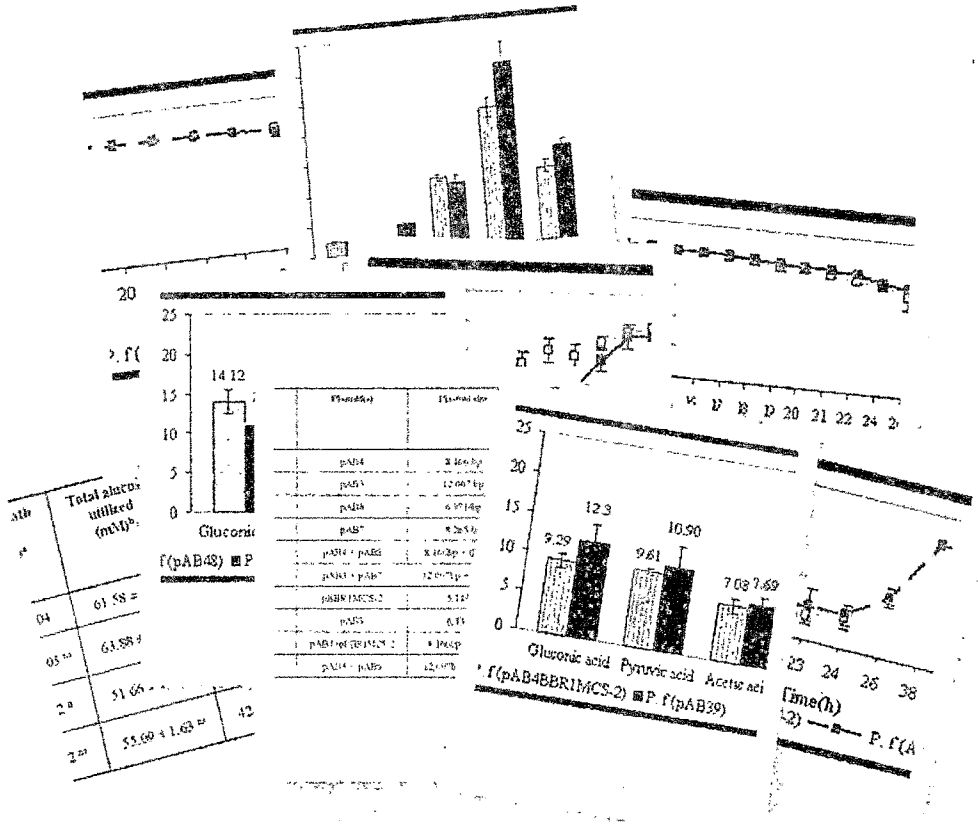

CHAPTER 6

Effect of simultaneous overexpression of *ppc-cs* genes on glucose metabolism of *P. fluorescens* ATCC 13525

"When you make the finding yourself - even if you're the last person on Earth to see the light - you'll never forget it."

-Carl Sagan

6.1: INTRODUCTION

Aspergillus niger (fungus) and *Candida* spp. (yeast) are known to secrete high amounts of citric acid, a phenomenon which is resultant of significant morphological, cytological and physiological changes largely governed at glycolysis and TCA cycle (Anastassiadis and Rehm, 2005; Papagianni, 2007). On the other hand, bacterial citric acid production quantitatively occurs at much lesser extent and is limited to a few strains of *Bacillus*, *Bradyrhizobium, Citrobacter* and *E. coli icd* mutants (Lakshmi and Helling, 1976; Carson et al., 1992; Gyaneshwar et al., 1998; Aoshima et al., 2003; Khan et al., 2006). *Bradyrhizobium* secretes citric acid in response to iron deprivation and also acts as a siderophore for iron uptake (Carson et al., 1992). Results in Chapter 5 of this study demonstrated ~25 fold increase in aerobic citric acid yield of *P. fluorescens* 13525 in response to 2 fold overexpression of *E. coli* citrate synthase (*cs*) gene. According to these results, increase in CS activity and citrate accumulation was accompanied by increased PYC activity in *Pf* (pAB7) which suggested increase in cellular demands for OAA. Similar reports for citric acid-accumulating *A. niger* cells demonstrate that the enhancement of anaplerotic reactions replenishing TCA cycle intermediates predisposes the cells to form citric acid (Legisa and Mattey, 2007).

PYC catalyzes the biosynthesis of OAA by fixing carbon dioxide into pyruvate at the expense of ATP. PPC is an alternate enzyme which can also synthesize OAA using PEP as a substrate instead of pyruvate. OAA biosynthesis catalyzed by PPC would be energetically preferable for the cell as it releases free Pi and is ATP-independent. Hence, an attempt was made to fulfill the additional requirement of OAA level arising during citric acid overproduction as a consequence of *cs* overexpression in *P. fluorescens* 13525, by simultaneously overexpressing *Synechococcus elongatus* PCC 6301 *ppc* gene. The detailed effects of ~12 fold enhancement in PPC activity on glucose metabolism of *P. fluorescens* 13525 has been discussed in Chapter 3. Negligible PPC activity in *Pf* (pAB4) suggested that PYC was the major anaplerotic enzyme and heterologous *ppc* overexpression thus would divert the glucose flux through otherwise negligible pathway.

The present work dealt with studying the effects of simultaneous overexpression of *ppc* and *cs* genes on overall glucose metabolism and physiology of *P. fluorescens* 13525 as well its effect on citric acid secretion.

