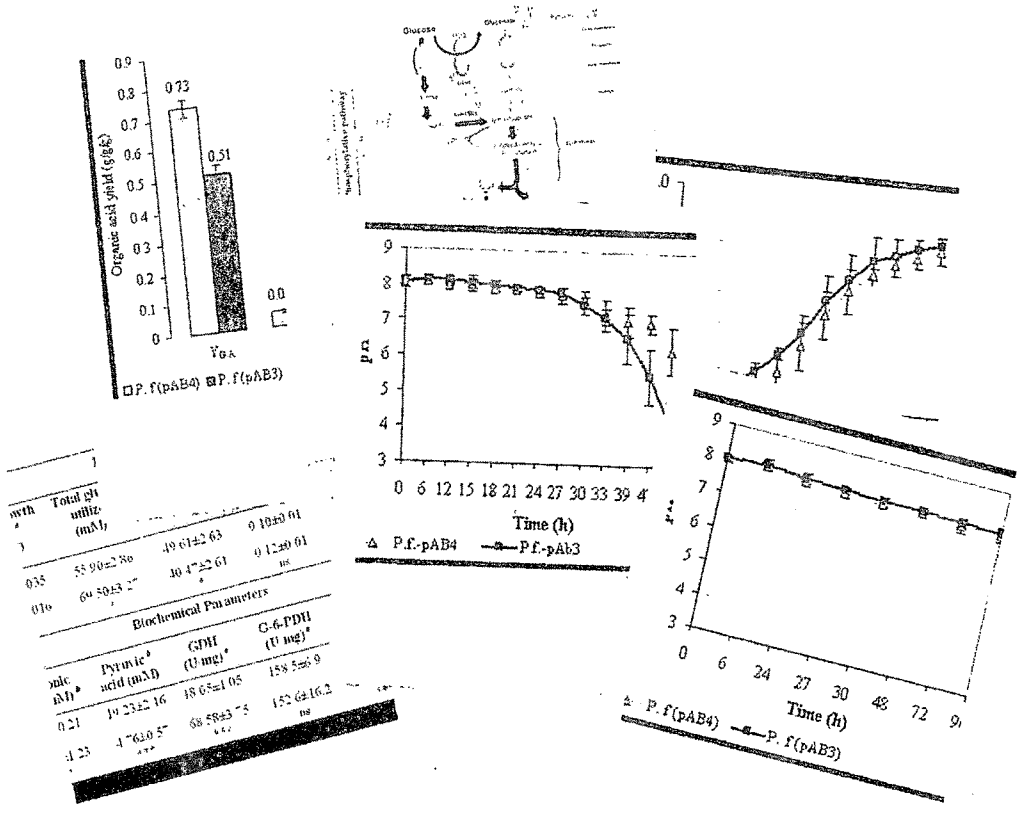


CHAPTER 4

Effect of Pi levels on glucose metabolism and organic acid secretion by *P. fluorescens* ATCC 13525 in the presence and absence of *ppc* gene overexpression

"Reason is the slow and torturous method by which those who do not know the truth discover it"

-Blaise Pascal

4.1: INTRODUCTION

Microbial metabolism responds to nutrient starvation by regulating specific pathways in response to specific environmental and metabolic signals. *E. coli* metabolism has been shown to alter depending on the carbon availability (Death and Ferenci, 1994). In the absence of glucose, cAMP receptor protein (CRP) and catabolite repressor/activator protein (Cra) in *E. coli* regulate the expression of genes allowing the use of alternate carbon sources by sensing the intracellular cAMP or fructose-1-phosphate and fructose-1,6-bisphosphate levels respectively (Saier and Ramseier, 1996). *E. coli* adapted a completely new metabolic circuit under glucose limitation in which PEPCk and glyxoylate shunt operate in parallel with normal TCA cycle, probably for convenient NADPH generation (Fischer and Sauer, 2003). On the other hand, due to the deregulated intracellular uptake of excess carbon source (sugars like glucose, xylose, lactose, arabinose, glycerol or gluconate) *E. coli* accumulates respective sugar phosphates which activate the methylglyoxal (MG) pathway (Weber et al., 2005). However, it represents an energetically unfavourable bypass to the glycolytic reactions of the lower EMP pathway. In pseudomonads, glucose levels govern the contribution of the two glucose catabolic pathways of which periplasmic direct oxidation pathway predominates in presence of excess glucose while intracellular phosphorylative oxidation prevails when glucose levels are low (Lessie and Phibbs, 1984). The metabolic pathways in pseudomonads also alter depending on the type of carbon source available.

Similarly, *E. coli* responds to phosphate (P) limitation by altering the global gene expression by using two component PhoR/PhoB sensory system (Wanner, 1996). The P-starvation induced Pho regulon governs a variety of genes including those responsible for Pi transport and maintaining intracellular Pi pool (Van Dien and Keasling, 1998). Additionally, Pho regulon has been also studied in other bacteria including *Sinorhizobium meliloti* and *Bacillus subtilis* (Allenby et al., 2005; Yuan et al., 2006). *E. coli* enters stationary phase upon P-starvation, like it does under carbon and nitrogen limiting conditions (Peterson et al., 2005). Additionally, *E. coli* under P-starvation conditions diverts the metabolic reserves towards biosynthesis of storage molecules such as polyphosphates (Kornberg, 1995; Rao and Kornberg, 1999). In addition to the genes under Pho regulon, global analysis of the protein expression profile under P-limitation in *E. coli* demonstrated drastic changes in the expression of genes involved in the central

carbon metabolism including citrate synthase (*glt*A), pyruvate formate lyase (*pfl*), phosphotransacetylase (*pta*), phophofructokinase (*pfk*) and PEP-PTS dependant glucose transport (*ptsIH*) (VanBogelen et al., 1996).

