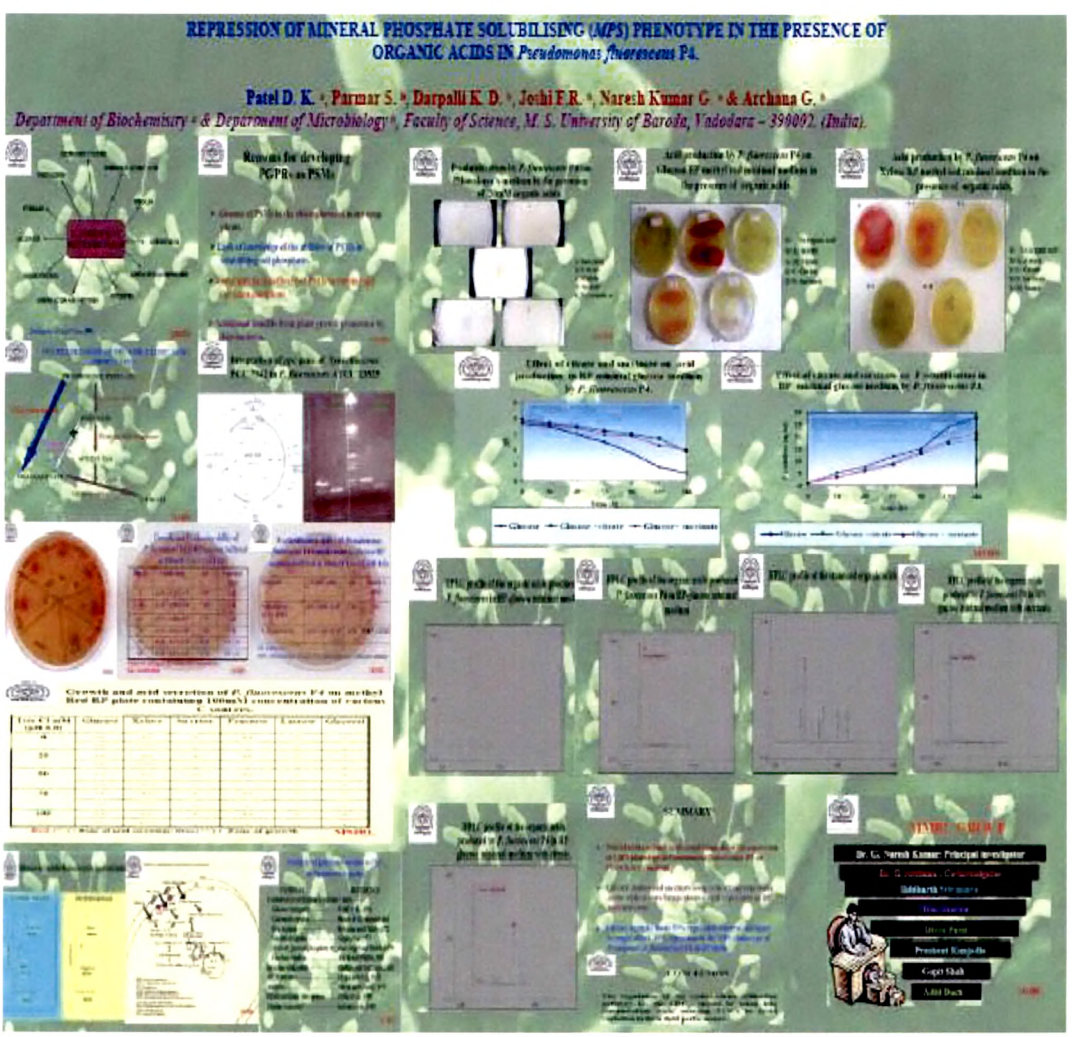
CHAPTER 3

Repression of Mineral Phosphate Solubilizing (mps) Phenotype in the Presence of Weak Organic Acids in Plant Growth Promoting Fluorescent *Pseudomonads*



3.1: INTRODUCTION

Phosphate (P) solubilization is an important activity of bacteria present in the rhizospheric soil. It contributes significantly to the plant growth and development. A variety of bacteria belonging to diverse genera and several groups of fungi have been implicated in the mineral phosphate solubilization (mps) (Gyaneshwar *et al.*, 2002). Among bacteria, *Erwinia, Enterobacter, Rahnella*, *Pseudomonas*, *Bacillus*, and *Rhizobium* are the most efficient phosphate solubilizing bacteria (PSB) (Rodriguez and Fraga, 1999). The major mechanism of mps is the secretion of organic acids synthesized by soil microorganisms and gluconic acid is most frequently encountered organic acid associated with PSB (Rodriguez and Fraga, 1999; Gyaneshwar *et al.*, 2002). Goldstein (1995) had proposed that the direct periplasmic oxidation of glucose to gluconic acid, and often 2-ketogluconic acid via the direct oxidation pathway forms the metabolic basis of the mps phenotype in some Gram negative bacteria. Moreover, the best-known microorganisms with plant growth promoting activity are reported to be bacteria belonging to the group of root-colonizing fluorescent *Pseudomonas* sp. (Van Veen *et al.*, 1997).

Unlike, enteric bacteria where the phosphoenolpyruvate-dependent phosphotransferase system (PTS) is the main carbohydrate uptake system and growth on glucose results in repression of other catabolic pathways, (Postma *et al.*,1993) in the case of *Pseudomonas* sp., low molecular weight organic acids such as acetate and intermediates of tricarboxylic acid cycle serve as the preferred carbon/energy sources over carbohydrates such as glucose, gluconate, glycerol, fructose, mannitol etc. and a strong repression of the enzymes of carbohydrate catabolism is observed in their presence (Collier *et al.,* 1996). Thus in the *Pseudomonads*, organic acids are utilized before the carbohydrates indicating that the preference for the carbon source and the catabolite repression control (CRC) is exactly opposite to that found in the enteric group of bacteria. Hence, the CRC exhibited by *Pseudomonas* sp. is called "reverse CRC". The molecular mechanism of carbon catabolite repression (CCR) has been extensively characterized in *E. coli* where it is mediated via the enzymes of the PTS which modulate the concentrations of cyclic AMP (cAMP) through adenylate cyclase activity. (MacGregor *et al.*, 1992). CCR in *Pseudomonads* however, does not involve cAMP as in *Enterobacteriaceae.* In fact, in *Pseudomonas putida* and *Pseudomonas aeruginosa*,

irrespective of the carbon source, the cAMP and adenylate cyclase levels are relatively constant and external addition of cAMP does not relieve the repression, indicating that *Pseudomonas* sp. exert CRC in a cAMP independent manner (MacGregor *et al.*, 1992; Collier *et al.,* 1996). Instead, catabolite repression seems to integrate different signals, a feature which increases the complexity of the system. Up to five different potential regulators have been related to catabolite repression in *P. putida*, namely, Crc (Morales *et al.*, 2004), Crp, called Vfr in *P. aeruginosa* (Suh *et al.*, 2002), CyoB (Dinamarca *et al.*, 2002), RelA (Jishage *et al.*, 2002), and the PTS system (Cases *et al.*, 2001). However, till date, the exact molecular mechanism establishing CRC is not been elucidated. In addition to the *hex* regulon, enzymes of several other catabolic pathways involved in metabolism of nitrogen, sulphur or other compounds such as amidase expression, alkylsulfatase, histidase, allantoinase etc. are found to be sensitive to CRC (Collier *et al.,* 1996).