6.2: EXPERIMENTAL DESIGN

6.2.1: Bacterial strains used in this study

Pseudomonas Strains	Relevant characteristics	Reference
Pf (pAB3)	*P. fluorescens* 13525 with pAB3 plasmid; Tc^r	Chapter 3
Pf (pAB4)	*P. fluorescens* 13525 with pAB4 plasmid; Tc^r	Chapter 3
Pf (pAB48)	*P. fluorescens* 13525 with pAB4 and pAB8; Km^r, Tc^r	This study
Pf (pAB37)	*P. fluorescens* 13525 with pAB3 and pAB7; Km^r, Tc^r	This study
Pf (pAB39)	*P. fluorescens* 13525 with pAB3 and pAB9 Km^r, Tc^r	This study
Pf (pAB4 BBR1MCS-2)	*P. fluorescens* 13525 with pAB4 and pBBR1MCS-2; Tc^r, Km^r	This study

Table 6.1: List of bacterial strains used. *P. fluorescens* 13525 double transformants were developed as described in text (Section 6.2.2). Detailed characteristics of these strains and plasmids are given in Section 2.1; 3.2.1 and 5.2.1. *P. fluorescens* 13525 transformants were grown at 30°C with tetracycline and kanamycin as and when required, at final concentrations varying for rich and minimal media (Section 2.2).

6.2.2: Development of *P. fluorescens* 13525 harboring *S. elongatus* PCC 6301 *ppc* and *E. coli cs* genes

The recombinant plasmids pAB7 and pAB9 were independently transformed in *Pf* (pAB3) (Section 2.4.2.2) to obtain *Pf* (pAB37) and *Pf* (pAB39), respectively. These resultant double transformants carried two plasmid pAB3 and pAB7/pAB9 harboring *ppc* and *cs* genes respectively. Similarly, pAB8 and pBBR1MCS-2 control plasmids were transformed in *Pf* (pAB4) to obtain respective double transformant controls *Pf* (pAB48) and *Pf* (pAB4BBR1MCS-2). All the double transformants were selected on tetracycline and kanamycin plates and were confirmed by fluorescence (Section 2.2; 2.3). The copy number of these plasmids in the *Pf* (pAB37), *Pf* (pAB48), *Pf* (pAB39) and *Pf* (pAB4BBR1MCS-2) was estimated as described in Section 2.13.

6.2.3: Determination of physiological and biochemical parameters

P. fluorescens 13525 double transformants were studied for growth and organic acid production profiles on M9 minimal medium with 100mM glucose as carbon source

(Section 2.2.3 and 2.9.2). The samples withdrawn at regular interval were analyzed for $O.D._{600nm}$, pH, extracellular glucose and organic acid (Section 2.9.3). Culture harvested at the time of pH drop was also subjected to intracellular citric acid estimation (Section 2.11.3). The physiological parameters were calculated as in Section 2.9.3. PPC, PYC, G-6-PDH and GDH were assayed in mid-log to late-log phase cultures while CS, ICL and ICDH activities were determined in the stationary phase cells (Section 2.10).

6.3: RESULTS

6.3.1: Estimation of plasmid copy number in *ppc* and *cs* transformants of *P. fluorescens* 13525

Copy numbers of pUCPM18 based plasmids pAB4, pAB3, pAB8 and pAB7 in comparison with pBBR1MCS-2 and its derivative pAB9, in single and double transformants of *P. fluorescens* 13525 are given in **Table 6.2.**

Pseudomonas Strain	Plasmid(s)	Plasmid size	Estimated Copy number	Estimated Total Plasmid DNA Content (kb)
Pf(pAB4)	pAB4	8,166 bp	14±4	~ 114
Pf(pAB3)	pAB3	12,007 bp	10±3	~120
Pf(pAB8)	pAB8	6,971 bp	16±4	~112
Pf(pAB7)	pAB7	8,265 bp	14±2	~116
Pf(pAB48)	pAB4 + pAB8	8,166bp + 6,971bp	8±2 + 10±3	~135
Pf(pAB37)	pAB3 + pAB7	12,007bp + 8,265bp	6±1 + 9±3	~146
Pf(pBBR1MCS-2)	pBBR1MCS-2	5,144bp	31±3	~159
Pf(pAB9)	pAB9	6,438bp	29±5	~186
Pf(pAB4BBR1MCS-2)	pAB4+pBBR1MCS-2	8,166bp + 5,144bp	10±3 + 23±4	~200
Pf(pAB39)	pAB3 + pAB9	12,007bp+ 6,438bp	8±2 + 21±5	~228

Table 6.2: Estimated copy numbers of various plasmids in *P. fluorescens* 13525. The plasmid copy numbers in all *P. fluorescens* 13525 transformants were calculated as described in Section 2.13

Copy numbers of pUCPM18 based plasmids pAB4, pAB3, pAB7 and pAB8 in *P. fluorescens* 13525 ranges 10-16. Unlike these plasmids, pBBR1MCS-2 based plasmids including pAB9 have comparatively higher copy number of ~30 in *P. fluorescens* 13525. On the other hand, in the *P. fluorescens* 13525 double transformants *Pf* (pAB48) and *Pf* (pAB37), the copy number of each plasmid reduced to almost half of that estimated in their respective single transformants. Similarly, the copy numbers of plasmid in *Pf* (pAB4BBR1MCS-2) and *Pf* (pAB39) also reduced as compared to their respective single transformants but to a lesser extent. Based on the varying sizes of all the plasmids, the total amount of DNA present in each transformant has been calculated (**Table 6.2**) which indicates that pBBR1MCS-2 based plasmids contain slightly higher amount of DNA than those containing pUCPM18 based plasmids.