As the P-starvation responsive genes appear to be involved in multiple metabolic pathways including the central carbon metabolism as mentioned above, bacteria might undergo major redistribution in the catabolic and anabolic fluxes in response to P-deficiency. ATP/PEP/Acetyl~P form an essential group of high energy P donor metabolites that regulate the cellular metabolism, the levels of which could be influenced by P status. High levels of acetyl phosphate activate Pho regulon even in P-sufficient conditions (Wanner and Wilmes-Riesenberg, 1992). Under Pi-insufficiency, *E. coli* activate MG pathway, which not only bypasses the Pi utilizing reactions but also benefits by generating one Pi per MG synthesized by MG synthase (Emmerling et al., 1999). Although MG pathway is present in *E. coli, Pseudomonas and Clostridium*, glucose is not catabolized exclusively by this pathway under P-limiting conditions (Totemeyer et al., 1998). But MG pathway might contribute significantly under P starvation as MG synthase is allosterically inhibited by P (Huang et al., 1999; Weber et al., 2005). Under aerobic P-starvation conditions, non-growing *E. coli* cells reduce NADH production by diverting the metabolic flux from NAD dependent PDH to NAD independent POX, which indirectly helps to decrease oxidative stress (Moreau, 2004).

Many aspects of the Pho regulon have been addressed in studies of *E. coli*; however, it is unclear how transferable this knowledge is to other bacterial systems. Studies on several pseudomonads including *P. aeruginosa, P. putida, P. aureofaciens* and *P. fluorescens* PfO-1 demonstrate conservation of major regulators of Pho regulon expression relative to *E. coli* (Monds et al., 2006). In *P. aeruginosa*, in addition to the regulatory genes *pho*U and *pho*B, *opr*P and *pho*A genes (encoding a phosphate-specific porin and an alkaline phosphatase respectively) are implicated in P-starvation response (Poole and Hancock, 1986; Kragelund et al., 1997). P-starvation induced loci were identified in rhizospheric *P. fluorescens* which respond to P status in the rhizosphere as well as in bulk soil (Kragelund et al., 1995; 1997).

P assimilation occurs mainly in the form of Pi in the energy and carbon metabolism reactions which in turn becomes the source of anabolic precursors. Hence, P

metabolism is closely intertwined with energy and central carbon metabolism. Enhanced PPC activity (key enzyme in the central carbon metabolism) in *P. fluorescens* 13525 has been demonstrated to alter the glucose metabolism (Chapter 3). The present work describes the influence of P levels on the glucose metabolism in *P. fluorescens* 13525 with high PPC activity.

4.2: EXPERIMENTAL DESIGN

The experimental plan of work is as follows-

4.2.1: Bacterial strains used in this study

Bacterial strains	Characteristics	Reference
Pf (pAB4)	*P. fluorescens* 13525 with pAB4 plasmid, Tc^r	This study, Ch.3
Pf (pAB3)	*P. fluorescens* 13525 with pAB3 plasmid, Tc^r	This study, Ch.3

Table 4.1: List of bacterial strains used. *P. fluorescens* transformants were grown at 30°C with tetracycline and kanamycin concentrations varying for rich and minimal media as in Section 2.2.

4.2.2: Growth and organic acid secretion in presence of varying Pi concentrations

Pf (pAB3) and *Pf* (pAB4) were subjected to batch cultivation on 100mM Tris (pH=8.0) buffered minimal medium containing 100mM glucose as the sole carbon source (Section 2.2.4) and KH_2PO_4 as the source of Pi. The growth and the pH profiles were obtained on three different Pi concentrations 0.1mM, 1.0mM, 10mM and 20mM and were compared with respect to specific growth rates and media acidification (Section 2.9).

4.2.3: Effect of heterologous *ppc* gene expression on *P. fluorescens* 13525 physiology and glucose metabolism on TrP1 medium

Growth and organic acid production profiles of *Pf* (pAB3) and *Pf* (pAB4) were monitored on 100mM Tris (pH=8.0), 100mM glucose and 1mM KH_2PO_4, which is referred to as TrP1 medium (Section 2.2.4; 2.9). The samples withdrawn at regular

interval were analyzed for $O.D._{600nm}$, pH_i, extracellular glucose and organic acids (Section 2.9). The physiological parameters like specific growth rate, total glucose utilization rate, glucose consumption, biomass yield and organic acid yields were determined as in Section 2.9. The PPC, PYC, G-6-PDH, GDH, ICL and ICDH activities were determined as described in Section 2.10 and were correlated with growth, glucose utilization and organic acid profiles to explain the metabolic and physiological status under the experimental conditions.

4.3: RESULTS

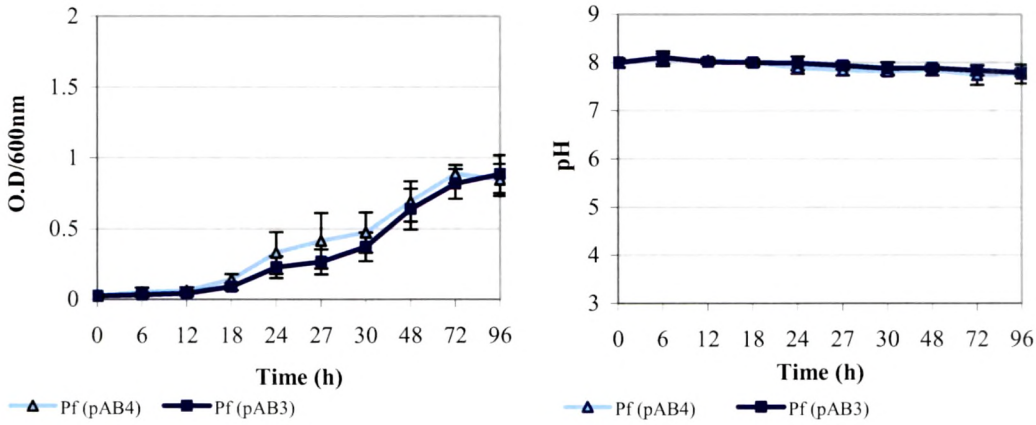
4.3.1: Effect of Pi levels on growth and media acidification of *P. fluorescens* 13525 transformants

Pf (pAB4) and *Pf* (pAB3) showed a Pi dependant variation in the growth (**Table 4.2; Fig. 4.1**). On 100mM glucose, growth rate of *Pf* (pAB4) was lowest on 0.1mM Pi which gradually increased with increase in Pi levels up to 20mM. However, the growth rate was not altered by *ppc* overexpression (**Table 4.2**). Significant media acidification was not seen only on 0.1mM Pi and the time required to drop the media pH to less than 5 reduced from 48h on 1.0mM and 10mM Pi to 30h on 20mM Pi (**Fig. 4.1**). Based on these results, 1mM Pi level was selected to study the effect of low glucose concentrations on growth and organic acid production. On 11.11mM (0.2%) and 50mM glucose, the growth was efficient and unaffected by *ppc* overexpression but media acidification was not seen.

