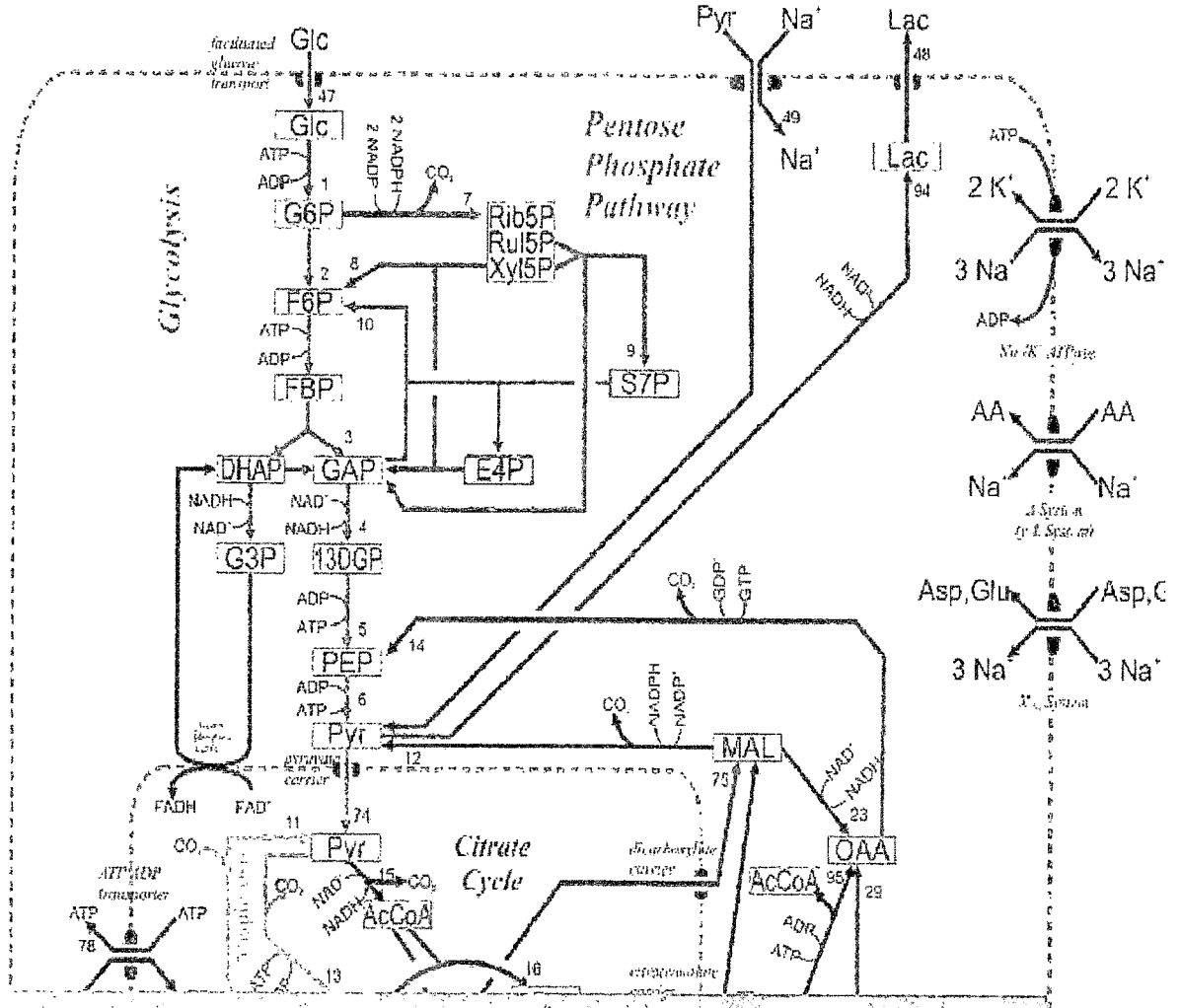

CHAPTER 10

Effect of overexpression of citrate synthase (*gltA*), phosphoenol pyruvate carboxylase (*ppc*), citrate transporter in *E. coli* MA1935 (*icd*) mutant on the growth and organic acid secretion.

10.1: Rationale of the present study.