The genus *Pseudomonas* is known for its diversity in habitat and physiology. Some *Pseudomonas* strains are known to be able to utilize over 100 different organic compounds as the sole source of carbon and energy, making them play an important role in nature as an efficient bioremediator of natural and xenobiotic environmental pollutants. Most of the time these recalcitrant compounds are present in nature as mixtures along with other conventional organic acids and carbohydrates as carbon source which might lead to hinderances in the efficient degradation of these compounds and unless the simpler carbon sources are completely depleted, the toxic aromatic compounds are not degraded (Collier *et al.,* 1996). CRC has been shown to regulate a number of functions in *P. aeruginosa* and *P. putida*, such as the toluene and xylene degradation in *P. putida* KT2440 by pWW0 plasmid (Duetz *et al.*, 1994), expression of branched-chain keto acid dehydrogenase (Hager *et al.*, 2000), the alkane degradation pathway in *P. oleovorans* and *Pseudomonas putida* GPo1 (Staijen *et al.*, 1999; Yuste and Rojo, 2001), phenol and chloroaeromatic degradative pathway of *P. putida* (Mcfall *et al.,* 1997; Petruschka *et al.*, 2001) and the glycine betaine catabolic enzymes in *P. aeruginose* PAO1 (Diab *et al.*, 2006).

In addition to bioremediation, *Pseudomonas* sp. colonize and enhance plant growth very efficiently by diverse mechanisms, thus forming a major group amongst plant growth promoting rhizobacteria (PGPR) (Latour *et al.*, 2003). The vicinity of the

plant root where the *Pseudomonas* sp. colonizes, however, are reported to be rich in mixtures of a variety of organic compounds and sugars which are exuded by the plant roots (de Weert *et al.*, 2002; Kravchenko *et al.*, 2003) and thus CRC is most likely to operate in the rhizosphere allowing the preferential utilization of most efficient carbon source. Present study investigates role of CRC mediated by the presence of low molecular weight organic acids on the P solubilisation phenotype to understand the performance of P solubilizing *Pseudomonads* in the rhizosphere.

3.2: MATERIALS AND METHODS

3.2.1: Bacterial Strains Used And Growth Conditions

Rhizospheric isolates belonging to the fluorescent *Pseudomonads* family namely *P. aeruginosa* M3 and SP1 were used for the study. The isolates were maintained routinely on Pseudomonas agar medium. *P. aeruginosa* M3 and SP1 were cultured on basal salts medium with 0.3% Casamino Acids overnight for inoculum preparation throughout the course of work. Cells were grown at 30°C for all the experiments. The organic acids selected for the repression studies are Na salts of malate and succinate at a concentration of 20 mM throughout the studies. For checking the effect of mixture of organic acids on the P solubilization 10 mM equimolar mixture of malate and succinate was used.

3.2.2: Characterization Of mps Ability Of *P. aeruginosa* M3 and SP1

mps ability of *P. aeruginosa* M3 and SP1 was determined on 100mM Tris-Cl (pH 8.0) buffered minimal medium with 100mM glucose as sole C source (Gyaneshwar *et al.*, 1998a). Commercial grade 'Senegal' Rock Phosphate (RP) was used as sole source of insoluble mineral phosphate and 1% methyl red as indicator dye for visualization of colour change on organic acid production.

Aliquots were withdrawn at required intervals. Absorbance at 600nm and pH drop were used as parameters for growth and acidification, respectively. Culture supernatant was collected when the pH dropped below 5.0 and used for identification and quantification of organic acids and for estimating soluble Pi. Results are Mean ± SD of three independent experiments performed in duplicates.

3.2.3: Identification Of The Rhizospheric Fluorescent *Pseudomonads* M3 And SP1 By 16S rDNA Gene Sequence Analysis

DNA of the isolates M3 and SP1 was used as template for PCR amplification of ~1,100bp region of 16S rDNA gene using the standard universal primers corresponding to 16S rDNA of *E. coli*, the sequences of which are as follows:

Primer Nucleotide sequence

Forward Primer: 5' AGAGTTTGATCCTGGCTCA 3'

Reverse Primer: 5' CTCGTTGCGGGACTTAACC 3'

The thermal profile set in the thermal cycler included initial denaturation at 94°C for 5 min. with internal denaturation at 94°C for 30 seconds, annealing at 58°C for 30seconds followed by extension at 72°C for 90seconds and final elongation for 10 min. The PCR product was then partially sequenced using 1107R primer. The partial 16S rDNA sequence was deposited to the GenBank and was subjected to standard bioinformatics tool for closest homology search. Molecular biology techniques were performed according to standard methods (Sambrook and Russel, 2001).

3.2.4: Carbon Catabolite Repression Studies With M3 And SP1

For solid plate experiments, *P. aeruginosa* M3 and SP1 were spotted on the RP solubilising minimal medium (Gyaneshwar *et al.*, 1998a) containing 20mM salts of week organic acids such as malate and succinate as well as an equimolar mixture i.e. 10mM each of both malate and succinate along with or without 35mM glucose. Medium containing both, organic acid as well as glucose, was designated as the repression medium.

For broth experiments, the above mentioned concentrations of organic acids and glucose were used in different combinations such as only glucose, only organic acids (single or in equimolar mixture) and glucose plus organic acid (repression medium). The growth and acidification were measured at regular intervals by monitoring the absorbance at 600nm and pH, respectively. Culture supernatant from all the combinations was collected at the time when pH was dropped below 5.0 in the medium containing only glucose and used to estimate the amount of Pi released. The buffering was reduced to 50mM Tris Cl (pH 8.0) in these experiments as the concentration of glucose was decreased to 35 mM. The experiments were performed for a minimum of three times in duplicates (i.e. n=6) and the results are expressed as Mean ± SD.

3.2.5: Enzyme Assays

P. aeruginosa M3 and SP1 were grown on 50mM Tris Cl (pH 8.0) buffered minimal medium with 35mM glucose and 1mM potassium dihydrogen phosphate as source of free Pi instead of RP. After the cells reached an O.D. at 600nm of 0.5, 20mM of the organic acid (Sodium salt) was added to the flask along with an additional 20mM glucose and allowed to grow further. This 0.5 O.D. was considered as time T1 (Initiation of repression conditions). Aliquotes were withdrawn at regular time intervals for enzyme assays till the cells entered stationary phase. Cells were harvested (5,000 x g for 10 minutes) from the medium, washed with sterile saline and resuspended in 50mM Tris Cl (pH 8.75) and whole cell suspension was used as source of periplasmic enzyme for GDH and GAD assays which were done according to Matsushita and Ameyama (1982) and Matsushita *et al.* (1982), respectively.

Units of activity of GDH and GAD are defined as nmol of 2, 6-Dichlorophenol-indo-phenol (DPIP) reduced per minute using glucose and gluconate as substrate, respectively. Specific (Sp.) activity is defined as units per mg protein. Repression was determined as percentage change in the original specific activity of each enzyme following the addition of organic acids to cells growing on glucose compared to that at time T1. The experiment is performed for a minimum of three times in duplicates (i.e. n=6) and the results are expressed as Mean ± SEM.