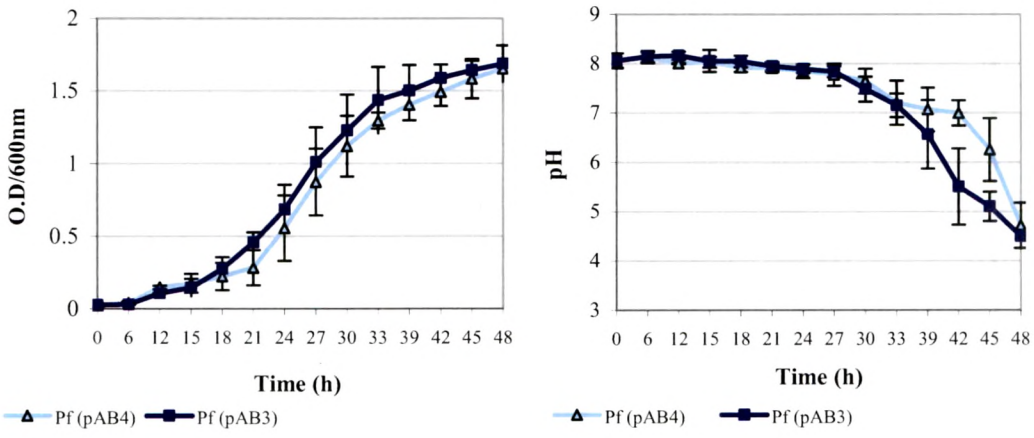
P. fluorescens strain	Sp. Growth rate, k (h^{-1})			
	0.1mM Pi	1.0mM Pi	10mM Pi	20mM Pi
Pf (pAB4)	0.11±0.02	0.25±0.02 ** [a]	0.34±0.03 * [a]	0.49±0.06 ** [a]
Pf (pAB3)	0.13±0.01 (ns)[b]	0.28±0.011 (ns)[b]	0.38±0.05 (ns)[b]	0.54±0.04 (ns)[b]

Table 4.2: Pi level dependant change in specific growth rates of *Pf* (pAB3) and *Pf* (pAB4). The values are represented as Mean±S.E.M of 4-5 independent observations. ***a***=Comparison of two successive *Pf* (pAB4) values to estimate significant change in specific growth rates with increase in Pi levels. ***b***= Comparison of *Pf* (pAB3) values with the corresponding values of *Pf* (pAB4) at each Pi level. * P<0.05; **P<0.01; ns= non significant.