Enhancing the anaplerotic pathway replenishing the TCA cycle intermediates has shown to direct the cellular metabolism to improve citrate production in *A. niger* (Legisa and Mattey, 2007). **Chapter 9** suggested that overexpression of *cs* gene (~1.8 fold) in *E. coli* MA1935 *icd* mutant enhanced citrate accumulation by ~3.25 fold. On the other hand overexpression of *ppc* gene (~3 fold) in *E. coli* MA1935 *icd* mutant (**Chapter 8**) directed the cells towards anaplerotic pathway with reduced citrate production. These results suggest that CS activity might limit the flux towards TCA. Hence, the present study deals with studying the effects of overexpression of *ppc* and *cs* genes together in *E. coli* MA1935 *icd* mutant on enhancing citrate accumulation.

10.2: WORK PLAN

10.2.1: Bacterial strains used for the present study.

E. coli strains	Genotype	References
DH5α pJE3	P_{tac} promoter, Tc^r, Ap^r	Present study
DH5α pVSppc	*ppc* gene under P_{tac} promoter in pTTQ18, Tc^r, Ap^r	Sharma, 2008
MA1935 pJE1:pVSD1	P_{tac} promoter, Chl^r, Km^r, Ap^r	Ch. 9
MA1935 pJE2: pVSD1	*gltA* gene, citrate transporter. Chl^r, Km^r, Ap^r	Ch. 9
MA1935 pJE1:pJE3:pVSD1	P_{tac} promoter, citrate transporter. Chl^r, Km^r, Tc^r, Ap^r	Present study
MA1935pJE2:pVSppc: pVSD1	*gltA* gene, *ppc* gene citrate transporter. Chl^r, Km^r, Tc^r, Ap^r	Present study

Table 10.1: *E. coli* strains used for the present study. The details of the plasmids and the concentrations of the antibiotics used are given in the table 2.2 and 2.3.

10.2.2: Incorporation of *ppc* gene carrying plasmid into *E. coli* MA1935 containing citrate transporter and *gltA* carrying plasmids.

pJE3 and pVSppc plasmids were isolated from *E. coli* DH5α and transformed in *E. coli* MA1935 containing citrate transporter and pJE1 and pJE2 plasmids respectively. The resultant triple transformants were selected on chloramphenicol, kanamycin, tetracycline and ampicilin containing plates (concentration as mentioned in table 2.3).

10.2.3: Monitoring the growth and physiological parameters of *E. coli* BL21(λDE3) *icd* mutant expressing *gltA* and *ppc* genes.

E. coli MA1935 transformants were grown on M9 minimal medium with micronutrients and 50mM glucose and 100mM glycerol as carbon source. IPTG (0.1mM) was used for induction. Samples were collected at regular interval for O.D. $_{600nm}$, glucose utilized and organic acid in the medium. The physiological parameters were calculated as described in (section 2.5). Enzyme assays were performed as per protocol mentioned in (section 2.6); ICDH, PPC, G-6-PDH, CS and ICL at stationary phase.

10.3: RESULTS

10.3.1: Monitoring the effect *ppc* and *gltA* overexpression on the growth and physiological parameters of *E. coli* BL21(λDE3) *icd* mutant in presence of citrate on glucose and glycerol as carbon source.

The triple transformants were referred as *E. coli* MA1935 pJE1:pJE3:pVSD1 (control) and *E. coli* MA1935pJE2:pVSppc: pVSD1 (test). Simultaneous overexpression of *ppc* and *gltA* genes in *E. coli* BL21(λDE3) *icd* mutant on glucose increased growth rate, but reduced glucose consumption rate and overall glucose consumed (**Table 10.2**) while on glycerol it increased growth rate as well as consumption of glycerol (**Table 10.3**).

E. coli strains	Growth rate μ (h^{-1})	Specific glucose consumption rate (Q glc) ($g.glc.g^{-1}h^{-1}$)	Total glucose utilized (mM)	Biomass ($Y_{dcw/glc}$) ($g\ dcw.g^{-1}\ glc.h^{-1}$)
MA1935 JE1:pJE3:pVSD1	0.24 ± 0.07	5.18 ± 0.17	33.0 ± 4	0.08 ± 0.01
MA1935pJE2:pVSppc: pVSD1	0.39 ± 0.02	2.07± 0.16	7.9 ± 2	0.31 ± 0.02
	***	**	***	***

Table 10.2: Physiological variables and metabolic data from *E. coli* MA1935:pVSD1 overexpressing *gltA* and *ppc* gene grown on M9 minimal medium. The results are expressed as Mean ±SD of 8-10 independent observations. Specific growth rate ($\mu(h^{-1})$) and specific glucose consumption rate (Q_{Glc}) and biomass ($Y_{dcw/glc}$) were determined from mid log phase of each experiment. Total glucose utilized was determined at the end of growth curve. * $p<0.05$, ** $p<0.01$, ns=non-significant.