3.2.6: Analytical Methods

Organic acids were analysed by LaChrom Merck Hitachi High Performance Liquid Chromatography (HPLC). The culture supernatant was filtered through 0.22μ nylon filter. The organic acids were separated using RP-18 column. The mobile phase consisted of 0.1% phosphoric acid with a flow rate of 1ml/min. Organic acids were detected by absorbance at 210nm. They were identified and quantitated by comparing the retention times and peak areas with standard acids.

Phosphate estimations were done according to Ames method using KH_2PO_4 as standard (Ames, 1964). Whole cell protein was estimated using modified Lowry's method (Peterson, 1979).

3.3: RESULTS

3.3.1: Characterization Of mps Ability Of *P. aeruginosa* M3 And SP1

The isolates M3 and SP1 were obtained from the rhizosphere of mungbean and sweet potato respectively by screening on buffered RP containing minimal medium with glucose as C source. On further characterization of the isolates in broth conditions, they were found to grow to an O.D. of 1.82 and 1.57 and acidify the medium to a pH of 3.8 and 4.36, respectively within 96 h. in the presence of 100 mM Tris Cl (pH 8.0) buffering **(Table 3.1).** The amount of Pi released under this condition was found to be 793 µM in the case of M3 whereas for SP1 it was 661 µM. The medium supernatant showed secretion of 37 mM and 26 mM of gluconic acid each for M3 and SP1, respectively **(Fig. 3.1).**

Table 3.1: Growth, acidification and rock phosphate solubilization by *P. aeruginosa* M3 and SP1 on 100 mM Tris Cl (pH 8.0) and 100 mM glucose containing RP minimal medium

	Initial O.D.	Final O.D.	Initial pH	Final pH	Pi (µM)
M3	0.04±0.005	1.82±0.26	8.11±0.04	3.8±0.03	793±141
SP1	0.01±0.001	1.57±0.21	8.18±0.09	4.36±0.38	661±22

3.3.2: Identification Of The Isolates M3 And SP1

The isolates M3 and SP1showed fluorescence when grown on Pseudomonas agar medium suggesting that they belonged to the broad class of fluorescent *Pseudomonads* **(Fig. 3.2).** For further characterization, 16S rDNA was amplified and 567 bp and 614 bp partial sequence was obtained for M3 and SP1, respectively which is given in **Fig. 3.3 (a).** Analysis of this sequence using Ribososmal Database Project (RDP) II online homology search program revealed maximum identity (100% for M3 and 94.7% for SP1) to *Pseudomonas aeruginosa* **(Fig. 3.3 b)** The GenBank accession number obtained for these partial 16S rDNA sequences is **EU711277** and **EU711278**, respectively for M3 and SP1.The accession profile is given in **Fig 3.3 (c).**

Fig. 3.1: HPLC Profile of *P. aeruginosa* M3 and SP1 on 100mM Tris Cl (pH 8.0) and 100 mM glucose RP minimal medium

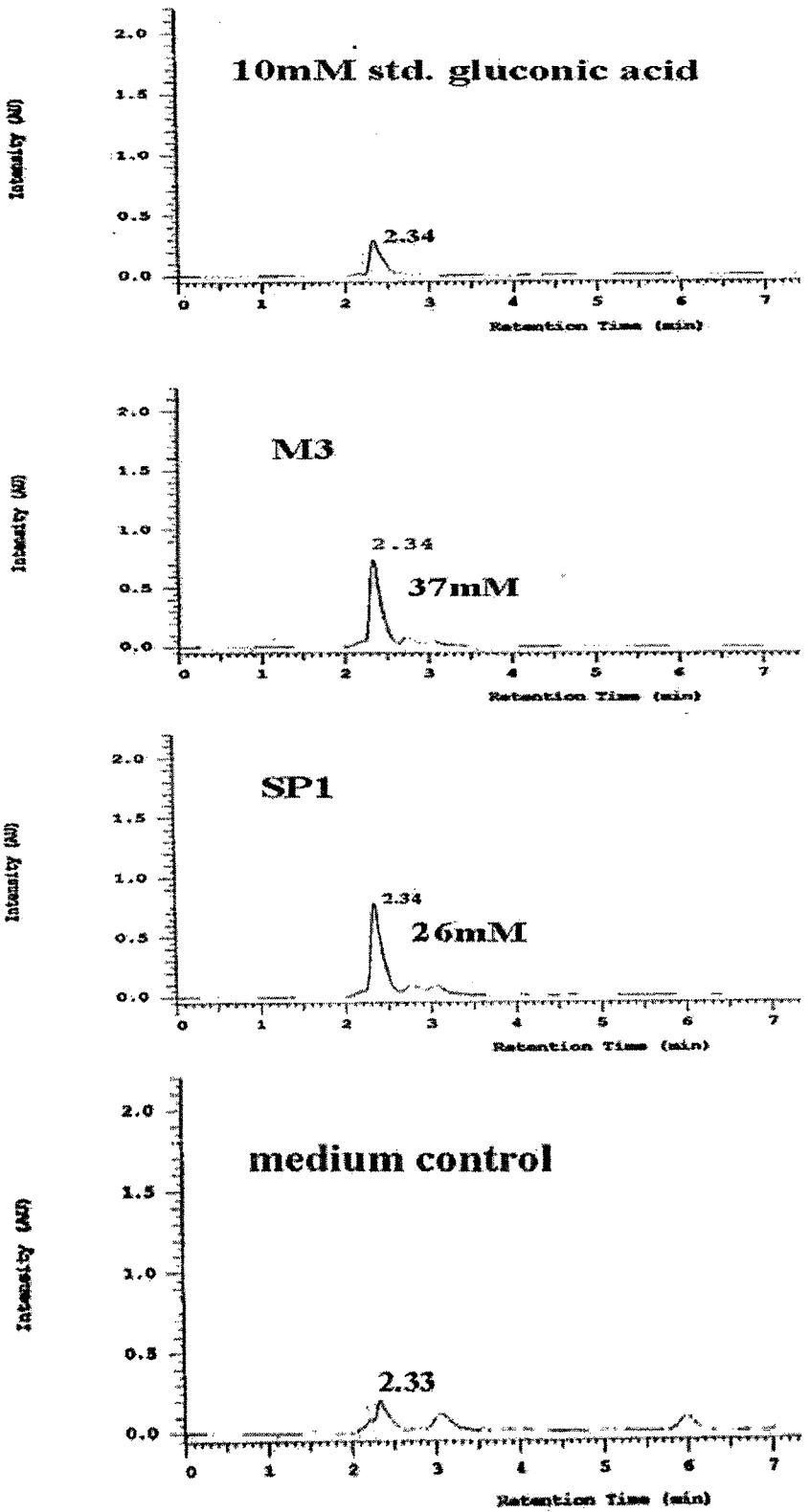


Fig. 3.2: Fluorescence of *P. aeruginosa* M3 and SP1 on Pseudomonas agar plate

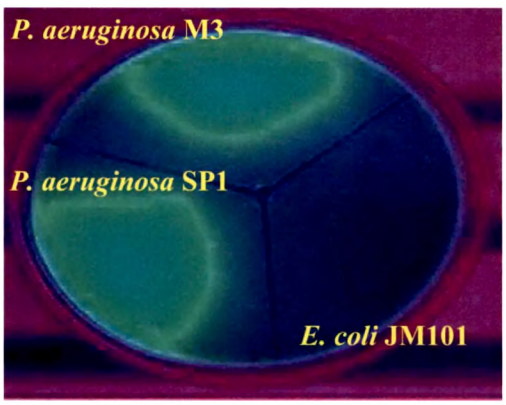


Fig. 3.3 a: Partial 16S rDNA sequence of *P. aeruginosa* M3 and SP1

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GAGCCCGGGGATTTCACATCCAAcTTGCTGAACCACCTACGCGCGC
TTTACGCCCAGTAATTCCGATTAAcGCTTGCACCCTTCgtATTACCGC
GGCTGcTGG