6.3.2: Simultaneous overexpression of *Synechococcus elongatus* PCC 6301 *ppc* and *E. coli cs* genes in *P. fluorescens* 13525

Pf (pAB37) demonstrated 12.79 ± 0.86U PPC and 97.69 ± 3.23U CS activities which were ~5.2 and ~1.4 fold higher than the control *Pf* (pAB48). On the other hand, *Pf* (pAB39) demonstrated 19.00 ± 1.17U PPC and 63.14 ± 4.42U CS activities which were ~5.1 and ~1.2 fold higher than the control *Pf* (pAB4BBR1MCS-2).

6.3.3: Effect of simultaneous *ppc-cs* overexpression on physiological properties of *P. fluorescens* 13525

Both *Pf* (pAB37) and the control *Pf* (pAB48) had similar growth profiles on excess glucose and could acidify the extracellular medium within 30h (**Fig. 6.1**). The physiological variables like specific growth rate, specific total glucose utilization rate, biomass yield, total amount of glucose utilized and glucose consumed after 30h in *Pf* (pAB37) remained unaffected as compared to the control *Pf* (pAB48) (**Table 6.3**). Similarly, *Pf* (pAB39) showed unaltered growth profile as compared to its control *Pf* (pAB4BBR1MCS-2) and both acidified the extracellular medium within 42h. All the above mentioned physiological parameters in *Pf* (pAB39) were unchanged as compared to *Pf* (pAB4BBR1MCS-2).

However there were significant differences found between the physiological parameters amongst the controls *Pf* (pAB48) and *Pf* (pAB4BBR1MCS-2). The specific growth and total glucose utilization rates of *Pf* (pAB4BBR1MCS-2) were lower by ~2

and 3.5 fold, respectively, as compared to *Pf* (pAB48). Concomitantly total glucose utilized reduced by ~1.2 fold. Glucose consumption and biomass yield in both *Pf* (pAB4BBR1MCS-2) and *Pf* (pAB48) was similar.

6.3.4: Effect of *ppc-cs* overexpression on the biochemical properties of *P. fluorescens* 13525

(i) Organic acid secretion

At the end of 30h and 42h respectively, gluconic, pyruvic and acetic acids levels and yields in the extra-cellular culture supernatant of *Pf* (pAB37) and *Pf* (pAB39) were unaltered as compared to their respective controls, *Pf* (pAB48) and *Pf* (pAB4BBR1MCS-2) (**Fig. 6.2 a, b, e, f**). On the other hand, while the extracellular citric acid levels and yields in *Pf* (pAB37) remained unaltered as compared to *Pf* (pAB48) (**Fig. 6.2c, d**); no citric acid was detected in the culture supernatants of *Pf* (pAB39) and *Pf* (pAB4BBR1MCS-2). Therefore, intracellular citric acid levels were estimated only in *Pf* (pAB48) and *Pf* (pAB37) and were demonstrated to be unaltered.

Amongst the controls *Pf* (pAB48) and *Pf* (pAB4BBR1MCS-2), gluconic, pyuvic and acetic acids levels and yields were similar. Citric acid was not at all detected in *Pf* (pAB4BBR1MCS-2) as mentioned above; while its levels in *Pf* (pAB48) were significantly high.

(ii) Alterations in PYC, GDH, G-6-PDH, ICDH and ICL activities.

Activities of key enzymes of glucose catabolism in *Pf* (pAB37) and *Pf* (pAB39) were assayed in response to the *ppc* and *cs* overexpression in order to correlate with the observed organic acid profile. All enzyme activities including GDH, G-6-PDH, PYC, ICDH and ICL remained unaltered in *Pf* (pAB37) and *Pf* (pAB39) as compared to their respective controls, *Pf* (pAB8) and *Pf* (pAB4BBR1MCS-2) (**Fig. 6.3**). As demonstrated in earlier chapters (Sections 3.3.8 and 5.3.5) PPC and ICL activities were negligible in all these transformants.

Amongst controls, *Pf* (pAB4BBR1MCS-2) exhibited ~4.8, 1.4, 1.4, 1.2 and 3.2 fold reduced GDH, PYC, CS, G-6-PDH and ICDH activities, respectively, as compared to that of *Pf* (pAB48).

