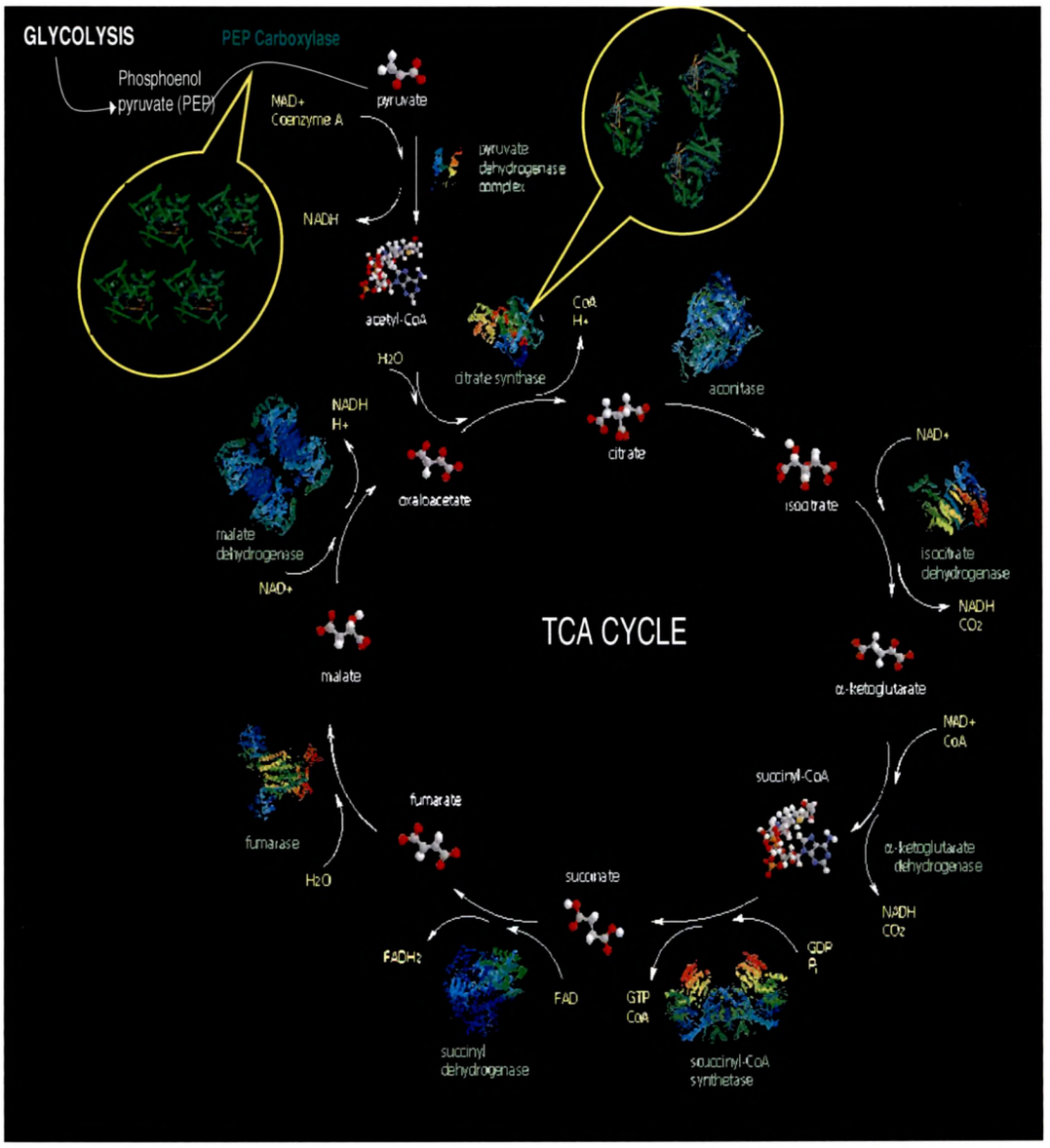
CHAPTER 5

Effect of heterologous overexpression of cs and ppc genes in Enterobacter asburiae PSI3



5.1 INTRODUCTION

Bacillus and *Pseudomonas* form the main bacterial mineral phosphate solubilizer genera (Illmer and Schinner, 1992), while *Aspergillus*, and *Penicillium* form the important fungal genera (Motsara et al., 1995). A majority of PSMs solubilize calcium phosphate (CaP) complexes which are predominant in alkaline soils by extrusion of protons (Roos and Luckner, 1984) or by the secretion of mono-, di- and tri-carboxylic acids (Cunningham and Kuiack, 1992; Illmer and Schinner, 1995; Nahas, 1996; Khan et al., 2006). Only a few have the ability to solubilize ferric and aluminium phosphates, predominant in acidic soils by chelation of Fe and Al ions (Sperber, 1957; Bolan et al., 1987; Kucey et al., 1989; Halder et al., 1991; Halder and Chakrabartty 1993; Omar, 1998). Complex formation depends upon the number and position of the carboxylic groups of the secreted organic acid (Bolan et al., 1994). Similar effects are also seen with the release of organic phytate complexed with Ca, Al or Fe and therefore their subsequent utilization of phytate by phytase enzyme (Hayes, 2000).

P-solubilisation by PSMs not only depends upon the quantity of organic acids but also on the nature of organic acids (Sanders and Tinker, 1971). Amongst organic acids, 20mM citric and 20mM oxalic acid are sufficient to release P and Fe in the soil solution from alkaline vertisols whereas 50mM of gluconic acid does not release the same amount of soluble P (**Table.5.1**; Gyaneshwar et al., 1998). In case of alfisols amended with RP, 10mM oxalic acid releases maximum P whereas 20mM of citric acid releases P equivalent to 50mM gluconic acid (Srivastava et al., 2006). Oxalic and citric acids are efficient in releasing P from alfisols and alkaline vertisols, respectively. Citric acid because of its better chelation ability confers additional benefit in the detoxification of aluminium which is prevalent in acidic soils (Kochian et al., 2002; Watanabe and Osaki, 2002), uptake of nutrients such as iron from the soil and extraction of soil adsorbed phytate salts for phytase hydrolysis.

Table 5.1: Efficacy of different organic acids at P-solubilization from alfisol supplemented with RP (40 mg g^{-1} soil) and alkaline vertisol

Organic acid	Concentration (mM)	Alkaline vertisol (Gyaneshwar et al., 1998)		Alfisol (Srivastava et al., 2006)	
		pH	Solution P (µM)	pH	Solution P (µM)
Water		8.94 ± 0.50	UD	6.69 ± 0.02	U.D.
Citric	10	4.34 ± 0.50	700 ± 2.0	3.45 ± 0.04	409 ± 8.0
Citric	20	3.98 ± 0.18	1170 ± 3.0	2.80 ± 0.03	850 ± 6.5
Oxalic	10	6.02 ± 0.54	620 ± 2.5	3.42 ± 0.05	1560 ± 4.0
Oxalic	20	5.56 ± 0.40	1290 ± 3.0	ND	ND
Tartaric	10	4.83 ± 0.82	330 ± 2.0	ND	ND
Tartaric	20	3.90 ± 1.10	730 ± 2.6	3.50 ± 0.02	550 ± 6.0
Gluconic	20	6.00 ± 0.20	500 ± 1.0	ND	ND
Gluconic	50	4.50 ± 0.50	650 ± 5.0	3.40 ± 0.05	800 ± 2.0
Succinic	100/50	5.80 ± 0.20	530 ± 0.6	3.64 ± 0.04	332 ± 7.0
Lactic	100/50	6.50 ± 0.30	630 ± 5.0	3.25 ± 0.02	920 ± 7.0
Acetic	100/50	6.00 ± 0.40	180 ± 3.0	3.80 ± 0.23	102 ± 2.0

UD: Undetectable; ND : not done

5.1.1 Effects of *cs* gene overexpression in plants

Citrate synthase condenses oxaloacetate and acetyl CoA to form citrate, catalyzing a crucial step at the branch point of oxidative, lipogenic, and anaplerotic pathways, depending upon the microorganism, in citric acid metabolism (Walsh and Koshland, 1985). Citrate synthase of *Pseudomonas aeruginosa* over expression in tobacco (*Nicotiana tabacum*; CSb lines) was suggested to enhance P-uptake and improve Al tolerance through citrate efflux (de la Fuente et al., 1997; López-Bucio et al., 2000). Later studies done by Delhaize et al., 2001 could not reproduce the same results and attributed the phenotype of citric acid secretion to Al toxicity rather than *cs* overexpression. Overexpression of mitochondrial citrate synthase in *Arabidopsis thaliana* improved growth on a phosphorus-limited soil and also could improve the

growth of carrot cells on Al-phosphate medium; the effect was attributed to enhanced secretion of citric acid (Koyama et al., 1999; 2000). On the other hand, overexpression of mitochondrial *cs* gene *CIT1* alone and in combination with a mitochondrial *MDH1* increased citrate content by 2-3 fold in and reduced Al sensitivity in yeast (*Sachharomyces cerevisiae*) and canola (*Brassica napus cv Westar*) (Anoop et al., 2003).