SP1

cgcATCTCACGACACGagCTGACGACAGCCATGCAGCACCTGTGTCT
GAGCTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCAGCATGT
CAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATG
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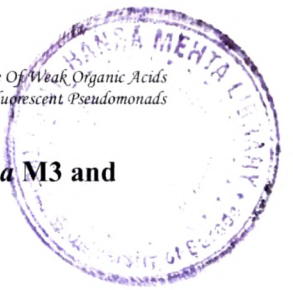

Fig. 3.3 b: Homology search result of partial 16S rDNA of *P. aeruginosa* M3 and SP1 using RDP II

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Ribosomal Database Project II

RDP HOME | BROWSER | CLASSIFIER | LIBCOMPARE | SEQMATCH | PROBE MATCH | TREE | myRDP | seqCART

RDP

Seqmatch :: Results for Query Sequences under Bacteria

Seqmatch: version 3
RDP Data: release 9.60
Data Set: both type and non-type strains, both environmental (uncultured) sequences and isolates, near-full-length sequences (≥1200 bases), good quality sequences
Comments: 180125 sequences were included in the search
The screening was based on 7-base oligomers
Query Submit Date: Wed Apr 30 07:05:22 EDT 2008

Match hit format: short ID, orientation, similarity score, S_ab score, unique common oligomers and sequence full name. More help is available.

Lineage:
Results for Query Sequence: unknown, 553 unique oligos

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phylum Proteobacteria (20)
class Gammaproteobacteria (20)
order Pseudomonadales (20)
family Pseudomonadaceae (20)
genus Pseudomonas (20)

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S000002377	-	not_calculated	1.000	1459	Pseudomonas aeruginosa; AL98; AJ249451
S000005690	-	not_calculated	1.000	1406	Pseudomonas aeruginosa; 42A2 NCBI 40045; AJ309500
S000007938	-	not_calculated	1.000	1302	Pseudomonas aeruginosa; type strain:ICMP 8647; AJ308297
S000008931	-	not_calculated	1.000	1452	Pseudomonas sp.; OLB-1; AJ387904
S000112106	-	not_calculated	1.000	1419	Pseudomonas thermaerum; T1; AB088116
S000128122	-	not_calculated	1.000	1390	Pseudomonas aeruginosa; KF702; AB109752
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S000333002	-	not_calculated	1.000	1425	Pseudomonas aeruginosa; X3; AY631240

Seqmatch :: Results for Query Sequences under Bacteria Page 1 of 2

skip to content

Ribosomal Database Project II

RDP HOME | BROWSER | CLASSIFIER | LIBCOMPARE | SEQMATCH | PROBE MATCH | TREE | myRDP | seqCART

RDP

Seqmatch :: Results for Query Sequences under Bacteria

Seqmatch: version 3
RDP Data: release 9.60
Data Set: both type and non-type strains, both environmental (uncultured) sequences and isolates, near-full-length sequences (≥1200 bases), good quality sequences
Comments: 180125 sequences were included in the search
The screening was based on 7-base oligomers
Query Submit Date: Wed Apr 30 07:05:22 EDT 2008

Match hit format: short ID, orientation, similarity score, S_ab score, unique common oligomers and sequence full name. More help is available.

Lineage:
Results for Query Sequence: unknown, 553 unique oligos

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phylum Proteobacteria (20)
class Gammaproteobacteria (20)
order Pseudomonadales (20)
family Pseudomonadaceae (20)
genus Pseudomonas (20)

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S000002377 - not_calculated 1.000 1459 Pseudomonas aeruginosa; AL98; AJ249451
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S000255100 - not_calculated 1.000 1456 Pseudomonas aeruginosa; AB126582
S000333002 - not_calculated 1.000 1425 Pseudomonas aeruginosa; X3; AY631240

Fig. 3.3 c: GenBank accession result of partial 16S rDNA sequence of *P. aeruginosa* M3 and SP1

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Thus, on the basis of their efficiency to solubilize significant amounts of RP under buffered conditions via gluconic acid production, the isolates M3 and SP1 were selected for further studies.

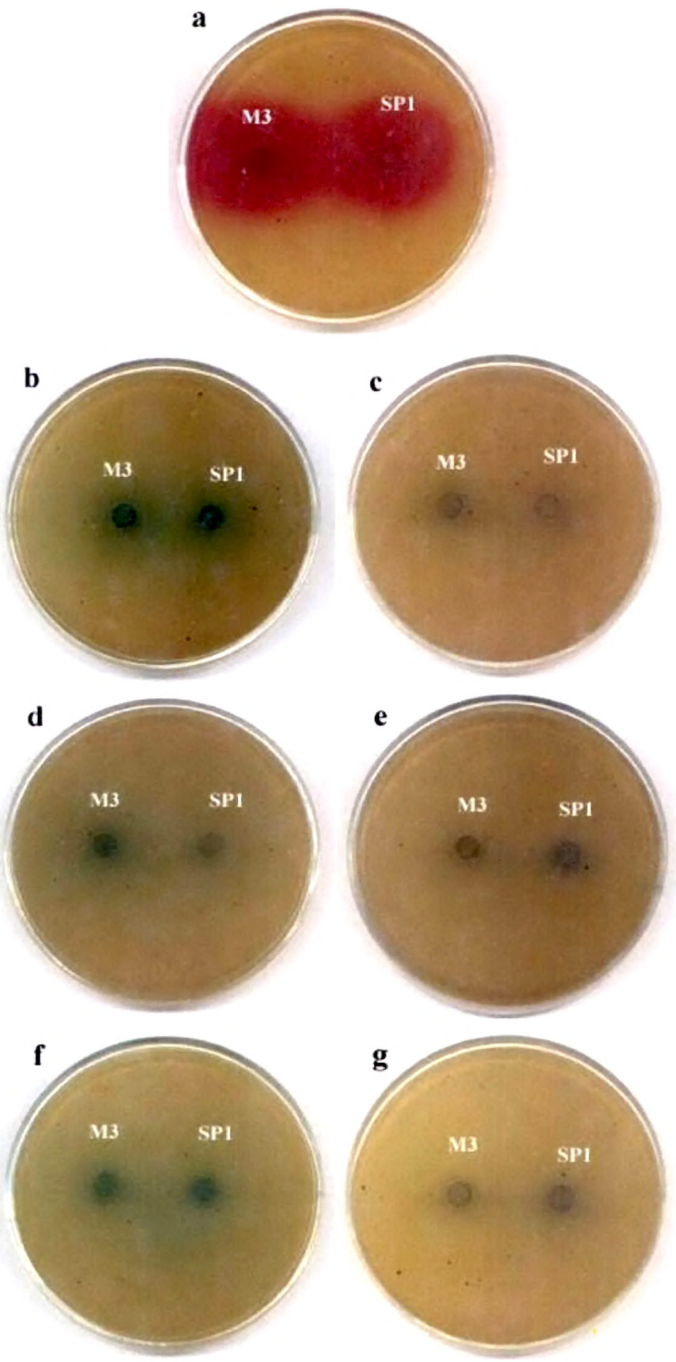
3.3.3: Carbon Catabolite Repression Studies With *P. aeruginosa* M3 and SP1