E. coli strains	Growth rate μ (h^{-1})	Total glycerol utilized (mM)
MA1935 JE1:pJE3:pVSD1	0.13 ± 0.007	28.8 ± 3.25
MA1935pJE2:pVSppc: pVSD1	0.30 ± 0.018 ***	56.8 ± 8.5 ***

Table 10.3: Physiological variables and metabolic data from *E. coli* MA1935:pVSD1 overexpressing *gltA* and *ppc* gene grown on M9 minimal medium with glycerol as carbon source. The results are expressed as Mean ±SD of 8-10 independent observations. Specific growth rate ($\mu(h^{-1})$) were determined from mid log phase of each experiment. Total glycerol utilized was determined at the end of growth curve. * $p<0.05$, ** $p<0.01$, ns=non-significant.

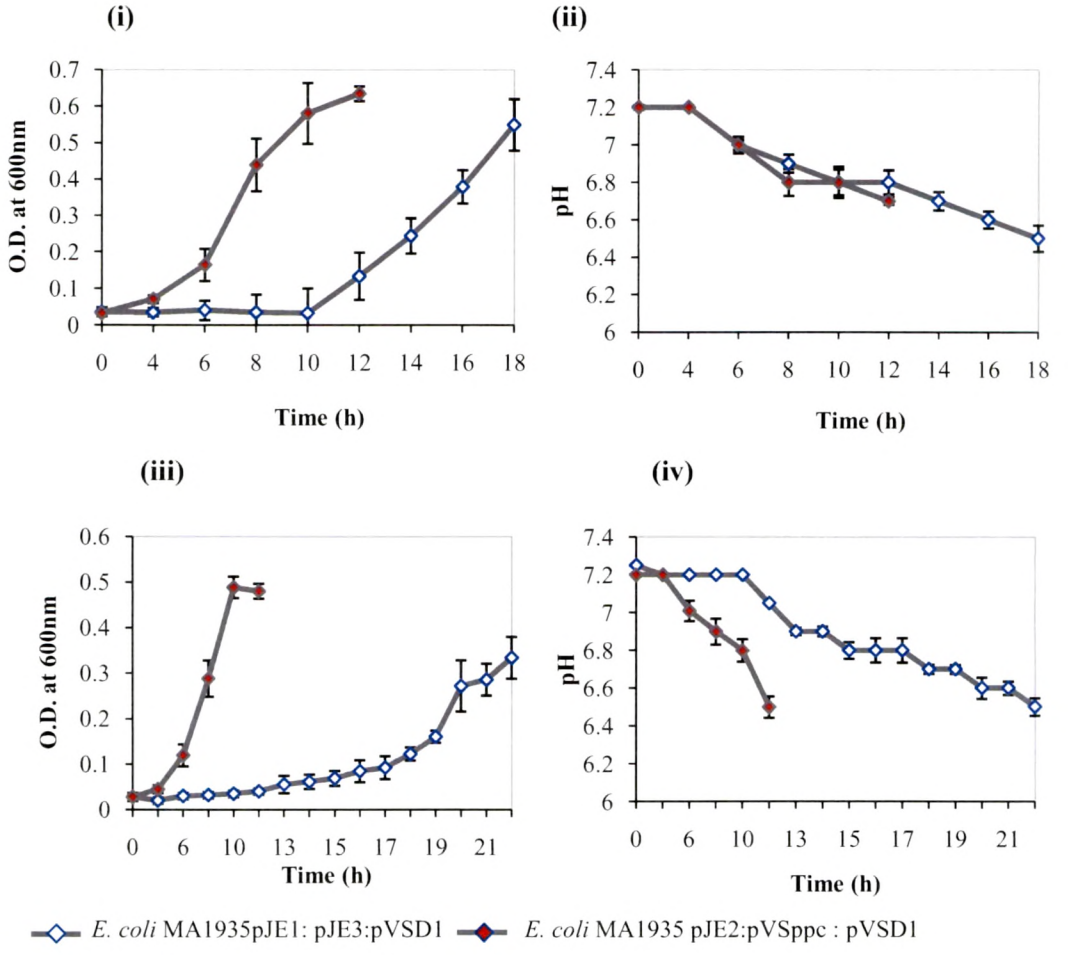

Fig 10.1: Growth characteristics of *E. coli* MA1935 overexpressing *ppc* and *gltA* genes on M9 minimal medium with 50 mM glucose and 100mM glycerol. (i) & (ii) $O.D_{600nm}$ and pH on glucose, (iii) & (iv) $O.D_{600nm}$ and pH on glycerol. All values plotted are represented as Mean ± SD for n= 4 to 7 observations.

10.2.2: Effect of *ppc* and *gltA* gene overexpression in *E. coli* BL21(λDE3) *icd* mutant in presence of citrate transporter on various enzyme activities and organic acid secretion.

Overexpression of *ppc* and *gltA* in *E. coli* BL21(λDE3) *icd* mutant demonstrated ~ 2.5 fold increase in PPC activity while ~4 fold increase in CS activity. An increase in G-6-PDH (~ 3 fold) and ICL (~ 4 fold) activities were observed in response to co-expression of

ppc and *gltA* (**Fig. 10.2 (i) and (ii)**). Similar results were observed on glycerol but the activities were low compared to that on glucose (**Fig. 10.2 (iii) and (iv)**).