5.1.2 Effect of *cs* over expression on microorganisms

Overexpression of *cs* genes in *A. niger* was not successful in increasing the citrate production (Ruijter et al., 1997; 2000). Overexpression of phosphofructokinase (*pfk*), and pyruvate kinase (*pk*) in *A. niger* also did not result in citrate secretion. The glucose uptake rate has been identified as an important factor in the rate of citric acid production which could increase the citric acid productivity by 45% as predicted by mathematical model in *A. niger* (Torres, 1994a; b). *A. niger* with increased levels of mitochondrial phosphate carrier could produce citric acid in the presence of carbon source excess with simultaneous growth limitation by nitrogen, mineral salts or thiamine (Karaffa and Kubicek, 2003).

E. coli lacking functional *cs* gene failed to utilize glucose unless supplemented with glutamate (or other TCA cycle intermediates) and had reduced growth as compared to the wild type (Gruer et al., 1997; Vandedrinck et al., 2001; De Maeseneire et al., 2006). However, overexpression or underexpression of *cs* gene in *E. coli* had no effect on growth on glucose but protein level gets strongly affected when acetate is used as a sole carbon source (Walsh and Koshland, 1985a; Vandedrinck et al., 2001). Conversely, *cs* overexpression in *E. coli* increased the maximum cell dry weight by 23% and reduced acetate secretion (De Maeseneire et al., 2006).

Isocitrate dehydrogenase (ICDH) mutants of *E. coli* K and B strains showed higher levels of citrate accumulation when grown on glucose with simultaneously more than two fold increase in CS activity (Lakshmi and Helling, 1976; Aoshima et

al., 2003). Similarly, accumulation of approximately 15 fold higher intracellular citrate is reported in *B. subtilis icd* mutant compared to the wild type in early stationary phase (Matsuno et al., 1999). The upregulation of *cs* coupled with the diminished activities of aconitase (ACN) and NAD-isocitrate dehydrogenase (NAD-ICDH) appeared to be instrumental in the accumulation of citrate in *Pseudomonas fluorescens* when grown on malate (Mailloux et al., 2008).

Metabolic studies suggested that citrate accumulation in *E. coli,* fungi and yeasts high citric acid yields could be attributed to host metabolism, glucose transport, flux though catabolic pathways and the regulatory mechanisms influenced by intracellular metabolite pools. However, role of CS in citrate accumulation is unclear.

5.1.3 Effects of PEP carboxylase (*ppc*) overexpression in *E. coli*

In *E. coli* PEP carboxylase (PPC) functions aerobically to replenish oxaloacetate consumed in biosynthetic reactions. Under fermentative conditions, PEP carboxylase also has a catabolic function by directing a portion of the PEP to succinic acid (Millard et al., 1996; Lin et al., 2004). One of the factors which could be limiting for citrate synthesis despite *cs* over expression is concentration of oxaloacetate (Anoop et al., 2003). Under anaerobic conditions, *ppc* overexpression in *E. coli* on glucose results in increase in succinic acid (Millard et al., 1996; Lin et al., 2004, 2005). Similar results were reported in *E. coli* overexpressing *ppc* or pyruvate carboxylase (*pyc)* genes suggesting that the cell adapts to these genetic alterations by adjusting the flux to lactate, ethanol and acetate (Gokarn et al., 2000). Overexpression of *ppc* under aerobic condition decreased glucose consumption rates and organic acid excretion, without change in growth and respiration rates therefore improving the growth yield. This result indicated that the wild-type level of PPC was not optimal for the most efficient glucose utilization in batch cultures (Chao and Liao, 1993). In presence of excess glucose, *ppc* overexpression and deregulation of the glyoxylate bypass did not

affect the growth and the glucose consumption rates but reduced the acetate excretion by 70% (Farmer and Liao, 1997; Abdel-Hamid et al., 2001).

5.1.4 Effects of *ppc* overexpression in other organisms

E. coli ppc gene when expressed in *Synechococcus* PCC 7942 *ppc* mutant showed lower PPC activity with reduced growth, chlorophyll-a (30%) content, 50% reduction in rate of NH_4^+ stimulated, dark carbon fixation and photosynthetic activity in low light intensity (Luinenburg and Coleman, 1993). Overexpression of *ppc* gene in combination with ornithine carbamoyltransferase and carbamoylphosphate synthetase genes triggered the biosynthesis of cyanophycin in *Acinetobacter sp.* strain ADP1 (Elbahloul and Steinbüchel, 2006). Overexpression of *ppc* gene resulted in lysine overproduction in *Clostridium glutamicum* containing feedback-resistant aspartate kinase while it did not contribute much in glutamate overproduction (Cremer et al., 1991; Shirai et al., 2007).

PPC and CS enzymes are highly regulated both negatively and positively depending upon the metabolic status of the organism. *Synechococcus elongatus* PCC 6301 PPC is known to be insensitive to the allosteric effectors including dioxane a non-physiological activator and L-aspartate (Ishijima et al., 1985; Kodaki et al., 1985). Cyanobacetrial PPC is not activated by acetyl-CoA (Luinenburg and Coleman, 1993). *S. elongatus* PPC is a homotetramer of ~95–110-kDa subunits, is more closely related to bacterial PPCs due to presence of conserved catalytic domain which is of bacterial type and lack of N-terminal phosphorylation domain typical of plant PPC (Kai et al., 1999; Sanchez and Cejudo, 2003; Xu et al., 2006; Sugita et al., 2007).

To check the effect of *ppc* and *cs* on the citric acid metabolism in *E. asburiae* PSI3, heterologous overexpression of above genes individually as well as in combination was carried out.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains and plasmids used in this study

Table 5.2: Bacterial strains and plasmids used in this study

Bacterial strains or plasmids	Genotype or relevant characteristics	Reference
***E. coli* strains**		
DH5α	*F' Phi80 Δ lacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+) phoA supE44 lambda-thi-1*	Sambrook and Russell, 2001
E. coli JWK3928	*lacI^q rrnBT14 Δ lacZWJ16 hsdR514 Δ araBADAH33, Δ rhaBADLD78* ***ppc****::Km*; Km^r	Peng et al., 2004
JWK3928 (pVSppc)	*E. coli* JWK3928 with pVSppc plasmid, Ap^r, Tc^r	This study
JWK3928 (pA172A)	*E. coli* JWK3928 with pA172A plasmid, Ap^r, Tc^r	This study
***E. asburiae* strains**		
E. asburiae PSI3	rhizosphere isolate from *Cajanus cajan*	Gyaneshwar et al., 1999
E. a (pJE1)	*E. asburiae* PSI3 containing pJE1plasmid, Ap^r, Km^r	This study
E. a (pJE2)	*E. asburiae* PSI3 containing pJE2 expressing *cs* gene of *E. coli*, Ap^r , Km^r	This study
E. a (pJE3)	*E. asburiae* PSI3 containing pJE3 plasmid, Ap^r , Tc^r	This study
E. a (pVSppc)	*E. asburiae* PSI3 containing pVSppc plasmid expressing *ppc* gene of *S. elongatus* PCC 6301, Ap^r, Tc^r	This study
E. a (pJE1, pJE3)	*E. asburiae* PSI3 containing two plasmids, pJE2 and pJE3	This study
E. a (pVSppc, pJE2)	*E. asburiae* PSI3 containing two plasmids pVSppc, pJE2	This study
Plasmids used		
pTTQ18	pUC18 derived vector having *tac* promoter; Ap^r	Stark, 1987
pME6010	pVS1-p15A shuttle vector, Tc^r	Heeb et al., 2000
pA172A	pBR322 with ~3.5kb *S. elongatus* PCC 6301 genomic DNA fragment harboring *ppc gene*	Kodaki et al., 1985
pVSppc	pTTQ18 with *S. elongatus* PCC 6301 *ppc* gene under P_{tac} and *tet^r* gene, Ap^r , Tc^r	This study
pJE1	pTTQ18 with *npt II* conferring Kanamycin resistance gene, Ap^r, km^r	This study
pJE2	pTTQ18 with *E. coli cs* gene under P_{tac} and *npt* II gene, Ap^r, km^r	This study
pJE3	pTTQ18 with tetracycline resistance gene, Ap^r, Tc^r	This study

Km =Kanamycin; Tc =Tetracycline; Ap =Ampicillin; r = resistant.

5.2.2 Media and culture conditions

E. coli and *E. asburiae* PSI3 were grown and maintained on luria broth and luria agar. Both the cultures were grown at 37°C, for growth in liquid medium, shaking was provided at 200 rpm. For plasmid bearing transformants appropriate rich media were supplemented with kanamycin at 25 μg ml^{-1}, tetracycline (30 μg ml^{-1}) streptomycin (10 μg ml-1) or ampicillin (50 μg ml^{-1}) (Sambrook and Russell, 2001) which was reduced to $1/4^{th}$ concentration when grown in minimal media.