When *P. aeruginosa* M3 and SP1 were spotted on minimal medium with RP as the sole Pi source, they showed a clear red zone indicating acidification on the methyl red containing minimal medium agar plates with 35 mM of glucose as the C source under 50 mM Tris Cl (pH 8.0) buffered conditions **(Fig. 3.4 a).** However no acidification was observed when low molecular weight organic acids such as malate, succinate or an equimolar mixture of malate and succinate was used as sole source of C **(Fig. 3.4 b, d, f).** In fact the region around the spotted culture turned slightly black indicating that the cells were experiencing nutritional stress and were unable to grow. Similarly, acidification was also found to be absent in the repression medium which contained glucose along with the organic acids **(Fig. 3.4 c, e, g).**

In order to quantitate the differences in the RP solubilizing ability observed under plate medium conditions by *P. aeruginosa* M3 and SP1 under repression conditions, liquid medium experiments were performed. Both, *P. aeruginosa* M3 and SP1 showed maximal growth when only glucose was provided in the medium as compared to that on the repression medium or only organic acids containing medium **(Table 3.2).** A drastic reduction in the media pH dropping ability was also observed by *P. aeruginosa* M3 and SP1 in the repression medium as compared to that on only glucose containing medium where the medium pH decreased below 5.0 within 72 h. **(Fig. 3.5 a and b).** Whereas, in the presence of only organic acids as the C source, *P. aeruginosa* M3 and SP1 failed to show pH drop in the 50mM Tris Cl (pH 8.0) buffered medium. On the contrary, the pH turned alkaline and increased above 8.0.

In glucose containing medium *P. aeruginosa* M3 released 153 µM Pi while *P. aeruginosa* SP1 showed 205 µM Pi release **(Table 3.2).** However, both the isolates failed to show P solubilization in the presence of only organic acids and repression medium as compared to that on glucose alone under 50 mM Tris Cl (pH 8.0) buffered conditions which was in agreement with the loss of pH reducing ability.

Fig. 3.4: Acidification by *P. aeruginosa* M3 and SP1 on methyl red containing minimal media agar plates with RP and 50 mM Tris Cl (pH 8.0)



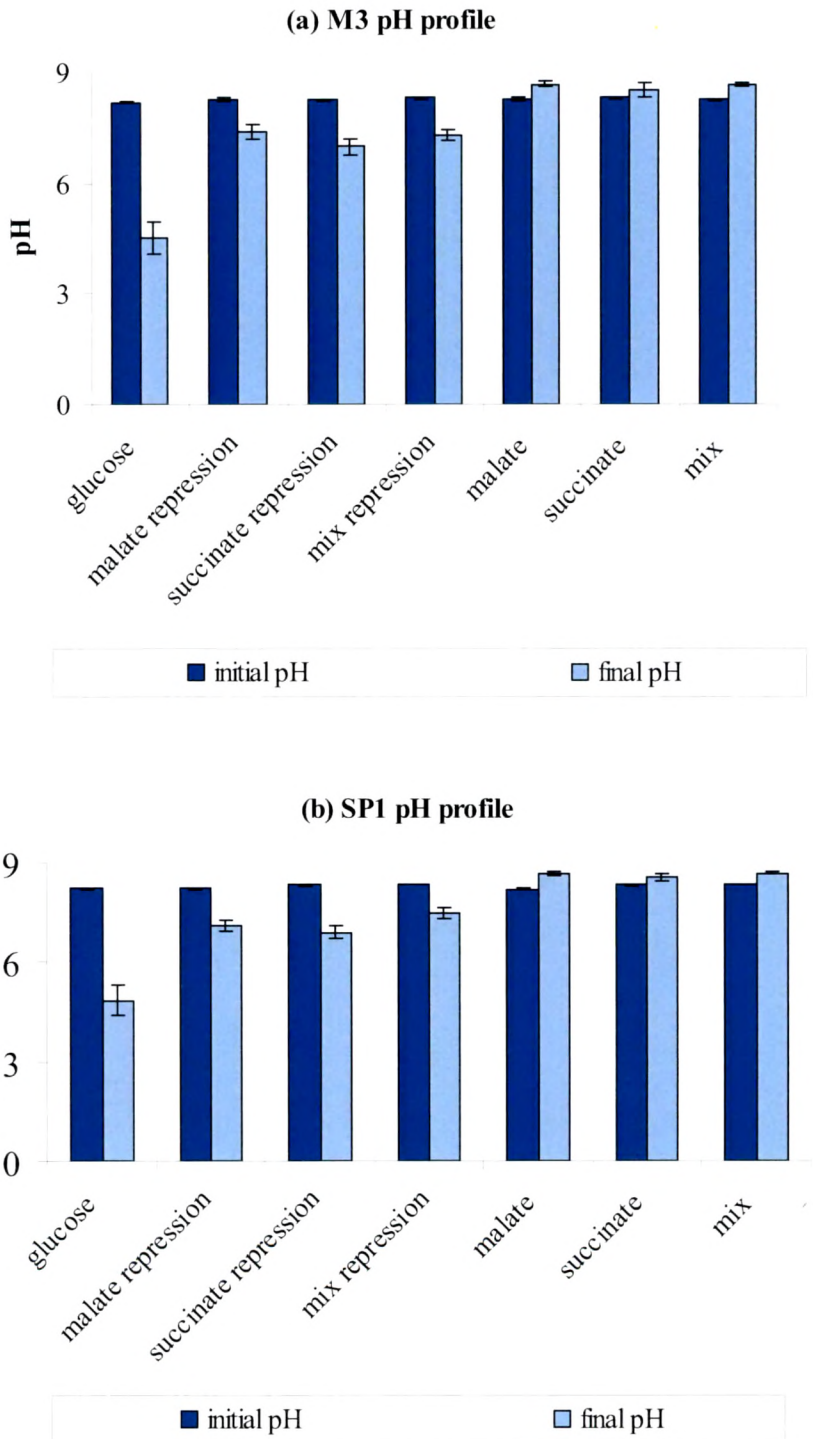
a: 35mM glucose; b: 20mM malate; c: 35mM glucose + 20mM malate ;
d: 20mM succinate ; e: 35mM glucose + 20mM succinate ; f: 10mM malate + 10mM succinate; g: 35mM glucose + 10mM malate + 10mM succinate

Table 3.2: Growth and RP solubilization by *P. aeruginosa* M3 and SP1 in the presence of organic acids in liquid medium

Sugar	Organic acid	Initial O.D.	Final O.D.	Pi (μM)
M3				
Glucose	None	0.04±0.002	1.67±0.116	153±30
	Malate	0.05±0.01	0.76±0.07	U.D.
	Succinate	0.05±0.004	0.81±0.14	U.D.
	Malate + Succinate	0.04±0.01	0.7±0.05	U.D.
None	Malate	0.04±0.01	0.64±0.06	U.D.
	Succinate	0.05±0.01	0.6±0.04	U.D.
	Malate + Succinate	0.04±0.01	0.61±0.04	U.D.
SP1				
Glucose	None	0.025±0.01	1.14±0.28	205±26
	Malate	0.02±0.01	0.86±0.06	U.D.
	Succinate	0.024±0.01	0.79±0.27	U.D.
	Malate + Succinate	0.023±0.01	0.73±0.06	U.D.
None	Malate	0.02±0.005	0.37±0.08	U.D.
	Succinate	0.02±0.01	0.53±0.04	U.D.
	Malate + Succinate	0.02±0.01	0.54±0.05	U.D.