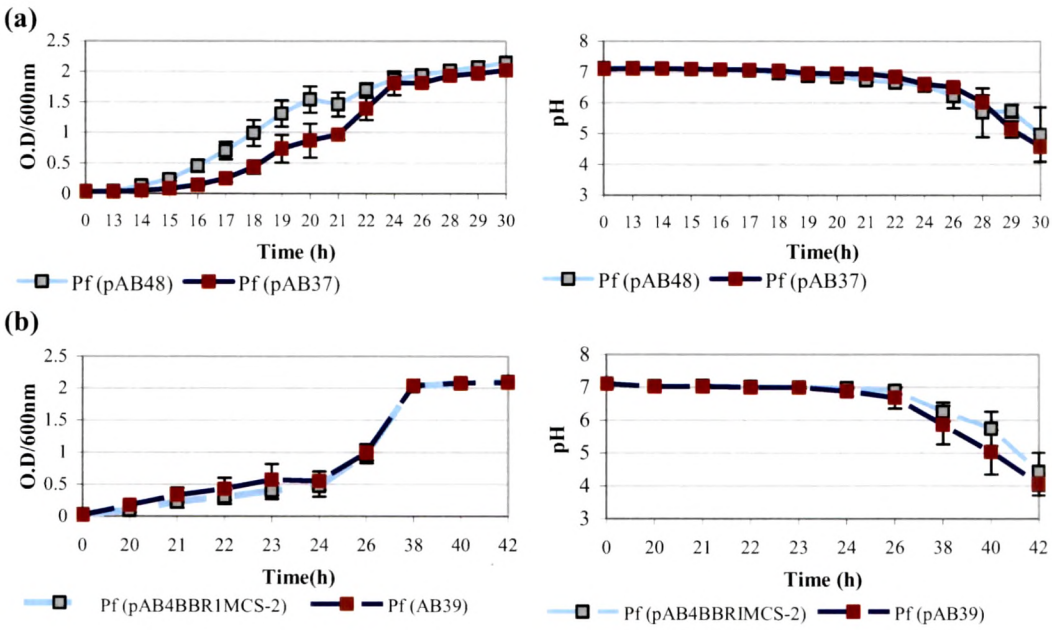

Fig. 6.1: Growth and pH profiles of *ppc-cs* transformants of *P. fluorescens* 13525. The growth and media acidification of **(a)** *Pf* (pAB48)/*Pf* (pAB37) and **(b)** *Pf* (pAB4BBR1MCS-2)/*Pf* (pAB39) was studied on M9 minimal medium with 100mM glucose (Section 2.2.3, 2.9). The values plotted as Mean±S.D of 6-8 independent observations.

***Pseudomonas* Strains**	**Sp. growth rate k (h^{-1})[a]**	**Total glucose utilized (mM)[b]**	**Glucose consumed (mM)[b]**	**Biomass yield $Y_{dcw/Glc}$ [a] (g /g)**	**Sp. glucose utilization rate Q_{Glc} [a] (g.g $dcw^{-1}.hr^{-1}$)**
***Pf* (pAB48)**	0.68 ± 0.04	61.58 ± 3.61	47.45 ± 3.93	0.14 ± 0.02	8.30 ± 1.57
***Pf* (pAB37)**	0.69 ± 0.03 ^{ns}	63.88 ± 2.56 ^{ns}	52.67 ± 3.05 ^{ns}	0.13 ± 0.02 ^{ns}	8.32 ± 1.32 ^{ns}
***Pf* (pAB4BBR1MCS-2)**	0.41±0.02 $^{\alpha}$	51.66 ± 1.54 $^{\gamma}$	42.37± 4.58 $^{ns'}$	0.08 ± 0.02 $^{ns'}$	3.82 ± 0.81 $^{\alpha}$
***Pf* (pAB39)**	0.38±0.02 ^{ns}	55.09 ± 1.63 ^{ns}	42.16 ± 4.14 ^{ns}	0.10 ± 0.01 ^{ns}	4.17 ± 0.81 ^{ns}

Table 6.3: Physiological variables and metabolic data from *ppc-cs* transformants of *P. fluorescens* 13525. The transformants were grown on 100mM glucose in M9 minimal medium (Section 2.2.3, 2.9). The results are expressed as Mean ± S.E.M of readings from 4-6 independent observations. [a]Biomass yield $Y_{dcw/Glc}$, specific growth rate (k) and specific glucose consumption rate (Q_{Glc}) were determined from mid log phase cultures. [b] Total glucose utilized and glucose consumed were determined at the time of pH drop (28h) as in Section 2.9.3. $^{\alpha}P<0.001$; $^{\beta}$ $P<0.01$; $^{\gamma}$ $P<0.05$; ns, ns'=non-significant. $^{\alpha, \gamma, ns'}$ denote comparison between *Pf* (pAB48) and *Pf* (pAB4BBR1MCS-2).