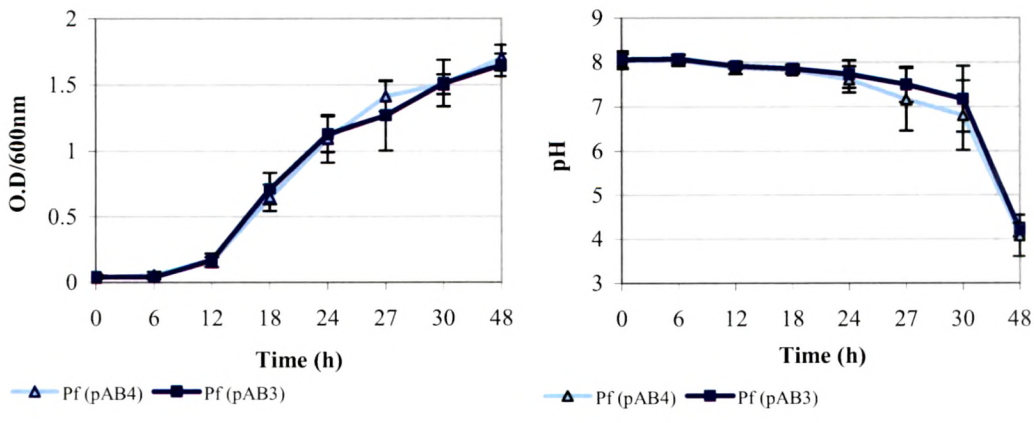
(a) 0.1mM KH_2PO_4



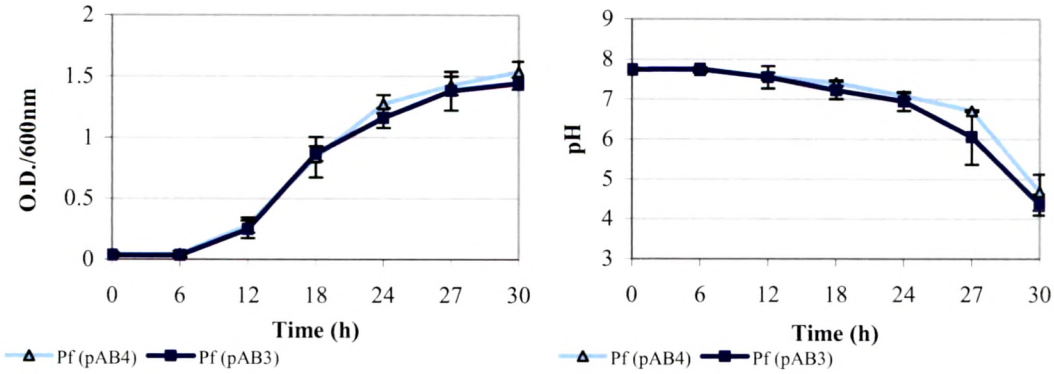
(b) 1.0mM KH_2PO_4



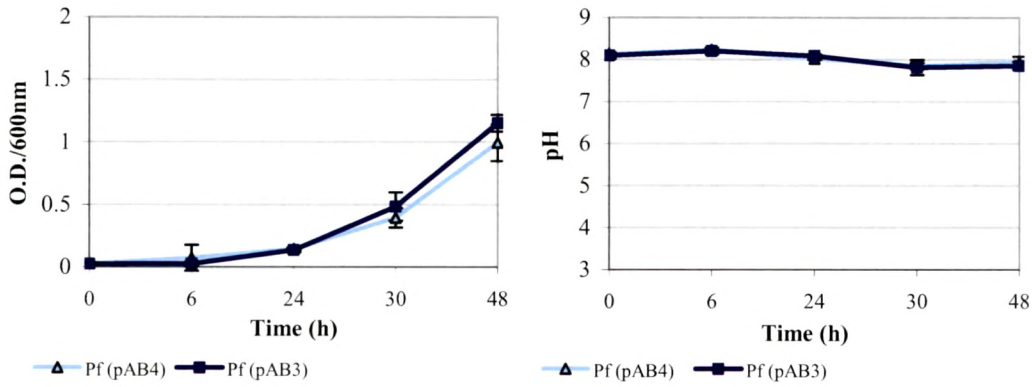
(c) 10mM KH_2PO_4



(d) 20mM KH_2PO_4



(e) 0.2% glucose + 1.0mM KH_2PO_4



(f) 50mM glucose + 1.0mM KH_2PO_4

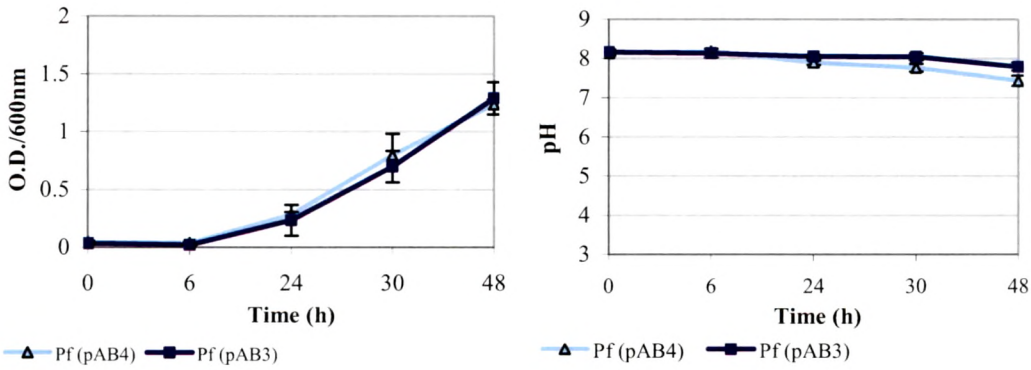


Fig. 4.1: Growth and organic acid production of *Pf* (pAB3) and *Pf* (pAB4) on different Pi and glucose levels. Values plotted are Mean±S.D of 3-5 and 8-10 independent observations for (a,c,d,e,f) and (b) respectively. Growth was determined as described in Section 2.9.3.

Based on these observations, the composition of the Tris minimal medium (TrP1) was selected to contain 1mM Pi and 100mM glucose to carry out all the further physiological experiments.

4.3.2: Effect of *ppc* overexpression on growth, glucose consumption and biomass yield on TrP1 medium

PPC activity in *Pf* (pAB3) on TrP1 medium was 46.26±3.59U protein as compared to 3.36±0.43U protein in the control *Pf* (pAB4). This 14 fold increase in PPC activity was similar to the ~12 fold increase in PPC activity of *Pf* (pAB3) demonstrated on M9 minimal medium (Section 3.3.5). As discussed above, *ppc* overexpression did not affect the growth as evident from the specific growth rates of *Pf* (pAB4) and *Pf* (pAB3) (**Table 4.3**). The specific total glucose utilization rate and the total amount of glucose used at the time of pH drop were unaltered but amount of glucose consumed and the biomass yield increased significantly in *Pf* (pAB3) by approximately 1.3 and 1.8 fold respectively. However, all these physiological parameters on TrP1 medium were significantly lower than on M9 medium, except the amount of total glucose utilized was higher on TrP1 medium.

Bacterial Strain	Sp. Growth rate[a] k (h^{-1})	Total glucose utilized (mM)[b]	Glucose consumed (mM)[b]	Biomass yield $Y_{dcw/Glc}$ (g/g)[a]	Sp. glucose utilization rate Q_{Glc} (g.g $dcw^{-1}.hr^{-1}$)[a]
Pf(pAB4)	0.25 ± 0.02	69.50 ± 3.27	40.47 ± 2.61	0.12 ± 0.01	3.26 ± 0.33
Pf(pAB3)	0.28 ± 0.01 ns	73.70 ± 3.25 ns	**52.39 ± 2.03** **	**0.21 ± 0.01** **	2.90 ± 0.34 ns

Table 4.3: Physiological variables and metabolic data from *P. fluorescens* 13525 *ppc* transformant grown on TrP1 medium. The results are expressed as Mean±S.E.M of 6-8 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 of each experiment. [b] Total glucose utilized and glucose consumed were determined at the time of pH drop (48h). The difference between total glucose utilized and glucose consumed, determination and calculations of above parameters is as explained in Section 2.9.3. * P<0.05, ** P<0.01, ns=non-significant

4.3.3: Effect of *ppc* overexpression on organic acid secretion in TrP1 medium

On TrP1 medium, levels of gluconic acid secreted by *Pf* (pAB3) were 1.3 fold lower as compared to *Pf* (pAB4) with specific gluconic acid yield, $Y_{G/G}$ significantly decreasing by 1.4 fold (**Fig. 4.2a and b**). Concomitantly in *Pf* (pAB3), pyruvic acid levels and yields increased by 2.2 fold each with a marginal increase by 1.8 fold and 1.5 fold in acetic acid levels and yields respectively.