U.D.-Un Detectable

Fig. 3.5: Acidification by *P. aeruginosa* (a) M3 and (b) SP1 in the presence of organic acids in RP containing minimal medium



3.3.4: Effect Of Organic Acids On GDH And GAD Enzyme Activities Of *P. aeruginosa* M3 And SP1

GDH activity of *E. asburiae* PSI3 in the presence of glucose with malate as the organic acid was done as a positive control in this round of experiments and it was found to increase under the repression medium conditions in contrast to *P. aeruginosa* M3 and SP1 **(Fig. 3.6)**

In the presence of malate a drastic decrease in the specific activity of GDH was observed in *P. aeruginosa* M3 **(Fig. 3.7.1).** There was around 70% reduction in the specific activity within 12 hr. of addition of malate when the cells reached stationary phase. However, there was no significant change observed in case of GAD specific activity. In case of *P. aeruginosa* SP1, addition of malate resulted in 75-80% reduction of GDH specific activity by the time the cells reached stationary phase. Unlike M3 however, *P. aeruginosa* SP1 showed a 3 fold increase in the GAD specific activity **(Fig. 3.7.2).** Similar results were obtained with succinate or equimolar mixture of malate and succinate in case of *P. aeruginosa* M3 as well as SP1 **(Fig. 3.8.1; 3.8.2; 3.9.1; 3.9.2).** No significant change in the GAD specific activity was seen in the presence of succinate or equimolar mixture of malate and succinate in case of *P. aeruginosa* SP1. In all the cases, a strong repression of GDH enzyme was observed on addition of the organic acids to the cultures growing on glucose. The repression was maintained till the cells reached stationary phase. The rate of decline in the specific activity suggests a simple dilution of the GDH enzyme activity during the continuous growth of the culture indicating that the organic acids do not inhibit the preformed enzyme activity. GAD activity was not found to be affected by the addition of malate or succinate indicating that it was not under the catabolite repression control mediated by malate and succinate.

Fig. 3.6: GDH activity of *E. asburiae* PSI3 in the presence of glucose with malate

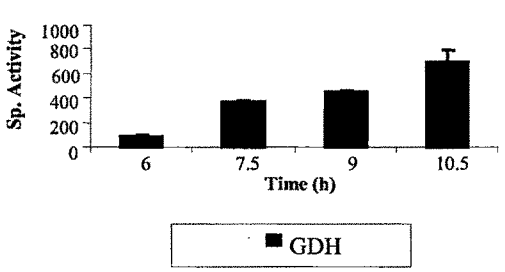
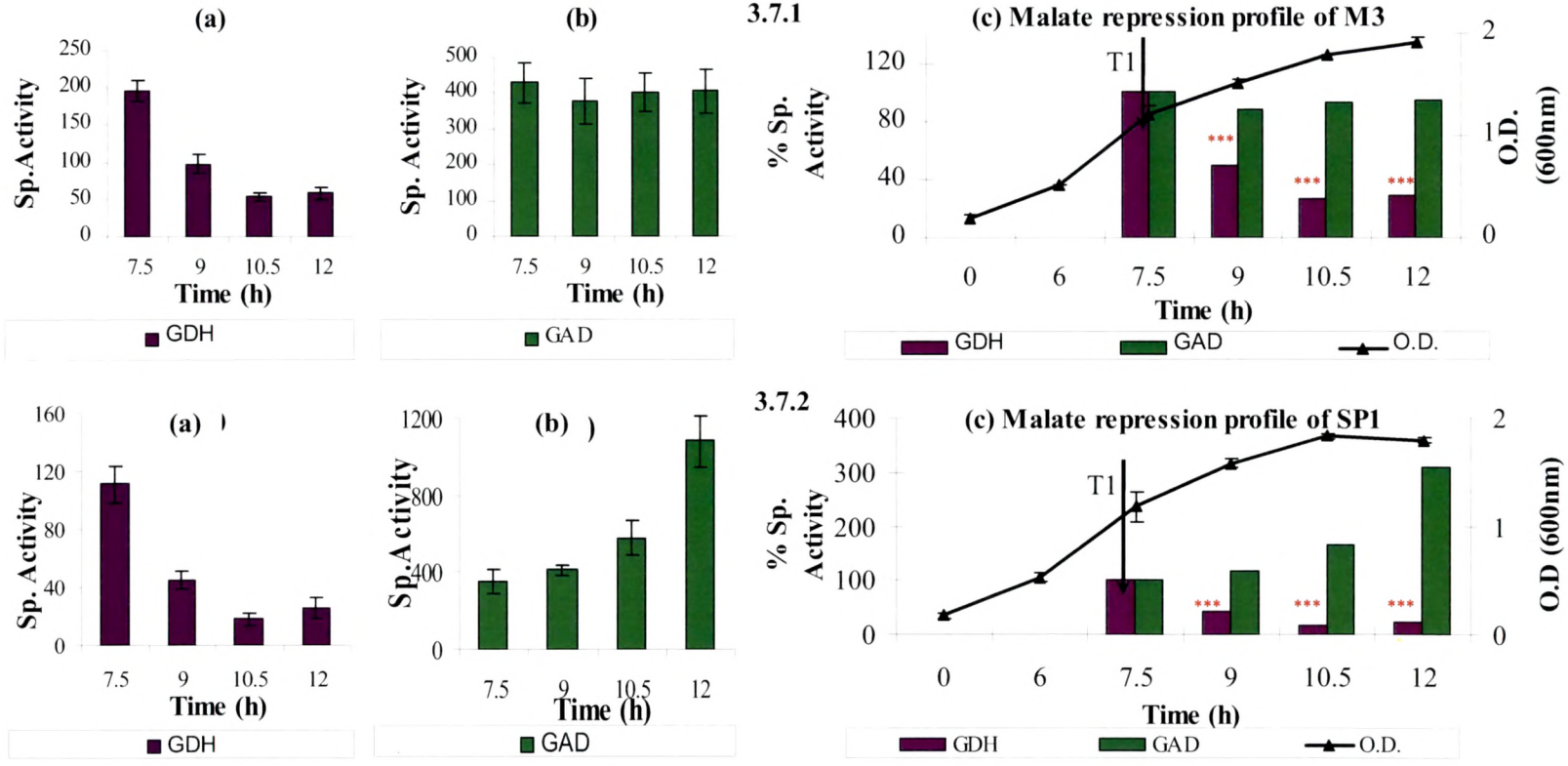
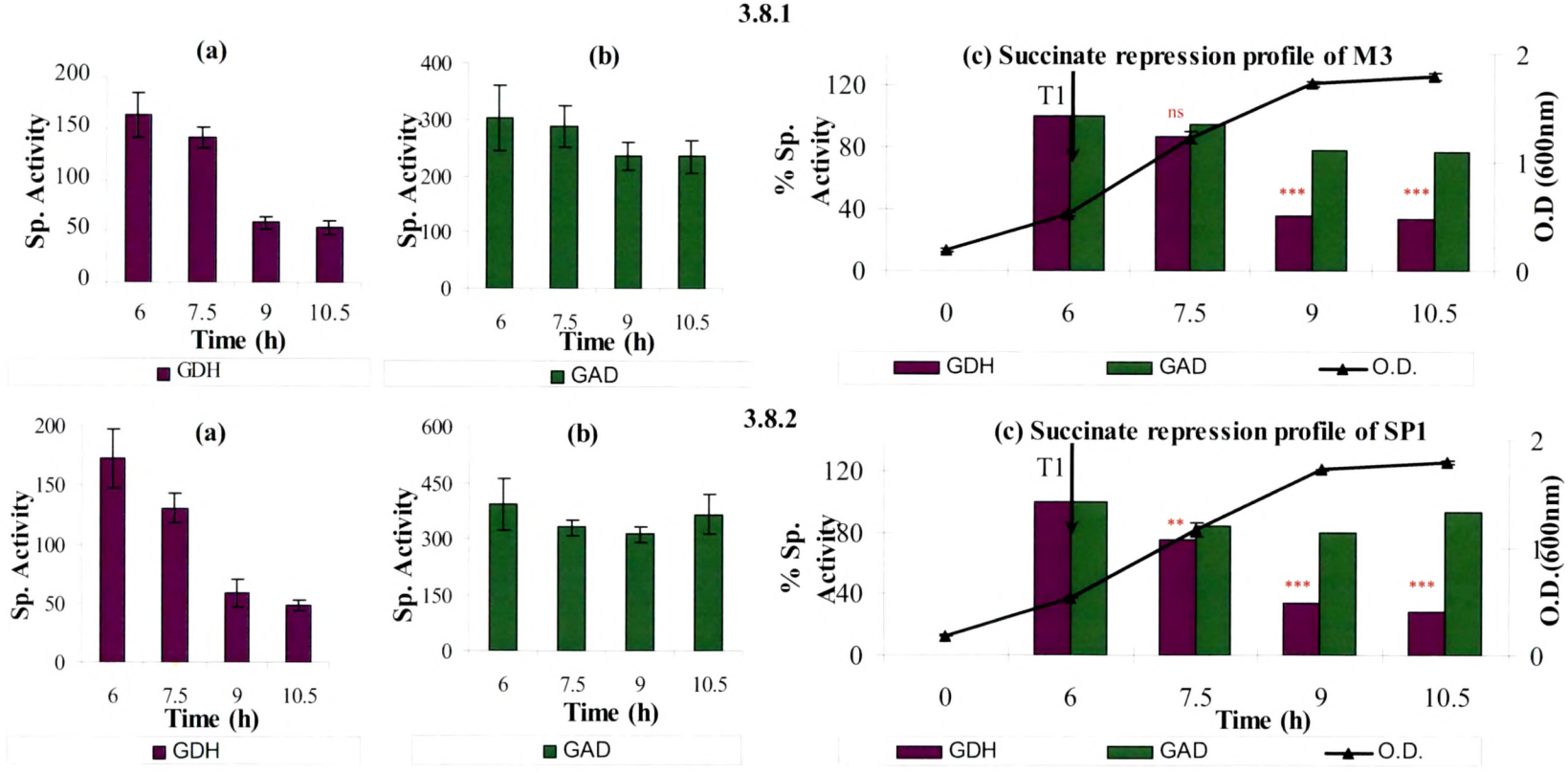