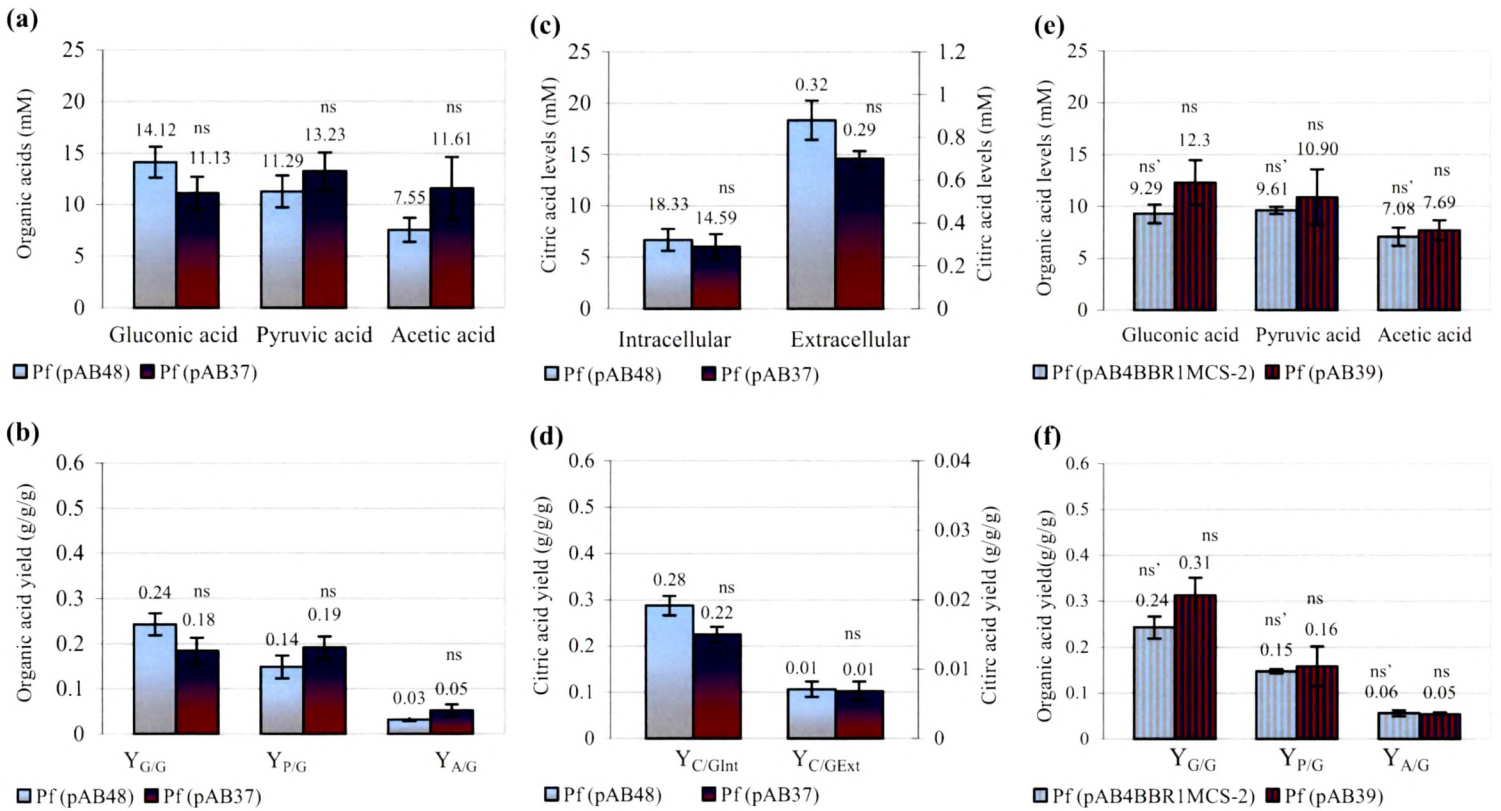

Fig. 6.2: Organic acid production from *P. fluorescens* 13525 overexpressing *ppc* and *cs* genes. (a) Gluconic, pyruvic, acetic and citric acids levels and **(b)** yields ($Y_{G/G}$ $Y_{P/G}$, $Y_{A/G}$ and $Y_{C/G\ ext}$ $Y_{C/G\ int}$) in *Pf* (pAB37), *Pf* (pAB48), *Pf* (pAB39) and *Pf* (pAB4BBR1MCS-2) {no citric acid detected in the latter two}. All organic acids are estimated from stationary phase cultures (at the time of pH drop) grown on M9 medium with 100mM glucose. Results are expressed as Mean ±S.E.M of 4-6 independent observations. ns, ns'= non-significant. ns- comparison with respective controls; ns'-comparison of two controls *Pf* (pAB4BBR1MCS-2) and *Pf* (pAB48).