5.2.3 P solubilisation phenotype

E. asburiae PSI3 single and double transformants of *cs* and *ppc* and their controls were grown overnight at 37°C in 3ml LB supplemented with appropriate antibiotics. The cells were centrifuged at 9200 x g for 2 min. Supernatant was discarded and cells were then washed thrice with physiological saline before finally suspending them in 500μl of saline. 3μl of the inoculum was then spotted on Tris minimal medium (Gyaneshwar et al., 1999) containing tri-calcium phosphate (TCP) 3mg ml^{-1}as sole P source in the presence of 100mM Tris (pH 8.0) and 100mM fructose or glycerol as sole carbon source. Appropriate antibiotics were added at $1/4^{th}$ the concentration added to rich media. Plates were kept at 37°C for one week to observe the zone of clearance around the growth.

5.2.4 Growth kinetics and acidification studies in liquid medium

Inoculum of *E. asburiae* PSI3 single and double transformants of *cs* and *ppc* and their respective controls were prepared according to Chapter 2 section 2.2.2.1. Cells were added to the 30ml of M9 minimal media containing (1L): Na_2HPO_4. $7H_2O$ 34g, KH_2PO_4 15g, NH_4Cl 5g, NaCl 2.5g containing 2mM $MgSO_4$. $7H_2O$, 0.1mM $CaCl_2$ and micronutrients containing (for 1L) $FeSO_4.7H_2O$ 3.5 mg, $ZnSO_4.7H_2O$ 0.16 mg, $CuSO_4.5H_2O$ 0.08 mg, H_3BO_3 0.5 mg, $CaCl_2.2H_2O$ 0.03 mg and $MnSO_4.4H_2O$ 0.4 mg, to get an initial $O.D._{600}$ of less then 0.03. The flasks were shaken at 37°C till the O.D. reaches saturation phase. 1ml of Aliquots were drawn at every 2 to3h interval. Absorbance at 600 nm and pH drop were used as parameters for growth pattern and acidification, respectively. Culture supernatant was used for identification of organic acids. Results are means of three independent experiments performed in duplicate.

5.2.4.1 Physiological parameters

The physiological parameters like specific growth rate, total fructose consumed, (as described by Chao and Liao, 1993), organic acid yield and organic acid was calculated as described in chapter 3 section 3.2.4.

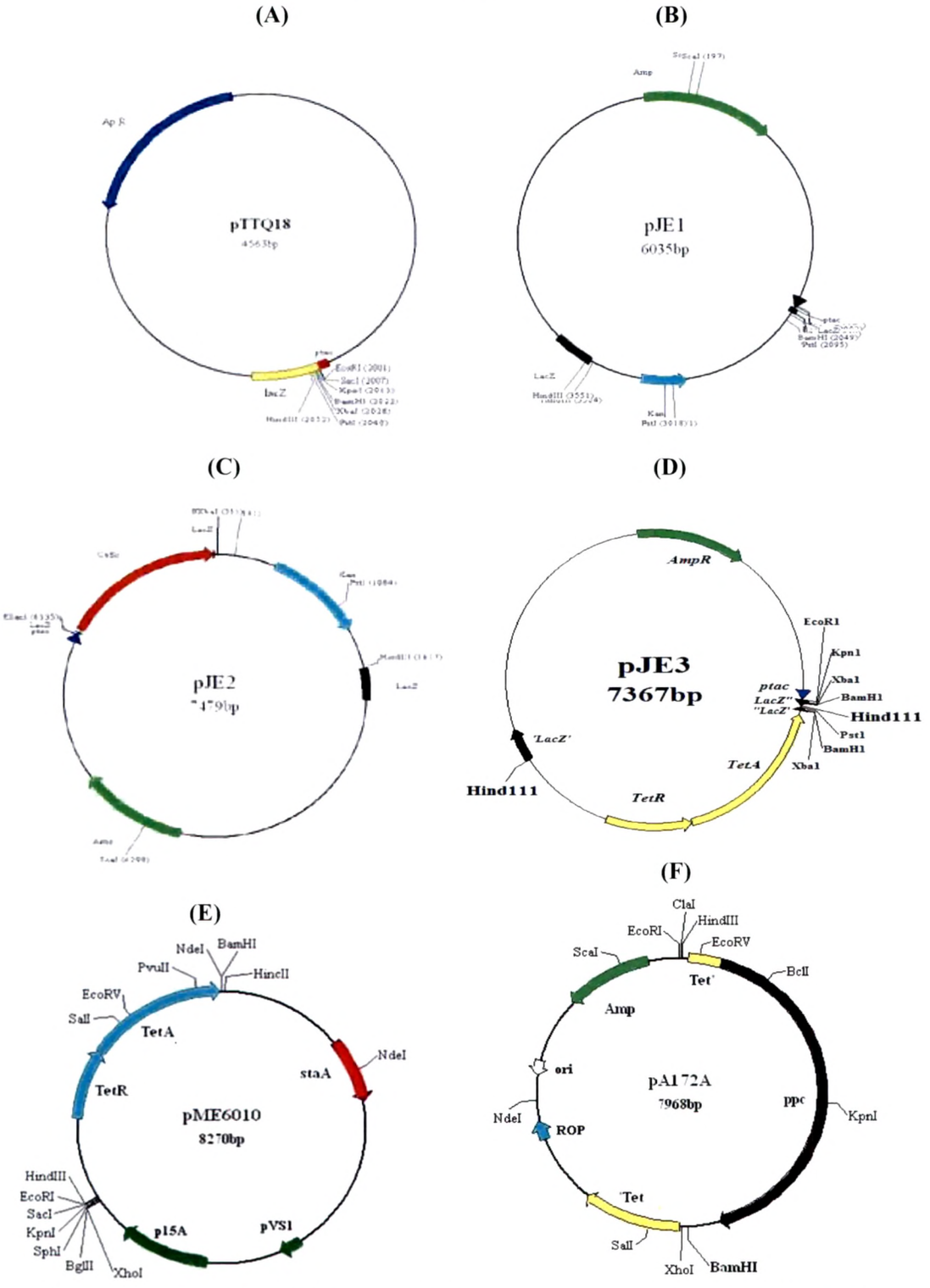
5.2.5 Molecular Biology tools and techniques

Plasmid extraction using the CTAB method, agarose gel electrophoresis, SDS PAGE, preparation of competent cells of *E. coli* and transformation were carried out as described by Sambrook and Russell (2001).

5.2.6 Description of plasmid used

pTTQ18 is a pUC based plasmid containing unique multiple cloning site and provides direct selection of recombinant plasmids based on blue-white selection strategy. *LacZα* gene in pTTQ18 is under the *tac* promoter which is strong and tightly regulated promoter in *Enterobactericeae* family (**Fig. 5.1 A**) (Stark, 1987). **pJE1, pJE2** and **pJE3** are derivatives of pTTQ18 containing kanamycin resistance gene marker, *cs* of *E. coli* under *tac* promoter with kanamycin resistance gene and tetracycline resistance gene respectively (**Fig. 5.1 B, C and D**). **pME6010** (GenBank accession number: AF118810) is a shuttle vector for use in plant associated gram-negative bacteria. It can replicate in *E. coli* owing to p15A (pACYC177) moiety while uses pVS1 replicon to replicate in *Pseudomonas* species (Heeb et al., 2000). It contains a unique and broad multiple cloning site downstream of a constitutive kanamycin resistance promoter (P_k) .It has a repressible tetracycline resistance (Tc^r) and has been shown to be 100% stable in biocontrol strain *P. fluorescens* CHAO for 100 generations without antibiotic selection (**Fig. 5.1 E**). **pA172A** plasmid contains *ppc* gene on ~3.5kb genomic DNA fragment of *S. elongatus* PCC 6301 inserted under the promoter of tetracycline resistance gene of pBR322 plasmid. This plasmid could functionally complement the *E. coli ppc* mutant (Kodaki et al., 1985) (**Fig. 5.1 F**).

Fig. 5.1: **Restriction maps of various plasmids used in this study**



Generous gift from **Dr. S. K. Apte**, BARC, Mumbai

Generous gift from **Dr. D. Haas**, Université de Lausanne, Switzerland

5.2.7 Construction of pVSppc (*ppc* gene of *S. elongatus* PCC 6301 under *tac* promoter)

Plasmid pA172A-Gm was used as a source of *ppc* gene of *S. elongatus* PCC 6301. pA172A-Gm was digested with EcoRI/BamHI and 4.0 Kb fragment containing *ppc* gene was gel purified and ligated to EcoRI/BamHI digest of pTTQ18 to generate 8.3Kb pVS1. Plasmid pVS1 was linearized with BamHI and ligated to 2.8Kb BglII/BamHI digested tetracyclin resistance fragment from pME6010 to generate 11.1 Kb pVSppc.