Fig. 3.7: GDH and GAD enzyme activities of *P. aeruginosa* (1) M3 and (2) SP1 grown in the presence of glucose with malate



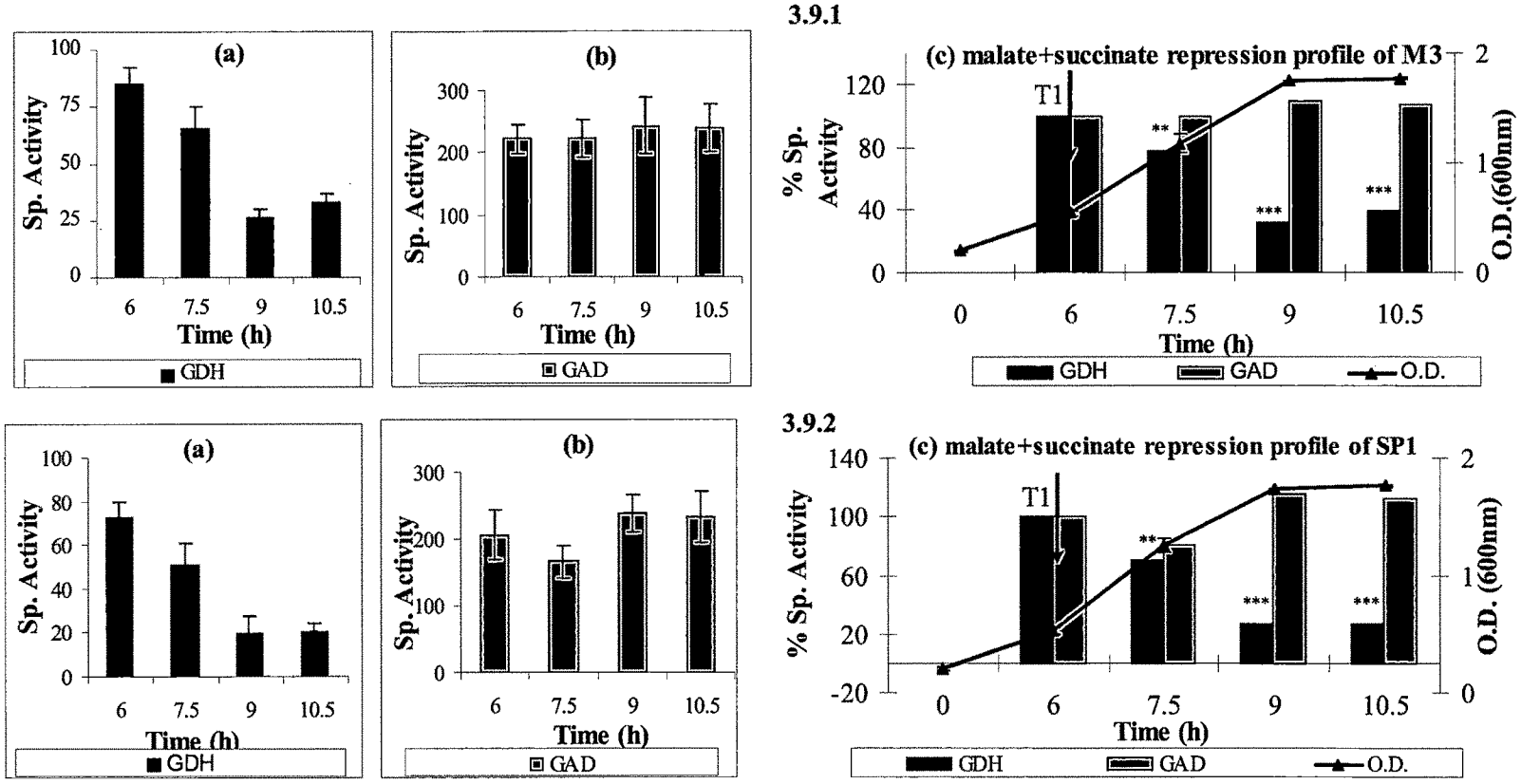
Change in the Sp. activities of GDH (a) and GAD (b) following the addition of 20 mM malate along with additional 20 mM glucose to cells growing on 35 mM glucose at an O.D. of 0.5; The data are also plotted as % change in the original Sp. activity of each enzyme relative to that at time T1which is considered to be 100%, along with the growth profiles of the cultures in the same medium (c); All the enzyme activities are represented in the units of nmoles/min/mg total protein; The values are expressed as Mean ± S.E.M of three independent observations performed in duplicates (n=6); *** P<0.001