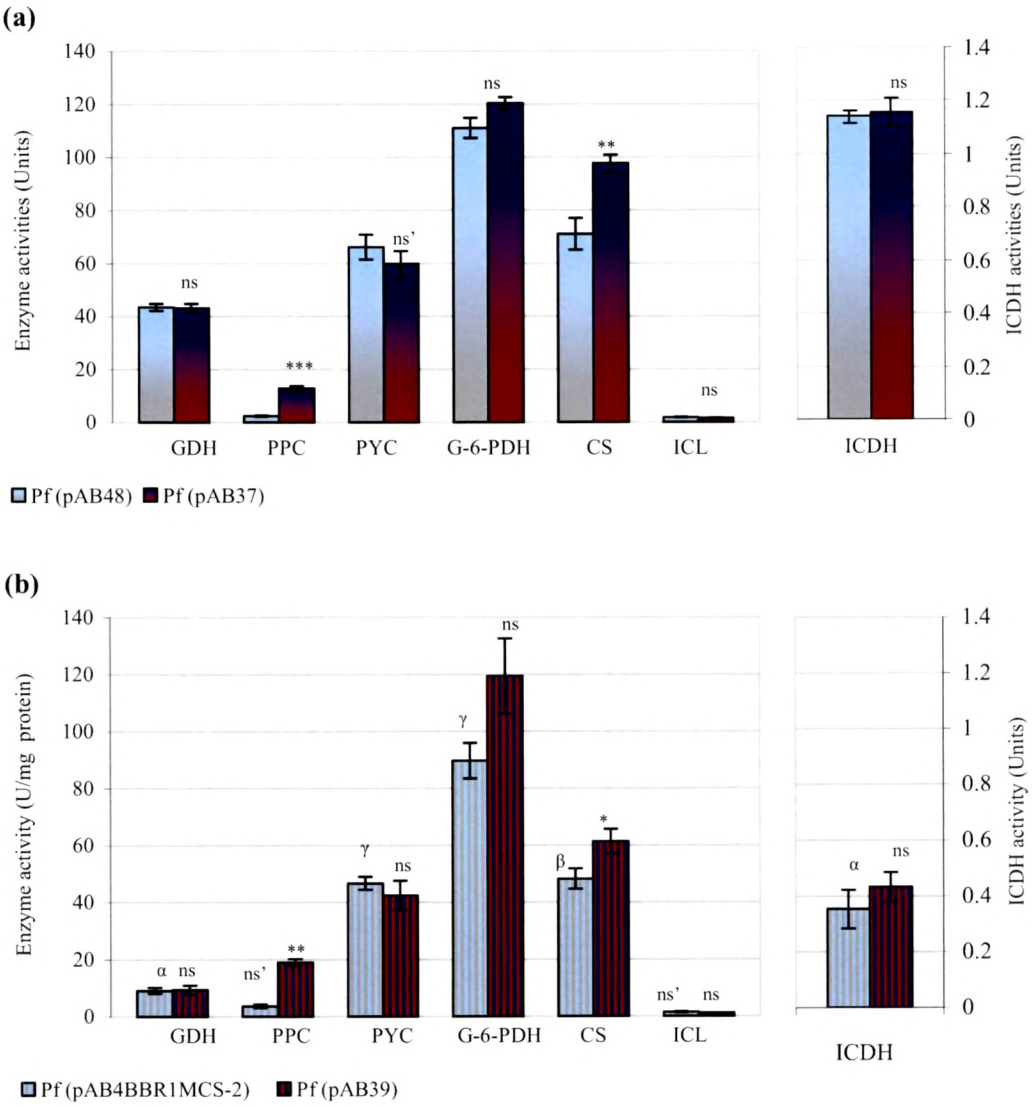

Fig. 6.3: Activities of enzymes CS, PPC, PYC, GDH, G-6-PDH, ICDH and ICL in *P. fluorescens* 13525 simultaneously expressing *ppc* and *cs* genes. The activities have been estimated using **(a)** *Pf* (pAB37), *Pf* (pAB48) and **(b)** *Pf* (pAB39), *Pf* (pAB4BBR1MCS-2) cultures grown on M9 medium with 100mM glucose (Section 2.2.3, 2.9). All the enzyme activities were estimated from mid-log phase to late log-phase cultures except CS, ICDH and ICL which were estimated in stationary phase. All the enzyme activities are represented in the units of nmoles/min/mg total protein, except ICDH activity which is depicted in the units of µmoles/min/mg total protein. The values are depicted as Mean ± S.E.M of 6-8 independent observations. ***,α P<0.001, ** P<0.01, *,β P<0.05, ns, ns'= non-significant. *Comparison of parameters with respective controls; α, β Comparison between parameters of two controls *Pf* (pAB8) and *Pf* (pBBR1MCS-2).

6.4: DISCUSSION

The present study is a continuation of the work discussed in Chapter 5 and describes yet another set of genetic modifications employed for improving the citric acid secretion demonstrated in *P. fluorescens* 13525. This study describes the influence of plasmid copy numbers on the metabolism of *P. fluorescens* 13525. pUCPM18 based plasmid controls, pAB4 and pAB8, replicate using the origin of pRO1614 which is similar to *ori* of *P. aeruginosa* (West et al., 1994). The copy number of these plasmids in *P. fluorescens* 13525 was found to be 10-16 and was independent of the nature of antibiotic resistance. These values are similar to the reported value of 13 copies of pRO1614 replicon based vectors in *P. aeruginosa* (Olsen et al., 1982; Farinha and Kropinski, 1990). Of the pUCPM18 based plasmids, pAB3 plasmid containing *ppc* gene had lowest copy number probably due to its largest size of about 12kb (**Table 6.2**). Increase in plasmid size is known to reduce plasmid copy number in *E. coli* (Smith and Bidochka, 1998). Presence of *ppc* and *cs* genes which constitutively overexpress did not affect the plasmid copy number.

On the other hand, pBBR1MCS-2 with a different origin of replication has comparatively higher copy number in *P. fluorescens* 13525 which is in accordance with the reported moderate copy number for this plasmid in *P. putida* (Pérez-Pantoja et al., 2000). In *Pf* (pAB48) and *Pf* (pAB37) since both the plasmids have same origin of replication, simultaneous presence of two pUCPM18 based plasmids redistributed the copy numbers of each plasmid so as to maintain similar amount of total DNA as in respective single transformants. Similar redistribution of plasmid copy numbers was also seen in *Pf* (pAB39) and *Pf* (pAB4BBR1MCS-2); however, the compatibility of pBBR1MCS-2 with pUCPM18 is not demonstrated.

Interestingly, the presence of one/two plasmids per se {as compared between controls *Pf* (pAB4), *Pf* (pAB8), *Pf* (pAB48), *Pf* (pBBR1MCS-2) and *Pf* (pAB4BBR1MCS-2) only} affected the basal central carbon metabolism as reflected by comparison of all the physiological and biochemical parameters of all the controls used in this study (**Table 6.4**). *E. coli* BL21 glucose metabolism was significantly affected due to presence of pOri1 and pOri2 plasmids which contained one and two ColE1 regions, and resultant copy numbers of 50 and 410 respectively (Wang et al., 2006). But

Parameter	*Pf* (pAB4)	*Pf* (pAB8)	*Pf* (pAB48)	*Pf* (pBBR1MCS-2)	*Pf* (pAB4 BBR1MCS-2)
Physiological parameters					
Sp. growth rate k (h^{-1})	0.75±0.04	0.69±0.04 ns	0.68±0.04 ns, ns'	0.33±0.03 1	0.41±0.02 ***, ns''
Total glucose utilized (mM)	55.90±2.86	66.88±3.84 *	61.58±3.61 ns, ns'	52.12±2.96 3	51.66±1.54 ns, ns''
Glucose consumed (mM)	49.61±2.63	60.02±2.27 *	47.45±3.93 ns, 2	45.50±2.21 2	42.37±4.58 ns, ns''
Biomass yield $Y_{dcw/Glc}$ (g /g)	0.10±0.01	0.15±0.02 ns	0.14±0.02 ns, ns'	0.10±0.02 ns	0.08±0.02 ns, ns'
Sp. glucose utilization rate Q_{Glc} (g.g $dcw^{-1}.hr^{-1}$)	10.10±1.43	7.10±0.74 ns	8.30±1.57 ns, ns'	2.63±0.62 1	3.82±0.81 ***, ns''
Biochemical parameters					
GDH	18.65±1.05	22.05±1.93 ns	43.4±1.33 ***, 1	6.92±0.80 1	9.12±1.31 **, ns''
G-6-PDH	150.82±6.46	85.02±5.23 ***	111.04±3.81 ***, 1	91.51±4.63 ns'	89.71±6.31 **, ns''
PYC	48.48±3.38	54.62±3.26 ns	66.80±4.74 **, ns'	45.02±3.89 ns'	46.69±3.61 ns, ns''
CS	70.48±2.13	51.41±3.06 ***	71.08±6.01 ns, 3	36.47±3.62 3	48.22±4.02 **, ns''
ICDH	1.24±0.07	1.09±0.09 ns	1.14±0.03 ns, ns'	0.24±0.07 1	0.35±0.04 ***, ns''
$Y_{G/G}$	0.17±0.01	0.13±0.04 ns	0.24±0.02 *, 3	0.14±0.04 ns'	0.24±0.02 ns, ns''
$Y_{P/G}$	0.32±0.04	0.13±0.03 **	0.14±0.03 *, ns'	0.11±0.01 ns'	0.15±0.01 *, ns''
$Y_{A/G}$	0.11±0.02	0.05±0.01 *	0.03±0.01 *, ns'	0.03±0.01 ns	0.06±0.01 ns, ns''
$Y_{C/G\ Int}$	0.30±0.03	0.15±0.02 ***	0.28±0.02 ns, 1	ND	ND
$Y_{C/G\ Ext}$	0.013±0.001	0.001±0.0002 ***	0.01±0.001 **, 1	ND	ND