5.2.8 Mutant Complementation Phenotype

E. coli ppc (JWK3928) mutant was used to confirm the functionality of the cloned *ppc* gene. The recombinant plasmid containing *ppc* under *tac* promoter and parent plasmid pA172A were transformed into *E. coli* (JWK3928). The *ppc* transformants were selected on kanamycin (12.5 μg ml^{-1}) and tetracycline (7.5μg ml^{-1}) or ampicillin (25 μg ml^{-1}). These transformants were grown under shaking conditions at 30°C on M9 minimal medium with 0.2% glucose as carbon source in presence and absence of 340μg ml^{-1} sodium glutamate. *ppc* gene under *tac* promoter was induced with 0.1mM IPTG. Lack of *ppc* gene in *E. coli* (JWK3928) exhibited glutamate auxotrophy (Izui et al., 1986).

5.2.9 Organic acid analysis

E. asburiae PSI3 and the transformants were grown in 30ml M9 minimal medium with 100mM fructose and glycerol as sole C sources up to late stationary phase. The cells were then centrifuged at 9200 x g for 3 min. The culture supernatant was filtered using nylon-66 membrane filters of 0.22 μm pore size and subjected to HPLC with the following specifications: HPLC (LC-AT 20, Shimadzu), reverse phase, ion pairing column (RP-18) column. The mobile phase consisted of 0.01N H_2SO_4 at a flow rate of 1 ml min^{-1}. Detection was performed by a PDA detector at 210 nm. The HPLC profile of the culture supernatants was analyzed by comparison with the elution profile of pure organic acids from HiMedia Laboratories Pvt Ltd., India.

5.2.10 Characterization of the expression of the *ppc* gene

E. coli DH5α harboring pVSppc and pA172A were grown overnight on luria broth, at 37°C with 200 rpm shaking. *E. coli* DH5α harboring pTTQ18 was used as control strain. The transformant bearing the pVSppc plasmid was grown in the presence and absence of the 0.1mM IPTG. The cells were pelleted down after centrifugating at 9200 x g for 3 min. and washed thrice to remove LB. Cells were finally suspended in distilled water and protein estimation by modified lowry method was carried out (Peterson, 1979). Equal amount of protein was loaded in all the wells and SDS-PAGE was performed according to Sambrook and Russell (2001).

5.2.11 PPC and CS Enzyme assays

E. asburiae PSI3 transformants were grown in M9 minimal media were harvested in stationary phase phase from 30ml of cell culture by centrifugation at 9200 x *g* for 2 minutes at 4°C. The preparation of cell free extracts for PPC and CS assays was carried out according to Kodaki et al., 1985. The cell pellet was washed once with 80mM phosphate buffer (pH 7.5) followed by re-suspension in same buffer containing 20% glycerol and 1mM DTT. The cells were then subjected to lysis by sonicating for maximum 1-2 minute in an ice bath, followed by centrifugation at 9,200x *g* at 4°C for 30 minutes to remove the cell debris. The supernatant was then used as cell-free extract for the enzyme assays.

5.2.11.1 PPC assay

PPC activity was estimated spectrophotometrically by monitoring the oxidation of NADH in a coupled assay with malate dehydrogenase (MDH) as described by Kodaki et al., 1985 with modifications. The coupled assay was divided in two steps. The assay mixture for first step of the reaction contained the following reagents in total volume of 0.9ml: Tris-SO_4 (pH 8.0) 100mM, potassium salt of PEP 2mM, $MgSO_4.7H_2O$ 10mM, $KHCO_3$ 10mM and the enzyme solution (~60µl cell lysate). The reaction was allowed to proceed for 20 min. at 30°C. The reaction was terminated using 25% TCA and complete protein precipitation was allowed on ice bath (~15 min.). The resulting mixture was centrifuged at 9200 x g for 30 minutes at 4°C. The recovered supernatant was brought to pH 8.0 using 10N NaOH. This mixture was

used as the source of OAA in second step of the reaction. The second reaction assay system contained 950 µl of reaction mix from first step of reaction, 5 units of malate dehydrogenase and 0.1mM NADH. The volume of assay system was adjusted to 1ml using distilled water. The rate of oxidation of NADH recorded as decrease in absorbance at 340nm which indicate concentration of OAA formed. The molar absorbance of NADH was taken as 6.22 mM^{-1} cm^{-1} at pH 8.0.

5.2.11.2 CS assay

CS activity was estimated by following the change in absorbance of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) at 412nm, due to its reaction with sulfhydryl group of CoA (Serre, 1969). The assay mixture per cuvette contained following reagents (for 1ml) : Tris-HCl (pH 8.0) 93mM, acetyl CoA 0.16mM, OAA 0.2mM, DTNB 0.1mM and cell lysate (~approximately 40µl). The reaction was started by addition of OAA. The molar absorbance coefficient was taken as 13.6 $mM^{-1}cm^{-1}$ at 412nm. The rate of increase in absorbance was used to calculate CS activity.

All the enzyme activities were determined at 30°C and were expressed per mg total protein. Total protein concentration of the crude extract as well as whole cell suspensions was measured by modified Lowry's method (Peterson, 1979) using bovine serum albumin as standard. Corrections were made for Tris buffer. Enzyme activities were calculated using following formula

$$\text{Units/mg} = \frac{\Delta A_{y\,nm}\,\text{min}^{-1}}{\epsilon \times \text{enzyme (sample) aliquot (ml)} \times \text{Total protein (mg ml}^{-1})}$$

where,

$\Delta A_{y\,nm}$ is the difference in the absorbance at any given wavelengths ($_{y\,nm}$) and € is the millimolar extinction coefficient at *y* nm.

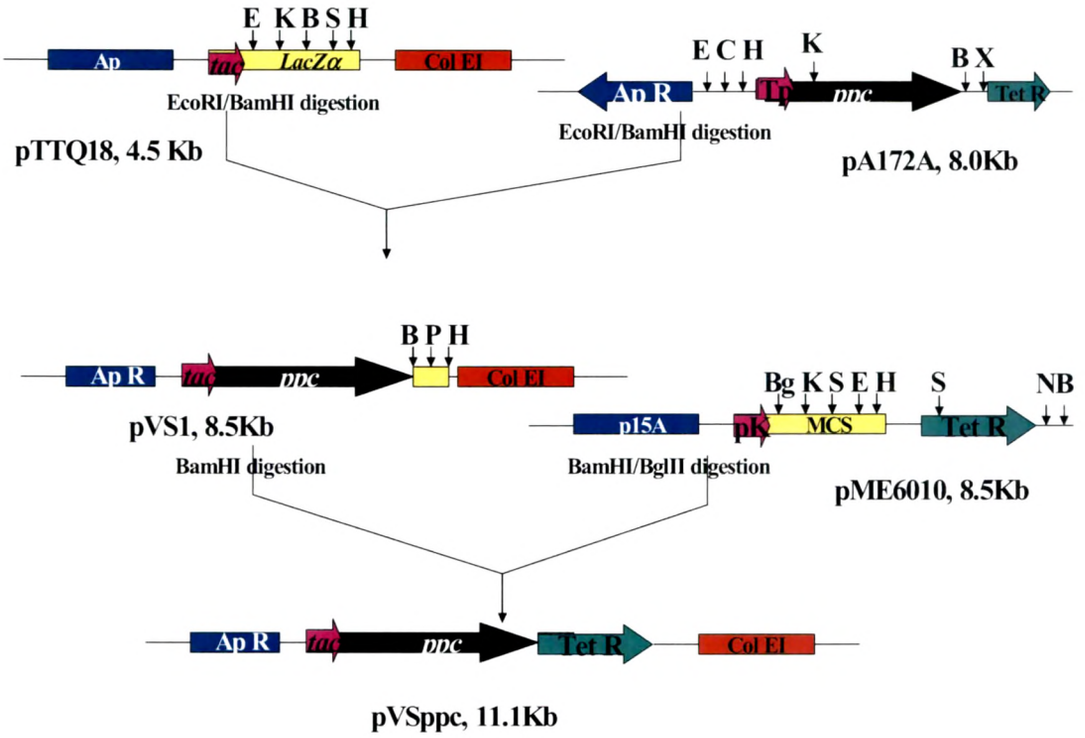
One unit of enzyme activity was defined as the amount of protein required to convert 1 nmole of substrate per minute unless stated.