As compared to M9 medium, gluconic acid secretion in *Pf* (pAB4) was higher on TrP1 medium while pyruvic and acetic acid secretion was lower. On the contrary, increased PPC activity of *Pf* (pAB3) in TrP1 medium led to higher secretion of pyruvic and acetic acids and decreased amount of gluconic acid. The levels of organic acids secreted are in accordance with overall glucose utilization and consumption pattern.

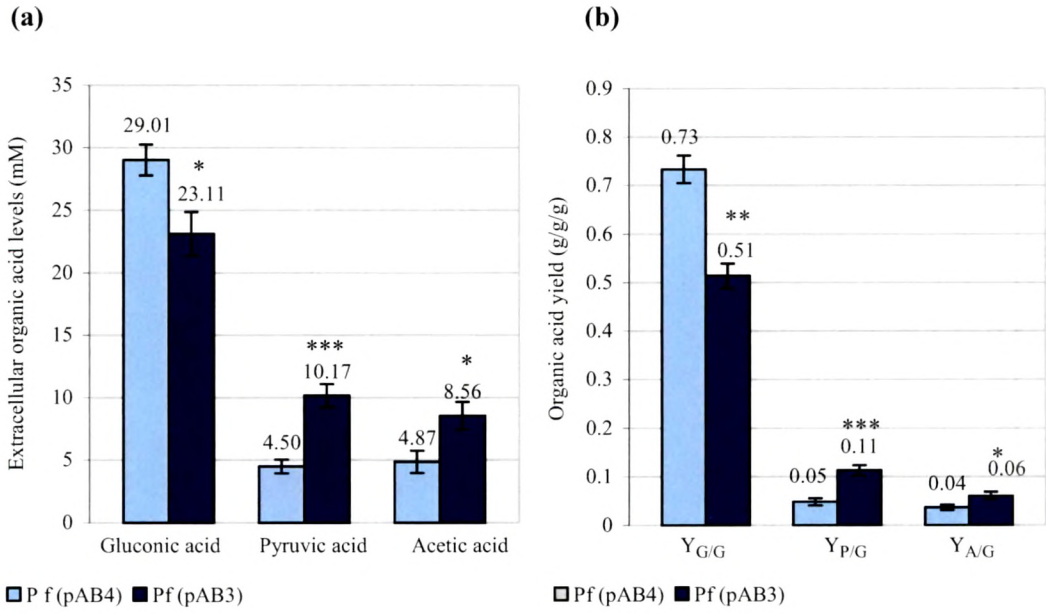


Fig. 4.2: Organic acid production from *P. fluorescens* 13525 *ppc* transformant. (a) Gluconic acid, pyruvic acid and acetic acid levels estimated in the extracellular medium. **(b)** Organic acid yields depicted as $Y_{G/G}$, $Y_{P/G}$ and $Y_{A/G}$ respectively and were estimated as described in Section 2.9.3. Organic acid yields are expressed as g/g of glucose utilized/g dry cell mass These organic acids are estimated from late stationary phase cultures of *Pf* (pAB4) and *Pf* (pAB3) grown on TrP1 minimal medium with 100mM glucose (samples drawn at 45-48h, Section 2.9.3). Results are expressed as Mean ±S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01, *** P<0.001.

4.3.4: Effect of *ppc* overexpression on PYC, GDH G-6-PDH, ICDH and ICL activities

The activities of key enzymes of periplasmic direct oxidation and intracellular phosphorylative pathways of glucose catabolism were determined in order to correlate the alterations in physiological variables and organic acid levels and yields in response to increased PPC activity. In *Pf* (pAB3) grown on TrP1 medium, GDH activity decreased by 2.6 fold whereas G-6-PDH activity (representing the phopshorylative pathway) increased by 1.6 fold (**Fig. 4.3**) as compared to similarly grown *Pf* (pAB4). Additionally, no change was found in PYC, ICDH and ICL activities of *Pf* (pAB3) as compared to the control. Amongst these ICL activity on glucose was very low. Compared to M9 medium, on TrP1 medium GDH activity in the control was higher but PYC and ICDH activities were reduced while ICL and G-6-PDH activities remained unchanged.

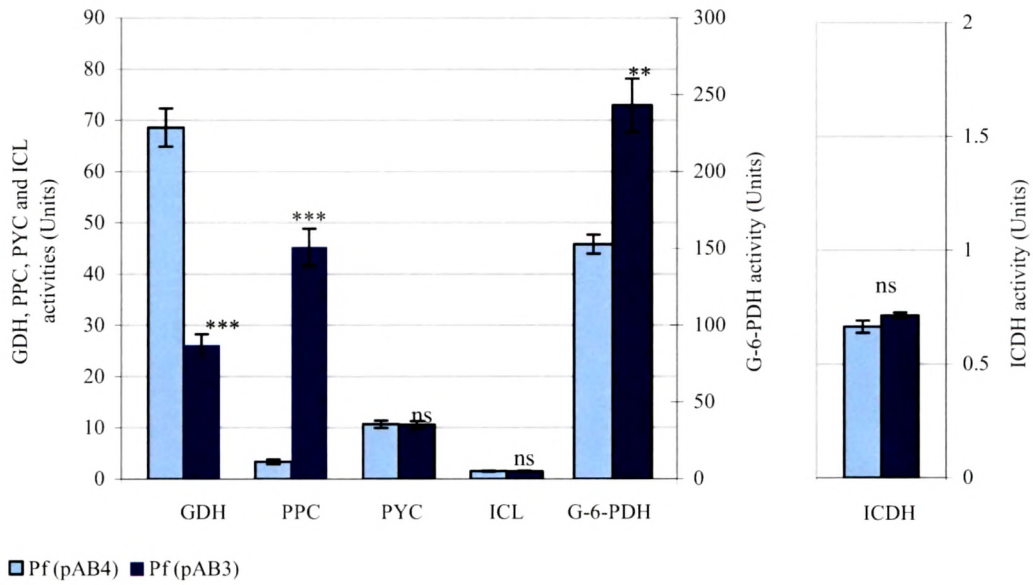


Fig. 4.3: Alterations in PYC, GDH G-6-PDH, ICDH and ICL activities in *P. fluorescens* 13525 *ppc* transformant. All the enzyme activities were estimated from cultures grown on TrP1 medium with 100mM glucose. All the enzyme activities were estimated from mid log phase to late log phase cultures except ICDH and ICL which were estimated in stationary phase (Section 2.10). 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. *** $P<0.001$, ** $P<0.01$, ns= non-significant.

