.01	7.50 ± 0.50	UD
2	-	0.05 ± 0.02	3.50 ± 0.30	1.30 ± 0.10
	+	0.20 ± 0.05	6.30 ± 0.30	0.10 ± 0.05
3	-	0.10 ± 0.04	4.00 ± 0.20	1.00 ± 0.10
	+	0.20 ± 0.04	7.80 ± 0.30	UD

+ Presence of buffer
- Absence of buffer

The buffer used was 100 mM Tris-HCl (pH 8.0).

Carbon source was 100 mM Glucose.

All the parameters were monitored after 48 h of incubation in case of buffered and after 6 h in case of unbuffered media.

Results are expressed as mean ± S.D. of three independent measurements.

Table 4.8

Growth and P release from alkaline soil by *mps*⁻ mutants with ammonium chloride as N source

	Time After Inoculation (Hours)				
	0	12	24	36	48
Mutant 1					
Culturable Count (cfu/ml)	5×10^3	8.0×10^4	6.0×10^6	1.0×10^7	2.0×10^7
pH	8.3	7.8	7.5	7.00	7.50
Soluble P (mM)	UD	UD	UD	UD	UD
Mutant 2					
Culturable Count (cfu/ml)	4×10^3	7.0×10^4	2.0×10^6	6.0×10^6	1×10^6
pH	8.3	7.5	6.5	7.0	7.5
Soluble P (mM)	UD	UD	UD	UD	UD
Mutant 3					
Culturable Count (cfu/ml)	5×10^3	2.1×10^5	6.0×10^6	1×10^7	2.0×10^7
pH	8.3	8.0	7.5	7.5	7.5
Soluble P (mM)	UD	UD	UD	UD	UD

UD : Undetectable.

4.4 : DISCUSSION

The high potency PS isolates secreting high concentration of gluconic acid showed the induction of GDH activity upon P starvation. The increase in GDH was responsible for the ability of these bacteria to drop the pH of the media. The induction required *de novo* protein synthesis and different concentrations of free P was required to induce the GDH activity with 5mM P inducing the activity to about 50% for isolates 1 and 2 and less than 25% for isolate 3. This indicates that the GDH of these isolates is regulated by the P concentration in the media and could be part of *psi* mechanism of these bacteria. The soil isolates were induced for P starvation in media condition used for GDH assay as they showed high activity of AP. It is known that various strains of *Rhizobium* are P limited at 100-150 μM concentration of P as determined by the induction of AP activity (Smart *et al.*, 1984).

In *E. herbicola* the P solubilizing property was shown to be inducible by P starvation. 20mM P was required to completely abolish HAP solubilization by *E. herbicola.* MPS gene involved in PQQ biosynthesis cloned from *E. herbicola* in *E. coli* also showed P induction/repression phenotype indicating it to be part of *psi* system. Similarily the DCP solubilization by *E. coli* is abolished high levels by P (Goldstein & Liu, 1987). However, the E.D. pathway enzymes are not induced by P starvation in *E. coli.* In *Pseudomonas,* GDH is induced by gluconate or 2-ketogluconate (Liese & Phibbs, 1989). The GDH of the soil isolates reported here was induced only by P starvation as the activity was similar in all the C sources used for the growth of the bacteria. The GDH activity in *P. fluorescens* was induced two fold during growth on gluconate as C source but was not induced by P starvation and was not limited by PQQ indicating that both the apoprotein and PQQ are required at high levels to produce high concentration of gluconic acid which is necessary to solubilize P from soil condition.

The GDH of these isolates was active with maltose as substrate and could not use 2-deoxyglucose indicating it to be like GDH-B of *A. calcoaciticus* which is a periplasmic protein. The whole cells also showed activity with galactose although it was substantially less as compared to glucose. GDH from *P. fluorescens* has less than 10% activity with galactose as a substrate whereas GDH from *P. cepacia* is as active with galactose as with glucose (Lessie & Phibbs, 1984). The isolates also did not show the presence of extra-choromosomal elements indicating that the GDH responsible for high gluconic acid production is localized in the choromosome. It is known that GDH is encoded in the chromosome in *A. calcoaceticus* (Cleton-Jansen *et al.*, 1988), *E. coli* (Cleton-Jansen *et al.*, 1990) and *Pseudomonas* (Lessie & Phibbs, 1984).

Since there was no induction of GDH of *P. fluorescens* which was not limited by PQQ, the soil isolates showing high levels of GDH activity are probably able to increase both apo protein and PQQ upon P starvation. It would be of interest to clone the GDH gene and the gene(s) involved in the overproduction of PQQ from these soil isolates. The cloned gene(s) could then be transferred to Plant Growth Promoting Rhizobacteria (PGPR) like *Rhizobium* and *Pseudomonas*. *Pseudomonas* are known to be active root colonizers of many plants and enhance plant growth by various mechanisms (O'Sullivan & O'Gara, 1992; Kloepper *et al.*, 1989). *Rhizobium* can be used as a P biofertilizer for legumes as well as for cereals since many reports show that rhizobium can colonize roots of non-legumes as efficiently as roots of legumes (Shimshick & Hebert, 1979; Al-Mallah, *et al.*, 1989, 1990; Gaur, *et al.*, 1980; Hoflich, *et al.*, 1995; Chabot *et al.*, 1996; Schloter *et al.*, 1997). The gene(s) from these bacteria will have the advantage that these would be overexpressed only under P deficient conditions.

The transposon mutants isolated would help in cloning these genes by making a genomic DNA library of the isolates in suitable vector and then complementing the mutants for the *mps+* phenotype. The analysis of mutants has revealed them to be deficient in PQQ biosynthesis and a regulatory gene which seem specific for PQQ biosynthesis since in this mutant GDH activity increased 2 fold which was further increased by addition of PQQ in the assay system. These *mps-* mutants failed to solubilize RP under buffered media condition and from soil because of the lack of ability to secrete gluconic acid implying that gluconic acid was indeed the active factor involved in P solubilization by these isolates under buffered media condition and from alkaline soil.

In conclusion the work presented in this chapter has shown the biochemical basis of high level of gluconic acid acid production by the high potency bacterial species isolated from soil using buffered screening media. Mutants in gluconic acid secretion have been isolated which would help in cloning of these high-potency *mps* genes from these isolates.