5.3 RESULTS

5.3.1 Construction of pVSppc containing *ppc* gene from *S. elongatus* PCC 6301

pVSppc was constructed as mentioned in materials and method. The schematic representation of the cloning procedures is depicted in **Fig. 5.2.** Plasmid was confirmed based on restriction digestion pattern shown in **Fig. 5.3 A and B.** Plasmid pVS1 was conformed by digesting the plasmid with BglII/BamHI with 2 sites of BglII present inside the *ppc* gene and 1 BamHI site in the MCS of pTTQ18 to give 2 fragments of 1.9Kb and 6.2 Kb**.** Digestion of pA172-Gm with EcoRI/BamHI gave 2 fragment of 5.5Kb and 4.0Kb. Release of tetracycline resistance fragment from pVSppc digested with BamHI/HindIII. The final restriction map was shown in **Fig. 5.4.**

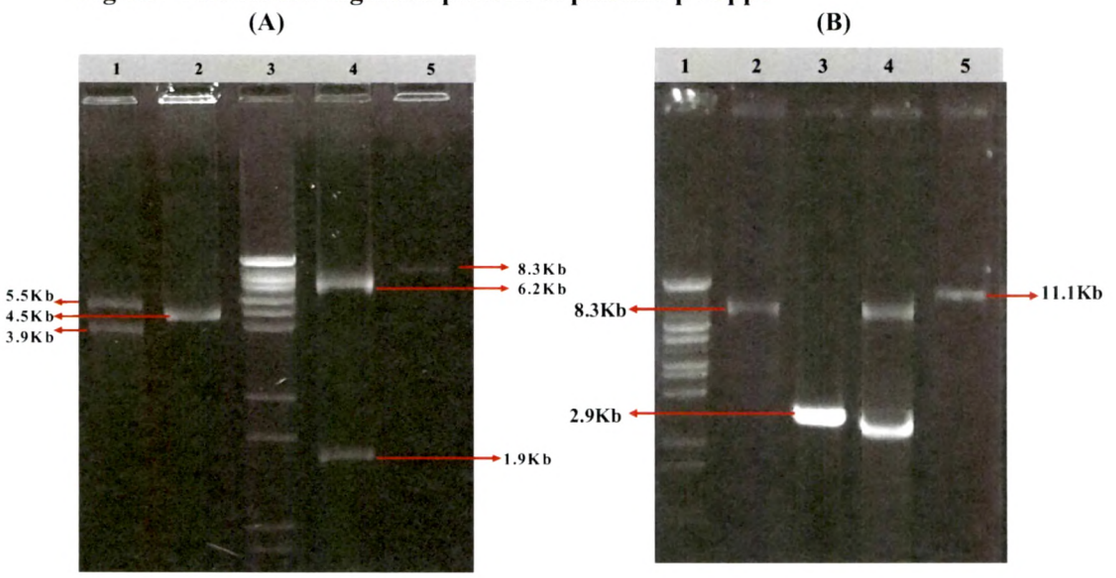
Fig 5.2: Schematic representation of construction of plasmid pVSppc



E=EcoRI; B=BamHI; K= KpnI; H=HindIII; S=SalI; P=PstI; X=XbaI; N=NdeI

tac=tac promoter; Tp=Tetracycline promoter; pK=Kanamycin promoter; Tet R= tetracycline resistance gene; Ap R= Ampicillin resistance gene; ColEI= origin of replication

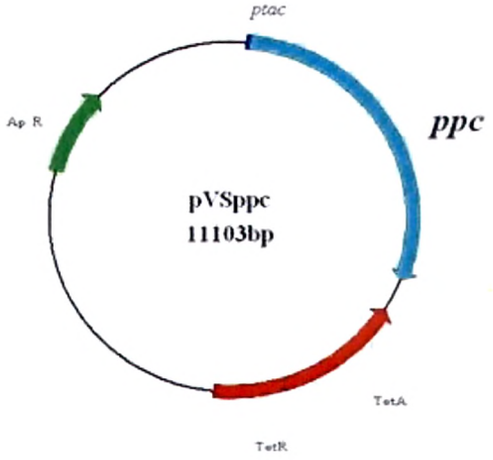
Fig 5.3: Restriction digestion pattern of plasmid pVSppc



A: Lane 1: pA172A-Gm digested with EcoRI/BamHI; Lane 2: pTTQ18 digested with EcoRI/BamHI; Lane 3: Molecular Weight Marker (MWM)-Lambda DNA cut with BstEII; Lane 4: pVS1 digested with BglII/BamHI; Lane 5: pVS1 digested with BamHI

B: Lane 1: Molecular Weight Marker (MWM)-Lambda DNA cut with BstEII; Lane 2: pVS1 digested with BamHI; Lane 3: 2.9Kb purified tetracycline fragment (pME6010 digested with BglII/BamHI); Lane 4: pVSppc digested with BamHI/HindIII; Lane 8: pVSppc digested with HindIII

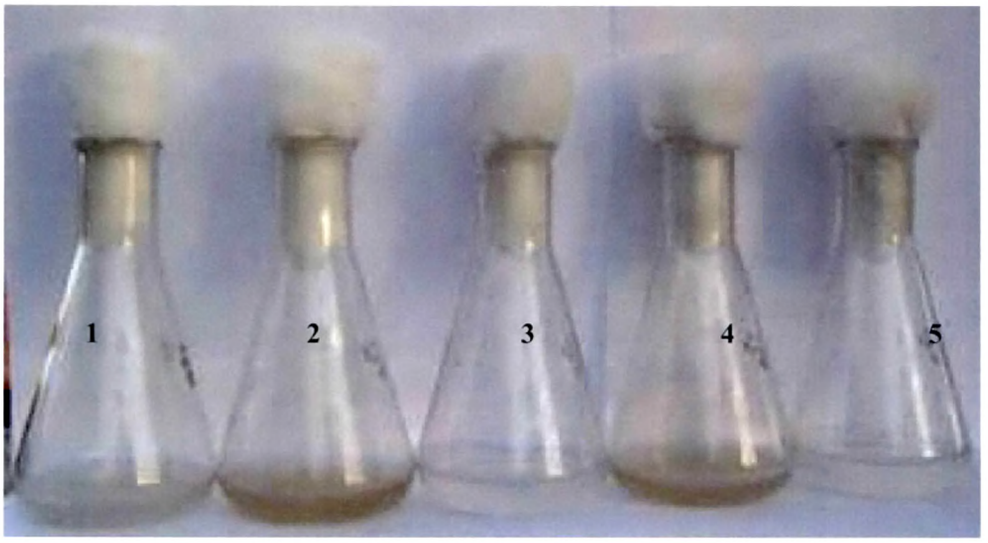
Fig. 5.4: Restriction map of pVSppc



5.3.2 Phenotype complementation of *ppc* mutant *E. coli* (JWK3928) by pVSppc

E. coli (JWK3928) harboring pA172A and pVSppc could complement the *ppc* mutant phenotype as indicated by the growth in the absence of glutamate in the medium. The control lacking either plasmid did not grow in the absence of glutamate. Glutamate supplementation in control could restore the growth (**Fig 5.5**)

Fig. 5.5: Phenotypic complementation of *E. coli* (JWK3928) by pVSppc plasmid

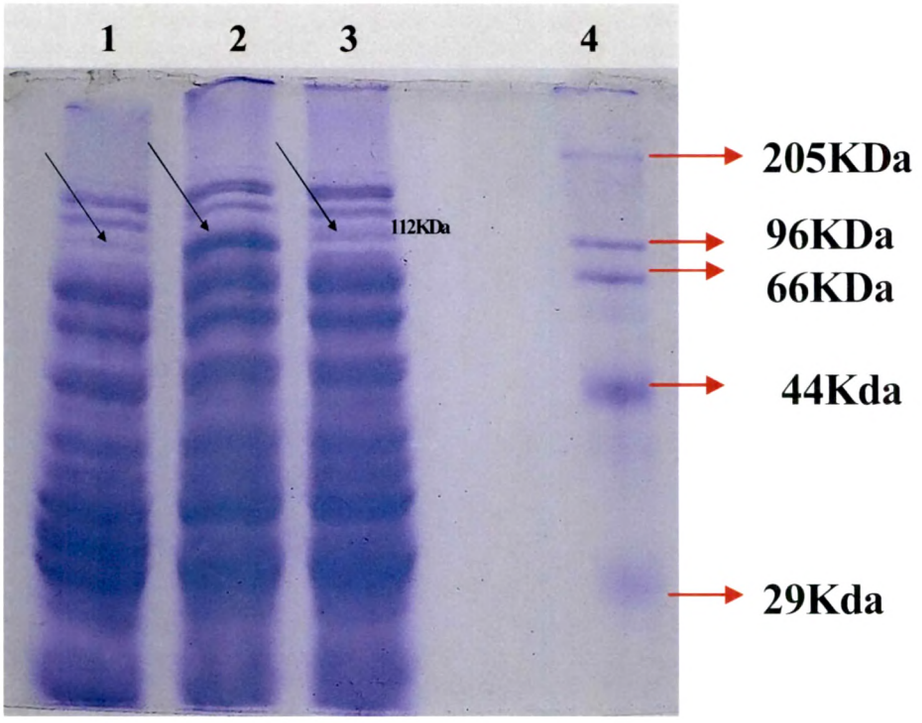


Flasks	1	2	3	4	5
plasmid	pVSppc	pA172A	pVSppc	-	PTTQ18
Glutamate	-	-	-	+	-
IPTG	+	-	-	-	-

5.3.3 Characterization of the expression of the *ppc* gene

SDS-PAGE gel shows protein band of *ppc* gene (114kDa) in *E. coli* DH5α (pVSppc) induced with 0.1mM of IPTG whereas a very faint band is seen in control and uninduced culture (**Fig 5.6**)

Fig. 5.6: SDS-PAGE gel of expression of the *ppc* gene



Lane 1: *E. coli* DH5α (pTTQ18); Lane 2: *E. coli* DH5α (pVSppc) with IPTG induction; Lane 3: *E. coli* DH5α (pVSppc) with out IPTG induction; Lane 4: Molecular Weight Marker (MWM)

5.3.4 PPC activity of *E. asburiae* PSI3 transformants

E. a (pVSppc) showed 46 ± 3.6 U and 30 ± 1.5U of specific PPC activity on glycerol and fructose respectively which were ~4.6 and ~2.2 higher compared to *E. asburiae* PSI3 WT which showed 10U ± 2.7U and 14 ± 2.5U of specific PPC activity on glycerol and fructose respectively. Double transformants of *E. asburiae* PSI3 i.e. *E. a* (pVSppc, pJE2) demonstrated 37 ± 5.3U and 28 ± 3.8U of specific PPC activity on glycerol and fructose respectively. PPC activity was ~3.7 and ~2 fold higher than controls (**Table 5.3**).