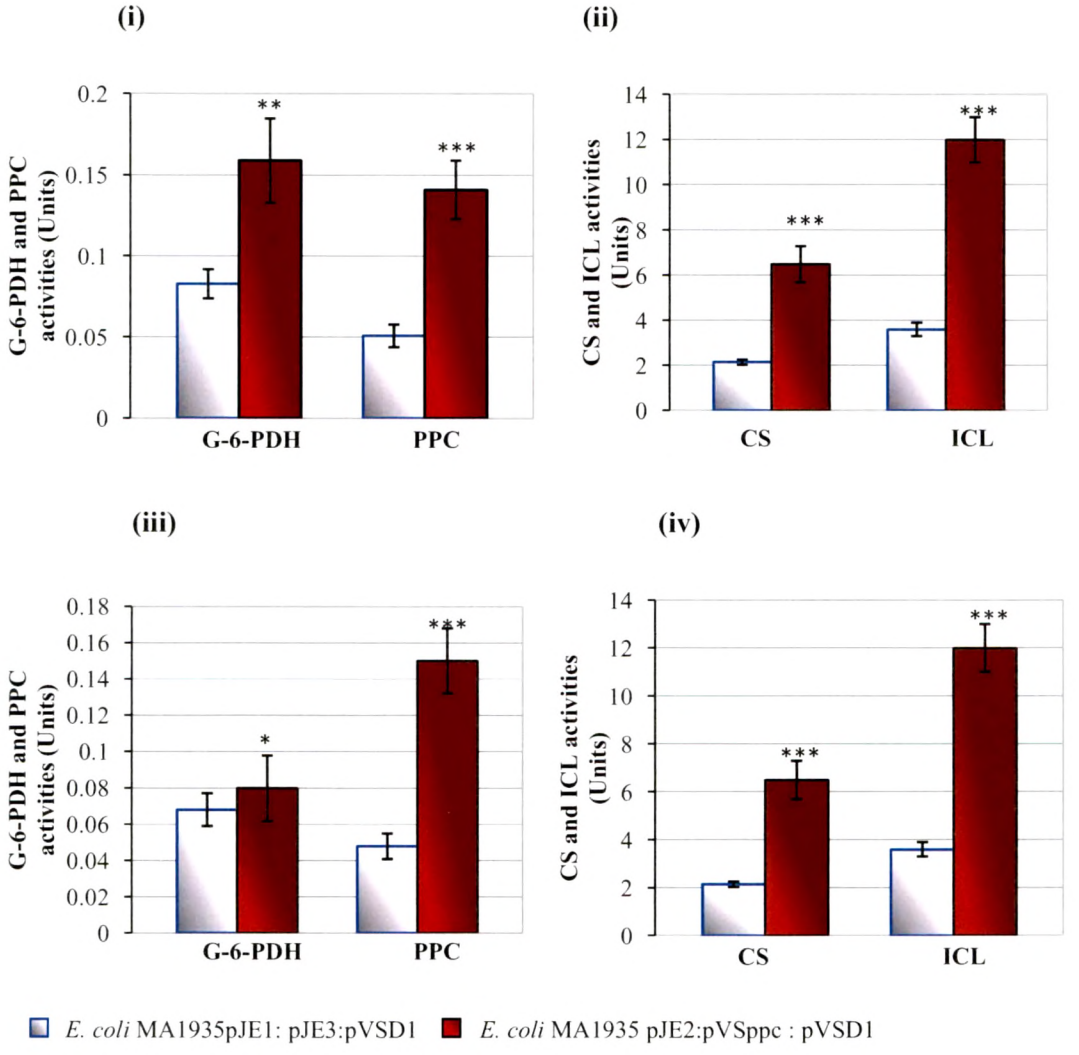

Fig. 10.2: Effect of *ppc* and *gltA* overexpression in *E. coli* BL21(λDE3) *icd* mutant on enzyme activities in presence and absence of citrate transporter on M9 minimal medium with 50 mM glucose and 100mM glycerol. (i) and (ii) enzyme activities on glucose (50mM) (iii) and (iv) enzyme activities on glycerol (100mM). Units for the specific activity are given as μmoles/mg. protein/min for PPC and G-6-PDH for CS and ICl Units are. nmoles of OAA utilised/mg. protein/min and nmoles/mg. protein/min respectively. All the values are represented as Mean ± SD for n= 5-8 observations. Units- μmoles/mg. protein/min. *p<0.05, **p<0.01, ***p<0.001.

E. coli strains	Intracellular citrate		Extracellular citrate	
	mM	Yield (g/l)	µM	Yield (g/l)
50 mM Glucose				
MA1935 JE1:pJE3:pVSD1	3.12 ± 0.43	0.018 ± 0.002	ND	ND
MA1935pJE2:pVSppc: pVSD1	8.04 ± 2.04	0.19 ± 0.01	26 ± 2	0.009 ± 0.002
	***	***	***	***
100 mM Glycerol				
MA1935 JE1:pJE3:pVSD1	2.1 ± 0.9	1.8 ± 0.07	ND	ND
MA1935pJE2:pVSppc: pVSD1	7.8 ± 1.4	3.8 ± 0.09	35 ± 2	0.08 ± 0.02
	***	***	***	***