Table 6.4: Comparison of physiological and biochemical parameters of *P. fluorescens* 13525 harboring various plasmids. Data for *Pf* (pAB4), *Pf* (pAB8) and *Pf* (pBBR1MCS-2) has been re-mentioned for the sake of convenient comparison. *, ns denotes comparison with *Pf* (pAB4); 1,2,3,ns' denote comparison with *Pf* (pAB8); ns'' denote comparison with pBBR1MCS-2. ***, 1 = P<0.001; **, 2 = P<0.01; *, 3 = P<0.05; ns, ns', ns''= non-significant.

Pf (pAB48) had same *ori* (pRO1614) on two independent plasmids unlike pOri2 which had 2 ColE1 *ori* regions on the same plasmid. On the contrary *Pf* (pAB4BBR1MCS-2) contains two different *ori* (pRO1614 and pBBR1) on two different plasmids, the compatibility for which is not clear so far as mentioned above.

Although growth rate, glucose consumption rate, total glucose utilization and biomass yield were unaffected in *Pf* (pAB48) as compared to *Pf* (pAB8) and *Pf* (pAB4), relative glucose distribution between direct oxidative and intracellular phosphorylative catabolic pathways was affected. Contribution of phosphorylative pathway in *Pf* (pAB48) reduced to 77% of the total glucose utilized as compared to ~90% in *Pf* (pAB4) and *Pf* (pAB8), respectively. Increased contribution of direct oxidation pathway in *Pf* (pAB48) correlated with significant increase in GDH activity (~2 fold) and gluconic acid yield (~1.4-2 fold) as compared to *Pf* (pAB8) and *Pf* (pAB4). Increased direct oxidation pathway might be advantageous in the presence of increased plasmid load in *Pf* (pAB48) as it is directly coupled to electron transport chain (ETC) by the virtue of electron transfer through co-factor PQQ, which might indirectly help ATP generation and benefit growth (van Shie et al., 1985). These results are not comparable with *E. coli* as it lacks the direct oxidation pathway. G-6-PDH activity in *Pf* (pAB48) was higher than in *Pf* (pAB8) but lower than *Pf* (pAB4) but this trend does not correlate with their similar growth rates as in *E. coli* (Wolf et al., 1979).

PYC activity in *Pf* (pAB48) was significantly higher than in *Pf* (pAB4) but was similar to that in *Pf* (pAB8). This variation in PYC activity was in accordance with low pyruvate secretion in *Pf* (pAB48) and *Pf* (pAB8) as compared to *Pf* (pAB4). On the contrary, CS activity in *Pf* (pAB48) was higher than in *Pf* (pAB8) but was similar to that in *Pf* (pAB4). In accordance with these activities, intracellular and extracellular citric acid yields in *Pf* (pAB48) are ~1.8 and 10 fold higher respectively, than that in *Pf* (pAB8). But, despite similar CS activity and intracellular citrate yields in *Pf* (pAB48) and *Pf* (pAB4) extracellular citric acid yield was ~1.3 fold lower than *Pf* (pAB4). However, these extracellular citric acid levels in *Pf* (pAB48) were ~3.5 fold lower than in *Pf* (pAB7) (Chapter 5). An interesting correlation was that presence of pAB4 appears to increase the citrate efflux which could be possible if tetracycline resistance gene (on pAB4) encoded tetracycline efflux pump facilitated citrate efflux. However, tetracycline efflux proteins are multidrug resistance (MDR) pumps which are known to secrete many

structurally unrelated compounds, usually hydrophobic and amphipathic and hence can show broad substrate specificity (more information available in Transport Classification Database at www.tcdb.org) (Saier, M. personal communication). Citrate is exceptionally hydrophilic, but it could get transported as much larger complexes possibly with heavy metals but no MDR pump is known so far known to transport citrate (Pao et al., 1998).

On the other hand, all the physiological and biochemical parameters estimated for *Pf* (pAB4BBR1MCS-2) exhibited no significant differences from those in *Pf* (pBBR1MCS-2) but showed prominent differences as compared to *Pf* (pAB4). *Pf* (pAB4BBR1MCS-2) had ~1.8 and ~2.6 fold low growth and glucose utilization rates as compared to *Pf* (pAB4) with similar glucose utilization profile and biomass yield. Presence of two plasmids with different *ori* did not significantly alter the glucose distribution between direct oxidation and intracellular phosphorylative pathways in *Pf* (pAB4BBR1MCS-2) as demonstrated in *Pf* (pAB48). As compared to *Pf* (pAB4), reduction in GDH, CS, G-6-PDH and ICDH activities of *Pf* (pAB4BBR1MCS-2) by 2, 1.5, 1.7 and 3.4 fold respectively, correlated with the reduction in the secretion of gluconic, pyruvic and acetic acids and lower growth rate. Similar differences between *Pf* (pAB4BBR1MCS-2) and *Pf* (pAB48) could be attributed to the presence of pBBR1MCS-2 with a different *ori* as compared to pAB8.