Table 5.3: PPC activity of *E. asburiae* PSI3 transformants

E. asburiae PSI3 transformants	FRUCTOSE	GLYCEROL
	nmole per mg of protein. min^{-1}	
E. a (WT)	10 ± 2.7	14 ± 2.5
E. a (pVSppc)	46 ± 3.6	30 ± 1.5
E. a (pVSppc, JE2)	37 ± 5.3	28 ± 3.8

Results are expressed as Mean ± S.D. of six independent experiments

5.3.5 CS activity of *E. asburiae* PSI3 transformants

E. a (pJE2) showed 20.0 ± 2.0U and 62.7 ± 7.1U of specific CS activity on glycerol and fructose respectively, which were ~1.5 and ~2.5 higher compared to *E. a* (pJE1) showing 13.4 ± 0.0U and 25.7 ± 4.0U. Double transformants of *E. asburiae* PSI3 i.e *E. a* (pVSppc, pJE2) demonstrated 18.3 ± 2.1U and 48.0 ± 6.1U of CS specific activity on glycerol and fructose, respectively which were ~1.3 and ~1.9 fold higher compared to its plasmid control which showed 14.2 ± 2.7U and 25.0 ± 0.6U (**Table 5.3**). cs specific activity.

Table 5.4: CS activity of *E. asburiae* PSI3 transformants

E. asburiae PSI3 transformants	Glycerol	Fructose
	nmole per mg of protein. min^{-1}	
E. a (pJE1)	13.4 ± 0.0	25.7 ± 4.0
E. a (pJE2)	20.0 ± 2.0	62.7 ± 7.1
E. a (pJE1, JE3)	14.2 ± 2.7	25.0 ± 0.6
E. a (pVSppc, JE2)	18.3 ± 2.1	48.0 ± 6.1

Results are expressed as Mean ± S.D. of six independent experiments

5.3.6 Growth kinetics of *E. asburiae* PSI3 *cs* and *ppc* transformants on M9 minimal media containing 100mM glycerol and 100mM fructose as sole carbon source.

E. asburiae PSI3 single or double transformants of *cs* and *ppc* when grown on M9 minimal medium with either 100mM of glycerol or fructose could not acidify the

medium till saturation (**Table 5.7).** All transformants of *cs* and *ppc* reached the saturation of growth (~1.9) but at different time points. *E. a* (pVSppc), *E. a* (pJE2)and *E. a* (pVSppc, pJE2) transformants showed better growth on 100mM glycerol as well as on 100mM fructose compared to their controls *E. a* (pJE3), *E. a* (pJE1)and *E. a* (pJE3, pJE1) **(Fig. 5.7 A, B and C)**. On glycerol specific growth rate of *E. a* (pJE2) was ~1.2 fold higher compare to *E. a* (pJE1) and ~3.3 and ~2.7 fold higher compared to *E. a* (pVSppc) and *E. a* (pVSppc, pJE2) respectively (**Table 5.5**). *E. a* (pVSppc) and *E. a* (pVSppc, pJE2) showed similar specific growth rate compared to controls. When grown on fructose specific growth rate of *E. a* (pVSppc) was ~1.6 fold higher compared to plasmid control and ~2 and ~1.2 fold higher to *E. a* (pJE1) and *E. a* (pVSppc, pJE2) respectively (**Table 5.6**). Time taken for all transformants on glycerol was less compared to fructose grown cells. Time taken even with in the glycerol grown transformants was different. For reaching ~1.9 $O.D._{600}$ of *E. a* (pJE1) took 14h compared to 20h for *E. a* (pJE3) and 24h for *E. a* (pJE3, pJE1). Similar results were seen when the transformants were grown on fructose i.e. 21h for *E. a* (pJE1) compared to 27h for *E. a* (pJE3) and 30h for *E. a* (pJE3, pJE1) **(Fig. 5.8 A, B and C).**

Table 5.5: Physiological variables of *E. asburiae* PSI3 and its various transformants grown on M9 medium with 100mM glycerol.

***E. asburiae* PSI3 transformants**	**Sp. growth rate k (h^{-1})[a]**	**Dry cell weight (g L^{-1})[c]**
E. a (pJE1)	0.66 ± 0.07	0.34 ± 0.01
E. a (pJE2)	0.77 ± 0.07	0.33 ± 0.01
E. a (pJE3)	0.27 ± 0.04	0.36 ± 0.01
E. a (pVSppc)	0.24 ± 0.06	0.36 ± 0.01
E. a (pJE1, pJE3)	0.29 ± 0.04	0.33 ± 0.01
E. a (pVsppc, pJE2)	0.29 ± 0.03	0.33 ± 0.01

Results are expressed as Mean ± S.D. of six independent experiments.

Fig. 5.7 Growth profile of *E. asburiae* PSI3 containing *cs* and *ppc* genes alone or in combination with their respective controls on glycerol as sole carbon source

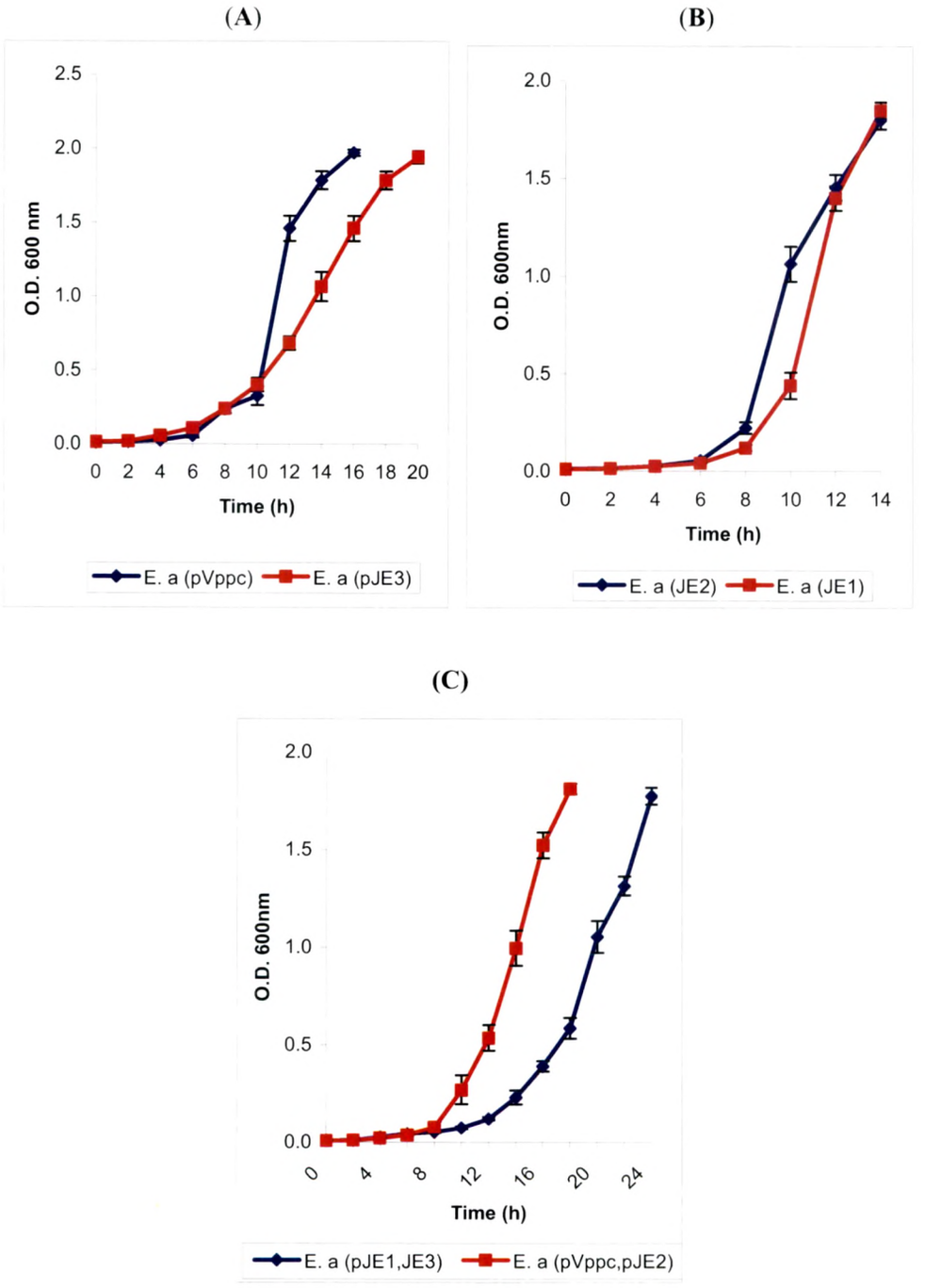


Fig. 5.8: Growth profile of *E. asburiae* PSI3 containing *cs* and *ppc* genes alone or in combination with their respective controls on fructose as a sole carbon source.