Table 10.4: Citrate accumulation in *E. coli* MA1935 expressing *gltA* gene and *ppc* under P_{tac} promoter on glucose and glycerol. The table depicts the citrate (intracellular) and (extracellular) in late stationary phase cultures of *E. coli* MA1935 transformants (plasmid control and test) grown on M9 minimal media with 50 mM glucose and 100mM glycerol as carbon source. All the values are represented as Mean ± SD of n=4-8 observations. *** p<0.001, ND-not detected.

E. coli strains	Extracellular Acetate	
	mM	Yield (g/l)
50 mM glucose		
MA1935 JE1:pJE3:pVSD1	ND	ND
MA1935pJE2:pVSppc: pVSD1	8.2 ± 2***	0.060 ± 0.003
100 mM glycerol		
MA1935 JE1:pJE3:pVSD1	ND	ND
MA1935pJE2:pVSppc: pVSD1	4.4 ± 0.9***	0.23 ± 0.007***

Table 10.5: Acetate secretion in *E. coli* MA1935 expressing *gltA* gene and *ppc* under P_{tac} promoter on glucose and glycerol. The table depicts the acetate secretion stationary phase cultures of *E. coli* MA1935 transformants (plasmid control and test) grown on M9 minimal media with 50 mM glucose and 100mM glycerol as carbon source. All the values are represented as Mean ± SD of n=4-8 observations. *** p<0.001, ND-not detected.