4.4: DISCUSSION

Physiology of bacteria is very well known to vary with the minimal and nutrient rich media in terms of growth rates and yield. But in conditions in which any single nutrient is present in very low (deficient) levels, the cell adapts sometimes by inducing novel pathways. Earlier study in *E. coli* shows that the genes involved in glucose metabolism do undergo altered expression profile in response to P-starvation (VanBogelen et al., 1996). However, not much is known about the effects of cellular metabolism when any nutrient is present at suboptimal levels but not deficient levels. The present study describes the glucose utilization pathways in *P. fluorescens* in presence and absence of *ppc* overexpression on two different minimal media *viz.* M9 and TrP1 media which chiefly differ in Pi levels in addition to minor difference in N levels. Chapter 3 described the effect of *P. fluorescens* 13525 overexpressing *ppc* gene of *S. elongatus* PCC 6301 in M9 minimal medium and the current work describes its effect on TrP1 medium.

Specific growth rates of *Pf* (pAB3) and *Pf* (pAB4) increased with Pi level, being significantly low at 1mM Pi than that at 20mM Pi (**Table 4.2**). This is contrary to earlier reports which demonstrated 1mM Pi to be sufficient for optimal growth of many wild-type strains of *E. coli* and *Pseudomonas* (Poole and Hancock, 1986, Monds et al., 2006). However in a recent work, 2mM Pi has also been used as P-limiting condition for *E. coli* (Noda et al., 2007). On the other hand, 0.066-0.2mM Pi is known to induce P-starvation response for *E. coli*, *Corynebacterium glutamicum* and several *Pseudomonas* strains (Poole and Hancock, 1986; VanBogelen et al., 1996; Rao et al., 1998; Ishige et al., 2003; White and Metcalf, 2004; Monds et al, 2006). Collectively, 1mM Pi could be regarded as limiting (suboptimal but not starvation) condition and such a suboptimal nutrient status could elicit adaptations that are significantly different from starving or unstressed conditions as demonstrated in *E. coli* (Ferenci, 1999). However, *ppc* overexpression in *Pf* (pAB3) did not significantly influence the growth rates on different Pi levels.

The Pi levels also seem to affect the total organic acid secretion from *Pf* (pAB4) and *Pf* (pAB3) as evident from varying media acidification profile. Absence of organic acid secretion at 0.1mM Pi while significant organic acid secretion at higher Pi levels suggests that at Pi level as low as 0.1mM metabolism is just sufficient to maintain

growth, albeit at very low rates. Higher Pi levels increasingly support growth by facilitating energy production and optimal functioning of metabolic pathways. Moreover, 0.1mM is known to elicit P-starvation response in *E. coli* which includes activation of MG pathway for glucose catabolism and accumulation of MG which is suggested to be a growth regulator (Huang et al., 1999). MG pathway is also known to occur in *P. saccharophila,* (Cooper, 1974) but has not been characterized in detail.

Lack of media acidification by *Pf* (pAB4) and *Pf* (pAB3) on low glucose could be attributed to the absence of gluconic acid secretion which could be due to the predominance of phosphorylative pathway of glucose catabolism over the direct oxidation pathway; the latter contributing significantly only at high glucose concentrations as demonstrated in *P. fluorescens* (Lessie and Phibbs, 1984). Similarly, in *E. coli* glucose levels affected the acetic acid secretion which was high in presence of excess glucose, while reduced at low glucose concentrations (Abdel-Hamid et al., 2001; Phue et al., 2005). In addition to gluconic acid, *Pf* (pAB4) and *Pf* (pAB3) also secreted pyruvic and acetic acids on high glucose and high Pi (discussed in Chapter 3) which are the products of intracellular glucose catabolic pathways. Hence in presence of high glucose, increase in organic acid secretion with increase in Pi appears to be a cumulative effect of increased direct oxidation pathway as well as saturation of phosphorylative pathway and is manifested as decrease in time taken for media acidification.

A 14 fold increase in PPC activity attained using pAB3 plasmid in TrP1 medium similar to that on M9 medium demonstrated that the *ppc* gene overexpression was independent of the media composition. Effects of *ppc* overexpression on the biomass yield, growth rate, total glucose utilization rates and total amount of glucose consumed in TrP1 medium were similar to that on M9 medium. However, increase in G-6-PDH activity concomitant with reduced GDH activity in *Pf* (pAB3) on TrP1 medium demonstrated increased phosphorylative oxidation and concomitantly reduced direct oxidation pathway, supporting 29% increase in glucose consumption. These results are also substantiated by reduced gluconic acid yield with concurrently increased pyruvic and acetic acid yields in *Pf* (pAB3) as compared to control. This is in contrast to the increased contribution of direct oxidative pathway in response to *ppc* overexpression in *Pf* (pAB3) demonstrated under M9 medium (Section 3.3.8).