In this context, comparison between *Pf* (pAB4) and *Pf* (pAB8) was also relevant as they had similar copy number and sizes (differing by ~1.0kb) yet showed significant differences in the basic metabolism (**Table 6.4**). In spite of similar growth and glucose consumption rates, the total glucose utilization in *Pf* (pAB8) was higher than in *Pf* (pAB4). However the relative contribution of direct and phosphorylative oxidation pathways in glucose catabolism was similar in these strains. Gluconic acid secretion by *Pf* (pAB4) and *Pf* (pAB8) correlated with GDH activity. Significantly lower CS activity in *Pf* (pAB8) correlated with low intracellular and extracellular citrate levels. These differences between *Pf* (pAB4) and *Pf* (pAB8) may be attributed, to some extent, to the presence of tetracycline and kanamycin resistance markers, respectively. Presence of kanamycin resistance gene was demonstrated to increase TCA flux in *E. coli* DH1 as compared to WT (Rozkov et al., 2004). On the other hand, tetracycline induced various ABC transporters, protein synthesis-related proteins, stress proteins and energy metabolism enzymes in *P. putida* KT2440 (Yun et al., 2006). Comparing *Pf* (pAB8) and

Pf (pBBR1MCS-2), both conferring kanamycin resistance, the latter had ~1.7 fold lower specific growth and glucose consumption rates which are in agreement with ~1.5 and 4.5 fold low CS and ICDH activities. Such plasmid load on the overall host metabolism is also reported for *E. coli* (Neubauer et al, 2002; De Gelder, 2007).

Physiological and biochemical parameters in the presence of plasmids also differ from *P. fluorescens* 1352 WT as demonstrated by our studies (Chapters 3, 5 and 8). In general, the presence of pUCPM18 based plasmids pAB4 and pAB8 increased the specific growth and glucose utilization rates by ~1.6 and 3 fold, respectively, as compared to WT while the presence of pBBR1MCS-2 did not alter these parameters. Increase in the growth rate due to presence of plasmids has also been demonstrated in *E. coli* (Rhee et al., 1997). GDH activities were elevated in presence of pAB4 and pAB8 but not in the presence of pBBR1MCS-2 while PYC activity increased as compared to the WT *P. fluorescens* irrespective of the nature of the plasmid. G-6-PDH activity remained almost similar except for increasing in *Pf* (pAB4).

The derivative strains expressing *ppc* and *cs* genes, *Pf* (pAB37) and *Pf* (pAB39) showed increased PPC and CS activities but the overexpression of both *ppc* and *cs* genes was relatively low as compared to *Pf* (pAB3) and *Pf* (pAB7)/*Pf* (pAB9) independently expressing *ppc* and *cs* genes, respectively. This reduction in PPC and CS activities in the double transformants correlated with decrease in the respective plasmid copy numbers as compared to their single transformants. Increase in PYC observed in *Pf* (pAB7) was not found in *Pf* (pAB37) and was similar to *Pf* (pAB48), the plasmid control. In spite of ~5 fold increase in PPC activity, the biomass yield remained unaltered in *Pf* (pAB37) and *Pf* (pAB39) while ~14 fold increase in PPC activity in *Pf* (pAB3) significantly increased the biomass yield (Chapter 3 and 4). On the other hand, *cs* gene overexpression had no effect on the biomass yield as compared to control (Chapter 5). Unaltered gluconic, pyruvic, acetic and citric acids secretion in *Pf* (pAB37) and *Pf* (pAB48) were in accordance with unaffected GDH, PYC, G-6-PDH and ICDH activities which in turn also correlated with similar glucose consumption and specific growth rates (**Table 6.3; Fig. 6.2; 6.3**). An additional 1.3 fold increase CS activity in *Pf* (pAB37) could not further alter the citrate levels as compared to *Pf* (pAB48). This suggests that probably PPC carried the glucose flux away from CS towards anabolism as supported by our earlier results where PPC overexpression has been demonstrated to increase the biomass yield in *P. fluorescens*.

Effect of *ppc-cs* overexpression was independent of the nature of the plasmid employed for overexpressing *cs* gene as the physiological parameters including specific growth and total glucose utilization rates and glucose consumption in *Pf* (pAB39) also were unaltered as compared to *Pf* (pAB4BBR1MCS-2). This is in contrast to the reduction in glucose consumption without any influence on specific growth rates in *Pf* (pAB3) and *Pf* (pAB7)/*Pf* (pAB9) (Chapters 3 and 5). These differences could be attributed to the metabolic effects exerted by presence of two plasmids per se as well as on the copy number and nature of plasmids present.

As concluding remarks, flux at anaplerotic node was one of the major factors influencing citrate accumulation in *P. fluorescens* (Chapter 5) as in *A. niger* (Papagianni, 2007). But, *ppc-cs* overexpression did not affect citric acid secretion, probably due to reduced effective overexpression of *cs* gene as compared to 2 fold in *Pf* (pAB7). Additionally, presence of two plasmids as such altered the *P. fluorescens* metabolism which might shield the effect exerted by the overexpressed genes. Present study also demonstrated that the physiology and metabolism of *P. fluorescens* 13525 was extensively modified by cumulative effects exerted by plasmid copy number, nature of origin of replication as well as the nature of antibiotic resistance gene.