Overexpression of *ppc* and cs in *E. coli* BL21(λDE3) *icd* mutant also increased intracellular citrate by ~ 2.5 fold while ~26 fold increase was found in exctracellular levels on glucose medium (**Table 10.4**). Similar results with respect to citrate accumulation were observed on glycerol although the levels were comparatively higher compared to that on glucose (**Table 10.4**). Overexpression of *ppc* and *gltA* showed high acetate secretion on glucose and glycerol (**Table 10.5**).

10.4: DISCUSSION

Earlier work on *ppc* overexpression in *E. coli* BL21(λDE3) *icd* mutant (Chapter 8) demonstrated increased growth rate without any change in glucose consumption rate and glucose utilization. On the other hand, *cs* overexpression in *E. coli* BL21(λDE3) *icd* mutant (Chapter 9) increased glucose consumption rate with low glucose utilization and unaltered growth rate. Co-expression of *ppc* (~ 2.5 fold) and *gltA* (~1.5 fold) genes in *E. coli* BL21(λDE3) *icd* mutant demonstrated high growth rate but reduced glucose consumption rate and glucose utilization. Hence, *ppc* overexpression improved growth rate while *gltA* overexpression reduced glucose utilization (**Table 10.6**).

Overexpression of *ppc* and *gltA* in *E. coli* BL21(λDE3) *icd* mutant increased intracellular citrate levels (8.04 ± 2.04) but less than that of *E. coli* BL21(λDE3) *icd* mutant overexpressing *gltA* gene (10.14 ± 1.5) (Chapter 9). Thus, *ppc* overexpression alone (Chapter 8) and in the presence of *gltA* overexpression appear to direct the flux towards anaplerotic reactions in *E. coli* BL21(λDE3) *icd* mutant. In addition, *ppc* overexpression helps in lowering acetate secretion, which is in agreement with earlier reports (Farmer and Liao, 1999), but higher than control. Overexpression of *gltA* in the *icd* mutant (Chapter 9) also demonstrated high acetate outflow compared to the control. Hence, present study of *ppc*

and *gltA* co-expression in an *icd* mutant demonstrated pyruvate flux towards acetate secretion leading to decreased citrate production.

Parameters	*E. coli* MA1935 *ppc* overexpression	*E. coli* MA1935 *gltA* overexpression	*E. coli* MA1935 *ppc* and *gltA* overexpression
Growth rate	High	unaltered	High
Glucose consumption rate	Unaltered	~ 2 fold high	~ 2 fold decrease
Glucose consumed	unaltered	reduced	Reduced
G-6-PDH	unaltered	unaltered	~ 3 fold increase
CS	~ 2 fold decrease	~ 2 fold increase	~ 4 fold increase
PPC	~ 3 fold increase	unaltered	~2.5 fold increase
ICL	~ 3 increase	unaltered	~ 4 fold increase
Citrate (intra cellular)	~ 4 fold decrease	~ 5 fold increase	~ 2.5 fold increase
Acetate	ND	~ 4 fold increase	~ 8 fold increase

Table 10.6: Effect of *ppc*, *gltA* gene overexpression in *E. coli* MA1935 *icd* mutant on glucose. ND- not detected.

The extracellular citrate levels were lower compared to the high intracellular citrate accumulation and with that observed in case of *E. coli* BL21(λDE3) *icd* mutant overexpressing *cs* gene demonstrating an inefficient citrate transport system. Overexpression of *ppc* and *gltA* in *E. coli* MA1935 *icd* mutant demonstrated ~3 fold increase in G-6-PDH activity and ~4 fold increase in ICL activity on glucose. High G-6-PDH activity correlated with the high growth rate which was in agreement with earlier reports where high growth rate resulted in high G-6-PDH activity (Wolfe et al., 1979). Overexpression of *ppc* also demonstrated increased ICL activity but the result remains unexplained.

Similar results with respect to growth rate, citrate accumulation and secretion was observed on glycerol. The levels of citrate (mM) were lower but the yields

(g.citrate/g.glycerol) were higher than that observed on glucose. This data is in agreement with the high flux observed on glycerol (Holmes, 2001). But increase in glycerol utilization in the triple transformants and the double transformant overexpressing *gltA* gene remains unclear.

Hence, the present study demonstrates that *ppc* overexpression (to meet the OAA demands) increases the flux towards anaplerotic reactions while it supports that CS activity plays a key role in citrate production.