Increased pyruvic acid secretion by *Pf* (pAB3) suggested that the downstream pathways utilizing pyruvate are limiting. This is supported by the fact that PYC activity, chiefly responsible for fixing pyruvate into downstream metabolic pathways, was unaltered. Moreover, relatively low PYC activity on TrP1 medium compared to that on M9 medium could also cause limitation of biosynthetic precursor OAA, which is overcome in *Pf* (pAB3) where elevated PPC activity increases the OAA supplementation without altering the inherent PYC activity. This metabolic change is further manifested as improved biomass yield. Unaltered ICDH activity in *Pf* (pAB3) suggested that the TCA cycle was unaffected which in turn supported the unaltered growth rates. Very low ICL activity suggested that glyoxylate pathway did not contribute much to the metabolism at the glucose concentration used, which was well in agreement with earlier report demonstrating repression of ICL activity in presence of glucose in *P. aeruginosa* PAO1 and *P. indigofera* (Howes and McFadden, 1962; Diaz-Perez et al., 2007).

Comparison of the physiological and biochemical parameters of *Pf* (pAB4) (control) on TrP1 and M9 minimal media (discussed in Chapter 3) highlighted some interesting and important effects imposed by media composition itself. It is noteworthy that M9 and TrP1 minimal media differed in Pi levels, which were as low as 1mM in TrP1 medium as compared to ~50mM on M9 medium, while ~2 fold lower $NH4^+$ content in TrP1 medium might not influence much. A comparative account of all biochemical and physiological parameters estimated for *Pf* (pAB4) (control) on TrP1 and M9 media is presented in **Table 4.4**. The specific growth rate of *Pf* (pAB4) on TrP1 medium with 1mM Pi was significantly lower than in 20mM Pi which suggested that differences in growth and metabolism of *Pf* (pAB4) in TrP1 and M9 media could be attributed to the differences in Pi levels.

Pf (pAB4) showed about 3 fold reduction in growth and total glucose utilization rates on TrP1 medium as compared to M9 medium which could be a direct effect of low Pi (**Table 4.4**). In order to maintain biomass generation and growth on low Pi, *Pf* (pAB4) increased the total glucose utilization by about 24%. However, the relative glucose flux distribution through direct oxidation and phosphorylative pathways differs in a Pi dependant manner. Of the total glucose used, ~89% and ~58% of the total glucose was metabolized via phosphorylative pathway in M9 (high Pi) and TrP1 (low Pi) media respectively. These results together with 3.7 fold higher GDH activity on TrP1 medium

indicate that on low Pi, *P. fluorescens* had increased contribution of direct oxidation pathway which might be preferable on low Pi because the GDH activity itself is linked to electron transport chain (ETC) by the virtue of electron transfer through co-factor PQQ. This reaction generates PMF in the membrane and indirectly helps ATP generation (van Schie et al., 1985). These results suggested that under low Pi, *Pf* (pAB4) minimized the energy intensive processes and thermodynamically favorable ATP generating reactions were enhanced.

Physiological Parameters						
Media	**Sp. Growth rate [a] k (h^{-1})**	**Total glucose utilized (mM) [b]**	**Glucose consumed (mM) [b]**	**Biomass yield $Y_{dcw/Glc}$ (g /g) [a]**	**Sp. Glucose utilization rate Q_{Glc} (g.g dcw^{-1} .hr^{-1}) [a]**	
M9	0.75 ± 0.04	55.90 ± 2.86	49.61 ± 2.63	0.10 ± 0.01	10.10 ± 1.43	
TrP1	0.25 ± 0.02 ***	69.50 ± 3.27 *	40.47 ± 2.61 *	0.12 ± 0.01 ns	3.26 ± 0.33 ***	
Biochemical Parameters						
Media	**Gluconic acid (mM) [b]**	**Pyruvic acid (mM) [b]**	**Acetic Acid (mM) [b]**	**GDH (U/mg) [c]**	**G-6-PDH (U/mg) [c]**	**PYC (U/mg) [c]**	**ICDH [b] (U/mg) [#]**
M9	6.31 ± 0.21	19.23 ± 2.46	16.44 ± 3.36	18.65 ± 1.05	150.8 ± 6.46	48.48 ± 3.38	1.24 ± 0.07
TrP1	29.01 ± 1.23 ***	4.76 ± 0.57 ***	4.87 ± 0.88 **	68.58 ± 3.75 ***	152.6 ± 16.2 ns	10.71 ± 0.72 ***	0.66 ± 0.03 ***

Table 4.4: Effect of media composition on basal physiological and biochemical parameters of *Pf* (pAB4). The values for M9 are as presented in Table 3.3 and Fig. 3.8; 3.9 while those for TrP1 medium are taken from Table 4.3 and Fig. 4.2; 4.3. The data is reported again for the sake for convenient comparison. Low or negligible values like acetic acid levels, PPC and ICL activities are not included. [a] Parameters are estimated in the mid-log phase; [b] parameters are estimated in the stationary phase; [c] Parameters estimated in the mid-log to late-log phase; the determinations and calculations for which are explained in details in the text. [#] ICDH activity was exceptionally expressed in the units of µmoles/min/mg total protein unlike other enzyme activities which are expressed in the units of nmoles/min/mg total protein. * P<0.02, *** P<0.0001, ns=non-significant.

Comparatively higher glucose utilization on low Pi was also reflected in altered organic acids secretion, although the nature of organic acids did not change with Pi levels. On low Pi, gluconic acid levels secreted were higher by about 4.6 folds as a result of higher GDH activity. Conversely, the pyruvic and acetic acid levels estimated in the medium were about 4 fold lower on low Pi as compared to high Pi conditions. Pyruvate being a product of intracellular metabolic reactions, overall reduction in the flux through phosphorylative pathway on low Pi could be responsible for the observed variations. PYC activity was largely reduced by 4.5 fold in low Pi conditions, which may be due to decreased ATP levels. Significantly lower ICDH activity on low Pi supported the reduced operation of TCA cycle and thereby observed lower growth rate.