Fig. 3.8: GDH and GAD enzyme activities of *P. aeruginosa* (1) M3 and (2) SP1 grown in the presence of glucose with succinate



Change in the Sp. activities of GDH(a) and GAD (b) following the addition of 20 mM succinate along with additional 20 mM glucose to cells growing on 35 mM glucose at an O.D. of 0.5 (c); The data are also plotted as % change in the original Sp. activity of each enzyme relative to that at time T1which is considered to be 100%, along with the growth profiles of the cultures in the same medium; All the enzyme activities are represented in the units of nmoles/min/mg total protein; The values are expressed as Mean ± S.E.M of three independent observations performed in duplicates (n=6); *** P<0.001, ** P<0.01, ns= non-significant

Fig. 3.9: GDH and GAD enzyme activities of *P. aeruginosa* (1) M3 and (2) SP1 grown in the presence of glucose with malate and succinate



Change in the Sp. activities of GDH (a) and GAD (b) following the addition of 10 mM malate plus 10 mM succinate (Total 20 mM organic acid) along with additional 20 mM glucose to cells growing on 35 mM glucose at an O.D. of 0.5 (c); The data are also plotted as % change in the original Sp. activity of each enzyme relative to that at time T1which is considered to be 100%, along with the growth profiles of the cultures in the same medium; All the enzyme activities are represented in the units of nmoles/min/mg total protein; The values are expressed as Mean ± S.E.M of three independent observations performed in duplicates (n=6); *** P<0.001, ** P<0.01

3.4: DISCUSSION

The rhizospheric isolates M3 and SP1 identified as *P. aeruginosa* released 76.12 mg/L and 63.45 mg/L of phosphate, respectively using RP as the sole source of insoluble P. This amount is at par with the P solubilizing ability of some of the reported PSB (Rodriguez and Fraga, 1999). *Pseudomonas striata* has been shown to accumulate 22 mg/L of free P from RP while *Burkholderia cepacia* and *Pseudomonas* sp. have been shown to release 35 and 52 mg/L of P from dicalcium phosphate, respectively. *E. asburiae* PSI3 and *Enterobacter intermedium*, belonging to the *Enterobacteriaceae* family have also been reported to solubilize 77 mg/L and 200 mg/L of RP, respectively (Gyaneshwar *et al.*, 1999; Hoon *et al.*, 2003). Thus, *P. aeruginosa* M3 and SP1 were found to be very efficient P solubilizers with an ability to release significant amounts of RP under 100 mM Tris Cl (pH 8.0) buffered conditions using glucose as a C source. The P solubilizing ability of *P. aeruginosa* M3 and SP1 was attributed to their ability to produce high concentrations of gluconic acid. The P releasing ability of several PSMs such as *Rahnella aquatilis, Erwinia herbicola, Pseudomonas cepacia* and *E. asburiae* have also been reported to be a result of their ability to secrete gluconic acid (Gyaneshwar *et al.*, 1999; Rodriguez and Fraga, 1999)

A drastic reduction in the zone of acidification and pH drop along with an elimination of RP solubilizing ability of *P. aeruginosa* M3 and SP1 was observed when they were grown in the presence of a mixture of glucose with low molecular weight organic acids under solid medium and broth conditions, respectively. This inability to solubilize P in the presence of malate and succinate can be attributed to the repression of the GDH enzyme which is responsible for the gluconic acid production in *P. aeruginosa* M3 and SP1. Periplasmic GDH mediated gluconic acid formation contributes as a predominant constitutive glucose catabolic pathway in *Pseudomonads* (Lessie and Phibbs, 1984). Thus, the direct oxidative pathway for glucose utilization involving GDH has been shown to play an important role in the P solubilizing ability of rhizospheric isolates belonging to the *Pseudomonadaceae* family (Babu-Khan *et al.*, 1995; Rodriguez and Fraga, 1999). Activities of the *hex* regulon enzymes involved in the hexose catabolism such as glucose -6-phosphate dehydrogenase (G-6-PDH), glucokinase, 2-keto-3-deoxy-6-phosphogluconate (KDPG) and glucose dehydrogenase (GDH) have been shown to be repressed in the presence of succinate or other organic acids (Hylemon and Phibbs, 1972). Moreover, glucose metabolism by intracellular phosphorylative pathway has been shown to be suppressed in *Pseudomonas*

putida CSV86 when grown in the presence of organic acids (Basu *et al.*, 2006; Basu and Phale, 2006). However, GAD was not found to be repressed in the presence of malate and succinate in *P. aeruginosa* M3 and SP1. Similarly, GDH of *E. asburiae* PSI3 was also not under the CRC of malate indicating that its P solubilizing ability will not be affected by the presence of organic acids in the rhizosphere.

Similar results were obtained even when the concentrations of the two organic acids, malate and succinate, were reduced by 50% and used as equimolar mixtures indicating that the catabolite repression of P solubilizing ability of *P. aeruginosa* M3 and SP1 persists even at lower concentration of each organic acid when they are present as a mixture. This observation is relevant from the rhizospheric point of view where the organic acids secreted by the root exudates are expected to be present as mixtures but in lower amounts (Jones, 1998). Low molecular weight organic acids form an integral part of plant root exudates (Jones, 1998) and plays an important role in the survival and competence of fluorescent *Pseudomonads* in the rhizosphere (Latour *et al.*, 2003). Growth and antifungal activity of plant growth promoting *Pseudomonas* strains in the plant rhizosphere was shown to depend on the sugar and organic acid composition of root exudates of tomato plants (Kravchenko *et al.*, 2003). Interestingly, the organic acids, majorly citrate and malate, but not the sugars of tomato root exudates were involved in the chemotactic response and thereby competitive tomato root-tip colonization by *P. fluorescens* WCS365 (de Weert *et al.*, 2002).

The phenanthrene-degrading activity (PDA) of *Pseudomonas putida* ATCC 17484 was found to be repressed after incubation with plant root exudates of oat (*Avena sativa*) and hybrid poplar (*Populus deltoids* x *nigra* DN34) (Rentz *et al.*, 2004). Carbon catabolite repression was apparently responsible for the observed repression of *P. Putida* PDA. In the case of *P. aeruginosa* M3 and SP, both malate and succinate seem to affect the P solubilizing ability to the same extent and manner indicating that the effect of repression can be extrapolated to other PSMs of the *Pseudomonads* group.Thus, there are chances that the P solubilizing ability of the rhizobacteria belonging to the *Pseudomonadaceae* family can be significantly affected by the preferential utilization of organic acids over glucose in the rhizosphere and hence the regulation of the carbohydrate utilization pathway i.e. the CRC should be taken into consideration while selecting PSMs to avoid failure in their field performance.