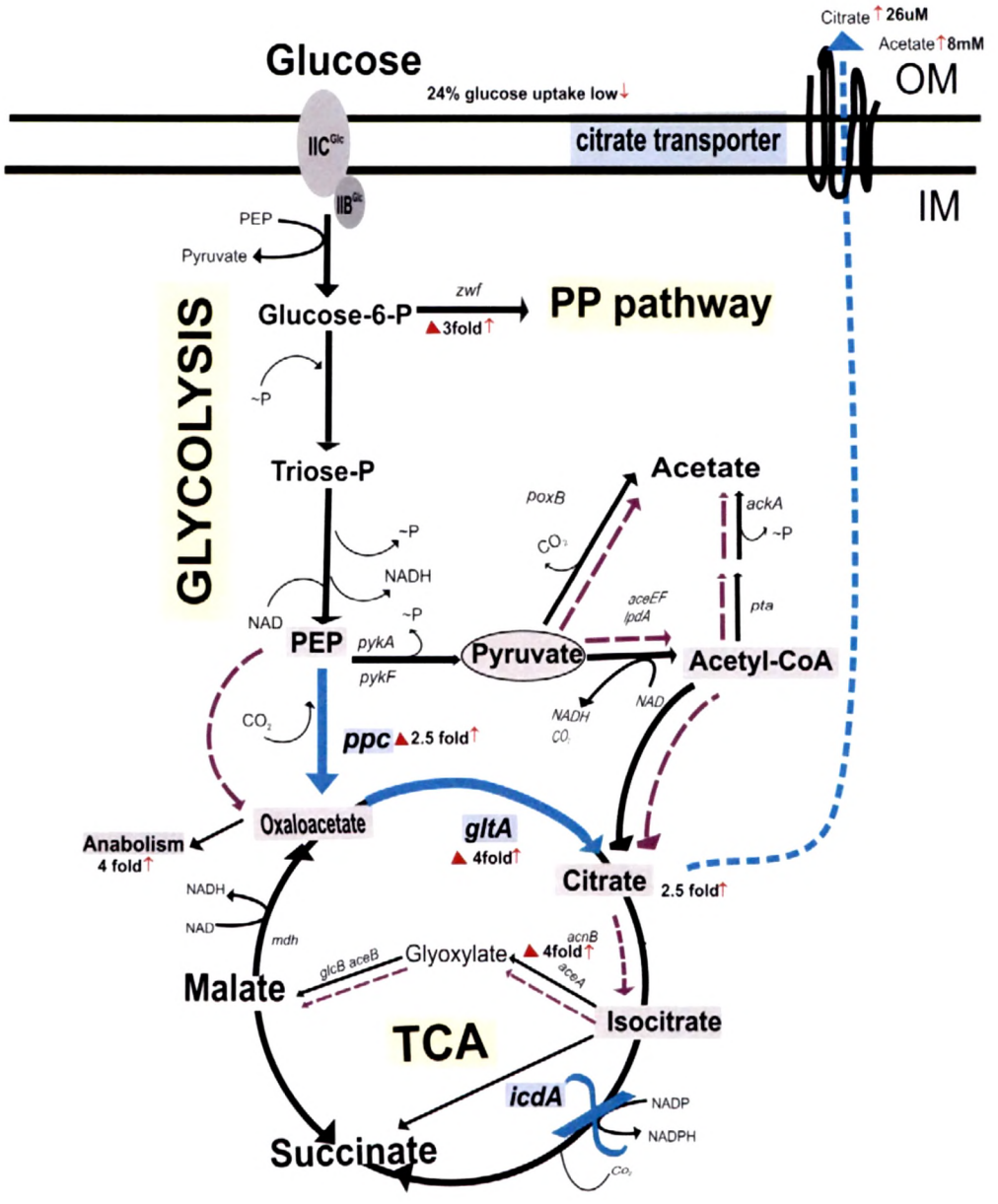

Fig 10.3: Summarized effects of *gltA* and *ppc* overexpresssion on glucose catabolism in *E. coli* MA1935 *icd* mutant. Numbers depicted along the arrows are the fold increase in response to *ppc* and *gltA* overexpression on M9 minimal medium with 50mM glucose. The genes upregulated are depicted as triangles. The metabolite concentrations are referred in μM and mM besides the arrows. Glucose utilized is depicted as percent utilization.