Our results suggest that Pi availability by itself significantly altered the contribution of the two glucose catabolic pathways in *P. fluorescens* 13525. Glucose flux through the direct and the phosphorylative oxidation pathways in pseudomonads was earlier demonstrated to be influenced by oxygen, glucose availability and temperature (Lessie and Phibbs, 1984) but the influence of Pi on the same has not been reported. Similarly, effects of *ppc* gene overexpression in *E. coli* varied with respect to oxygen sufficient (aerobic) and deficient (anaerobic) conditions (Chao and Liao, 1993; Farmer and Liao, 1997; Gokarn et al., 2000; Abdel-Hamid et al., 2001). Our results demonstrate that effects of *ppc* overexpression in *P. fluorescens* 13525 also are influenced by Pi status (**Fig. 4.4**). Based on the fact that PPC reaction per se liberates one free Pi molecule, it could be explained that increased PPC reaction might generate more conducive conditions in presence of lower Pi thereby resulting into more profound effects on the overall cellular physiology and metabolism.

In presence of excess Pi, reduced pyruvate and acetate secretion with improved biomass yield in presence of *ppc* gene overexpression suggested that physiological levels of PYC activity at the anaplerotic node of *P. fluorescens* 13525 was not optimal for efficient carbon utilization. On the other hand, *ppc* overexpression on lower Pi not only apparently pulled the glucose flux towards intracellular phosphorylative pathway but also served the anaplerotic functions when inherent PYC activity was compromised. Hence, on low Pi when direct oxidation pathway benefited *P. fluorescens* 13525 probably by facilitating electron transport, *ppc* overexpression benefited the physiology by enhancing the anabolic reactions.

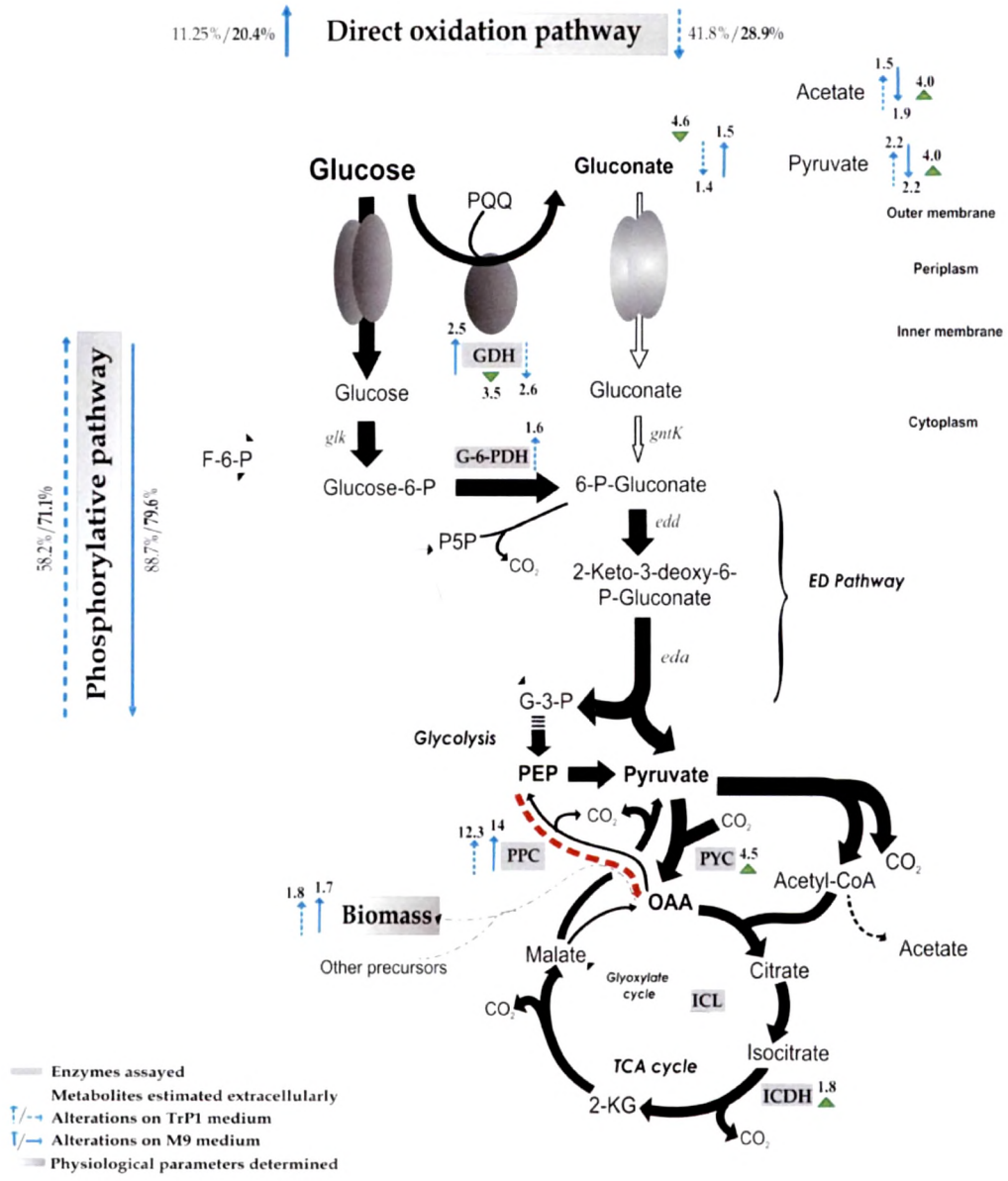


Fig. 4.4: Summarized Pi-dependent effects of *S. elongatus* PCC 6301 *ppc* gene overexpression on glucose catabolism in *P. fluorescens* 13525. Numbers depicted on the arrows represent the fold change in response to *ppc* overexpression on M9 and TrP1 minimal media. Numbers on the arrow heads (Δ) depict the fold variations in *Pf* (pAB4) on M9 as compared to TrP1 medium. Unchanged parameters are not indicated. The values (%) against solid and dashed arrows (indicating M9 and TrP1 media respectively) represent the percentile glucose (calculated out of the total glucose utilized) distributed in phosphorylative and direct oxidation pathways in *Pf* (pAB4)/***Pf* (pAB3)**.