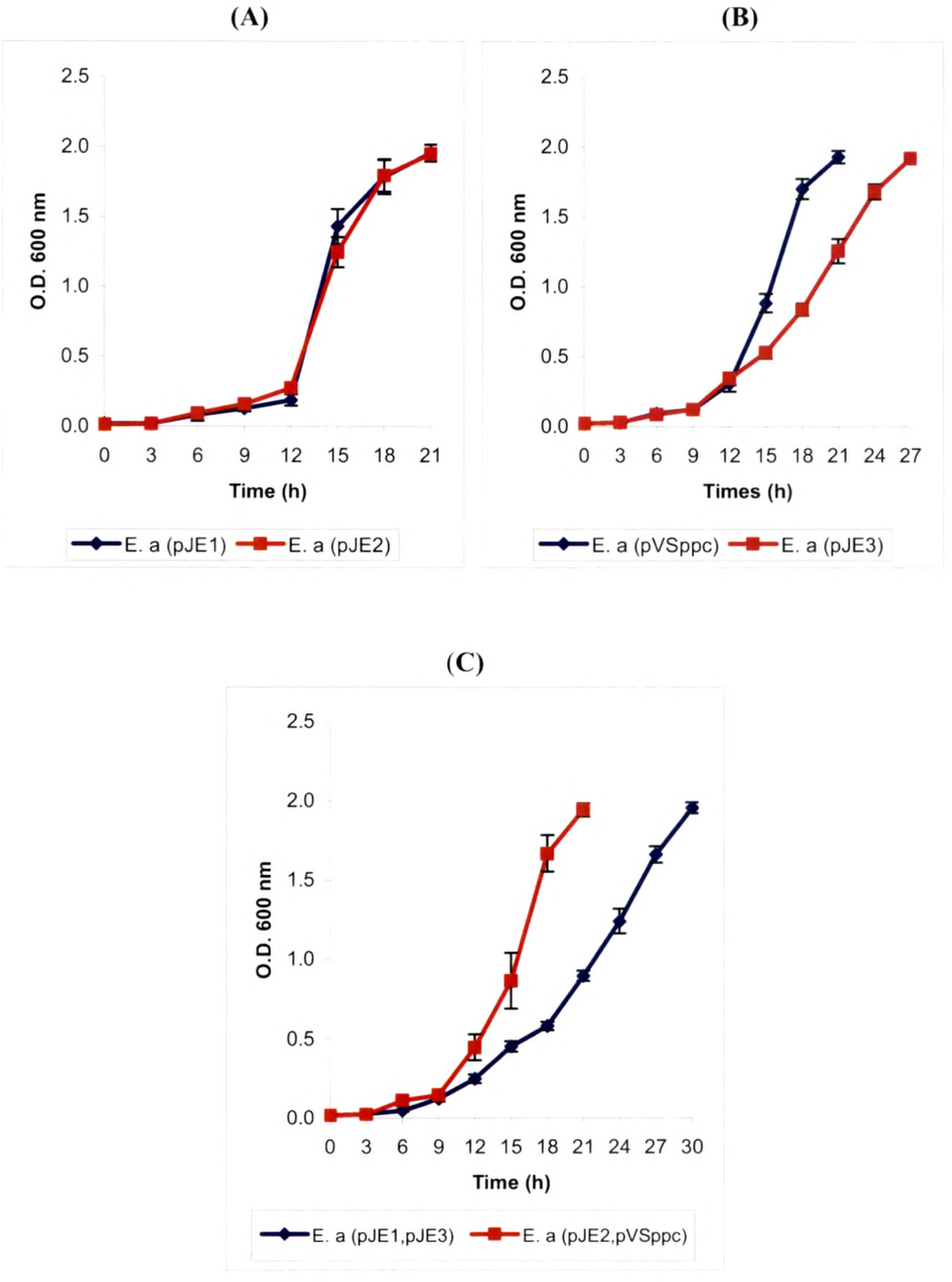


Table 5.6: Physiological variables of *E. asburiae* PSI3 and its various transformants grown on M9 medium with 100mM Fructose.

E. asburiae PSI3 transformants	Sp. growth rate k (h^{-1})[a]	Dry cell weight (g L^{-1})[c]
E. a (pJE1)	0.20 ± 0.03	0.36 ± 0.01
E. a (pJE2)	0.19 ± 0.07	0.36 ± 0.01
E. a (pJE3)	0.24 ± 0.04	0.35 ± 0.01
E. a (pVSppc)	0.39 ± 0.06	0.35 ± 0.01
E. a (pJE1, pJE3)	0.19 ± 0.30	0.36 ± 0.01
E. a (pVsppc, pJE2)	0.31 ± 0.03	0.36 ± 0.01

Results are expressed as Mean ± S.D. of six independent experiments

Table 5.7: pH profile of *E. asburiae* PSI3 transformants

E. asburiae PSI3 transformants	100mM glycerol		100mM fructose	
	Initial pH	Final pH	Initial pH	Final pH
E. a (pJE1)	7.09 ± 0.03	6.95 ± 0.01	7.03 ± 0.02	6.94 ± 0.02
E. a (pJE2)	7.02 ± 0.02	6.94 ± 0.01	7.05 ± 0.01	6.97 ± 0.03
E. a (pJE3)	7.01 ± 0.03	6.99 ± 0.02	7.06 ± 0.02	6.95 ± 0.02
E. a (pVSppc)	7.03 ± 0.02	6.90 ± 0.02	7.03 ± 0.02	6.99 ± 0.02
E. a (pJE1, pJE3)	7.04 ± 0.03	6.94 ± 0.03	7.09 ± 0.01	6.98 ± 0.02
E. a (pVsppc, pJE2)	7.06 ± 0.01	6.95 ± 0.02	7.01± 0.02	6.93 ± 0.02

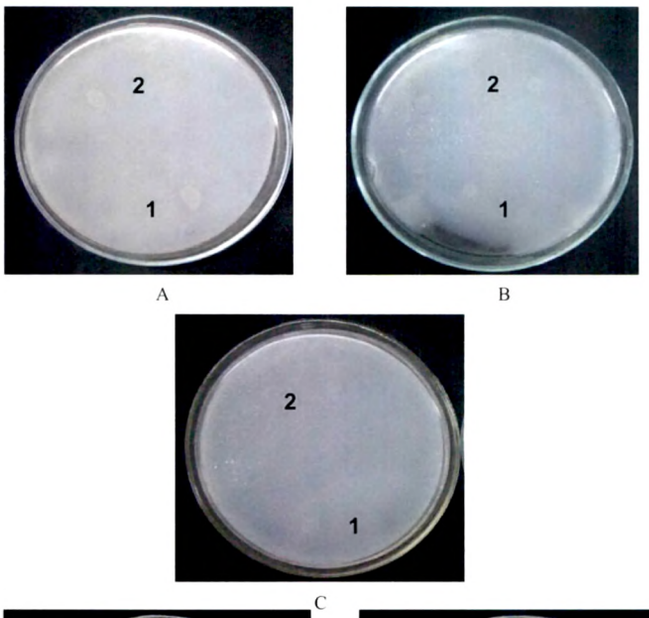
Results are expressed as Mean ± S.D. of six independent experiments

5.3.7 P-Solubilisation ability by plate assay method

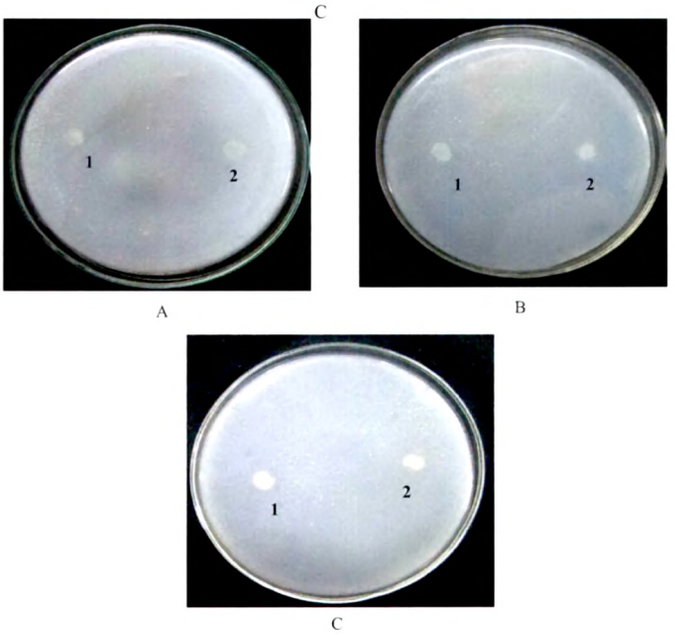
E. asburiae PSI3 single or double transformants of *cs* and *ppc* did not show zone of clearance when grown in presence of either 100mM glycerol or fructose with appropriate antibiotics after growing at 37°C for 5 days (**Fig. 5.9 A and B**).

Fig. 5.9: P solubilisation of *E. asburiae* PSI3 transformants on Tris minimal medium with TCP as sole P source and glycerol and fructose as sole carbon source

Glycerol: A



Fructose: B



P- solubilisation by *E. asburiae* PSI3 containing *cs* and *ppc* plasmids on Tris minimal media with (**A**) glycerol and (**B**) fructose as sole carbon source and TCP as a sole P source (A 1 and 2) *E. a* (pJE1) and *E. a* (pJE2), (B 1 and 2) *E. a* (pJE3) and *E. a* (pVsppc) and (C 1 and 2) *E. a* (pJE1, pJE3) and *E. a* (pJE2, pVSppc)

5.3.8 Organic acid analysis

HPLC analysis of the medium supernatant was done to determine the nature and amount of organic acids secreted by *E. asbiriae* PSI3 and its *cs* and *ppc* transformants. Approximately 2.6 mM (0.15 g L^{-1}) and 2.4 mM (0.14 g L^{-1}) of acetic acid was secreted when fructose and glycerol were used as C source, respectively. *E. asburiae* PSI3 bearing plasmids showed ~ (0.7 fold to ~5.6) fold higher level of acetic acid compared to untransformed cells grown on fructose. There was ~1mM (0.06 g L^{-1}) acetate secretion in *E. asburiae* PSI3 bearing pJE1, pJE3 and (pJE1, pJE3) plasmid on glycerol. On fructose, among the plasmid bearing strains *E. a* (pJE2), *E. a* (pVSppc) and *E. a* (pJE2, pVSppc) showed 5.1mM, 3.8 mM and 5.9 mM of acetic acid which was ~2.7, ~3.8 and ~2.2 fold lesser then their controls. When grown on fructose, ~1.8mM (0.21 g L^{-1}) and 2.3mM (0.27 g L^{-1}) succinic acid was also detected in culture supernatant of *E. a* (pVSppc) and *E. a* (pJE2, pVSppc), respectively.

5.4 DISCUSSION

Present study deals with over expression of *cs* and *ppc* alone and in combination. Attempts were made to genetically modify microorganisms to secrete citric acid which include over expression of *cs* and mutation of *icdh* gene (Ruijter et al., 2000; Kabir and Shimizu, 2004). Over expression of *cs* in *Aspergillus niger* did not result in higher secretion though the *cs* expression was 11 fold higher (Ruijter et al., 2000). On the other hand biomass level decreased, glucose consumption and citric acid yield remained unchanged. Homologous *cs* gene overexpression from *tac* promoter in *E. coli* increased CS activity upto 2 fold but it increased to 50 fold when *cs* gene was expressed from its own promoter on minimal medium containing glucose (Walsh and Koshland, 1985a). Mutant complementation of *E. coli* with *Streptococcus mutans cs* resulted in ~1.3 fold increase (Cvitkovitch et al., 1997). Thus it appears that CS activity is controlled at the expression as well as protein level by complex processes which are not very clear. *cs* over expression in *E. coli* resulted in elimination of acetate production, increase in dry cell weight mass and biomass yield (De Maeseneire et al., 2006). Many reports on *cs* overexpression in plants have resulted in alleviation of aluminium toxicity but it is because of citrate accumulation by *cs* is controversial (de la Fuente et al. 1997, 1998; Koyama et al., 2000; Delhaize et al. 2001; Kihara et al., 2003).

The present study deals with the similar strategy of heterologously over expression of citrate synthase of *E. coli* in *E. asburiae* PSI3. Approximately ~1.5 and ~2.5 fold increase in CS activity was observed on glycerol and fructose respectively compared to controls which was comparable to other reported literature (Cvitkovitch et al., 1997). Citrate was not detected when transformants were grown on either fructose or glycerol but specific growth rate was increased ~1.2 fold in case glycerol grown cells whereas there was no change in fructose grown cells. Approximately 2.4 mM (0.14 g L^{-1}) and 2.6 mM (0.15 g L^{-1}) of acetate was produced in wild type grown on glycerol and fructose respectively. However, there was ~5 fold and ~2 fold increase in acetate levels in *E. asburiae* PSI3 bearing control plasmid and plasmid bearing citrate synthase gene which could be attributed to of high carbon influx for protein synthesis and for maintenance of the plasmid (Shimizu et al. 1988; Seeger et al. 1995; Lin and Neubauer 2000; Wang et al., 2006; De Gelder, 2007). Changes in respiratory activity have been shown to increase due to IPTG induction (Bhattacharya et al. 1997; Lin and Neubauer 2000). However, very low concentration of acetate was detected with cells containing control plasmid grown on glycerol. There was no acetate detected in case of cells bearing *cs* gene. This could be attributed to the fact that glycerol being 3 carbon compound gears the cell towards gluconeogenesis which is in supported by the results of *E. coli* grown cells (Hohmes, 2001; Martínez et al., 2008). Decrease of ~2.7 fold in acetate levels in *cs* overexpressing cells compared to control could be attributed to the fact that CS direct metabolites into the TCA cycle and therefore diminishes the pool of PEP, pyruvate and acetyl CoA, to participate in acetate formation (De Maeseneire et al., 2006).

PPC plays an anapleurotic role in replenishing oxaloacetate (OAA) and keeping the TCA cycle intermediates from depletion (Peng et al., 2004). By converting PEP to OAA, PPC prevents the accumulation of pyruvate and provides OAA, which is further converted by CS. Therefore *ppc* over expression was carried out to lower the pyruvate pool and to overcome the limitation of oxaloacetate which is the precursor for citrate synthesis. Several reports of over expression of *ppc* gene under aerobic conditions has resulted in ~1.8 fold increase in case of *Acinetobacter* sp. (Elbahloul and Steinbuchel, 2006) to ~10 fold increase in case of *Corynebacterium glutamicum* (Cremer et al., 1991; Shirai et al., 2007). Over expression of *ppc* in present study showed ~4.6 and ~2.2 fold higher PPC activity compared to control when grown on

fructose and glycerol respectively. This was in comparison with the report where enzyme was present to a high level when cells were grown under glycolytic conditions and to a low level when grown under gluconegonic conditions (Izui et al., 1981). Over expression of *ppc* resulted in decrease in the acetate secretion by ~3.7 fold supported by many reports where over expression leads to decrease in acetate secretion and increase in the specific growth rate (Chao and Liao, 1993; Farmer and Liao 1997; Gokarn et al. 2001; Lin et al. 2005; De Maeseneire et al., 2006). Specific growth rate in *E. a* (pVSppc) increased ~1.6 fold compared to its plasmid control on fructose grown cells could be attributed to the fact that elevated acetate concentrations are highly detrimental for growth rate and recombinant protein yield (De Anda et al. 2006). On glycerol there is no change in the specific growth rate (**Table 5.5**). The specific growth rate was also high ~2 fold compared to *cs* over expressed cells *E. a* (pJE2) which shows ~1.3 fold higher acetate production. *ppc* over expression in *E. coli* under aerobic conditions increases the flux towards the succinate production by decreasing the pyruvate pool (Farmar and Liao, 1997; Lin et al.., 2004). Low concentration 2.3mM (0.27 g L^{-1}) of succinic acid was detected in *ppc* over expressing cells.

OAA could be a limiting for *cs* activity (Anoop et al., 2003), however till date there are no reports of simultaneous over expression of these two genes in bacteria. Therefore simultaneous over expression of *cs* and *ppc* was carried out for efficient citrate formation. Double transformants *E. a* (pVSppc, pJE2) showed ~2.2 fold decrease in acetate level compared to control on fructose grown cells, which was ~1.5 and ~1.1 fold higher compared to single *ppc* and *cs* transformants. This could be due to over expression of two proteins simultaneously leading to metabolic load on the cells. However, low concentration of acetate (~1mM) was secreted by *E. asburiae* PSI3 bearing control plasmids grown on glycerol which were similar to single transformants. ~1.8mM (0.21 g L^{-1}) of succinic acid was secreted in double transformants which was ~1.3 fold lower compared to *ppc* transformant which could be attributed to channeling of metabolic flux towards citrate synthesis. Specific growth rate on glycerol did not change much compared to single *ppc* tranformants. On fructose the rate ~1.6 fold higher compared to control and little lower compared to single tranformants which could be due to increased acetate secretion due to metabolic load caused by two antibiotic resistance gene product. No citrate was

detected in the supernatant indication that the metabolism of *E. asburiae* PSI3 does not change much with over expression of these two enzymes and apart from over expression suppression of enzymes lower in cycle showed be attempted as been reported (Lakshmi and Helling, 1976; Matsuno et al., 1999; Kabir and Shimizu, 2004).