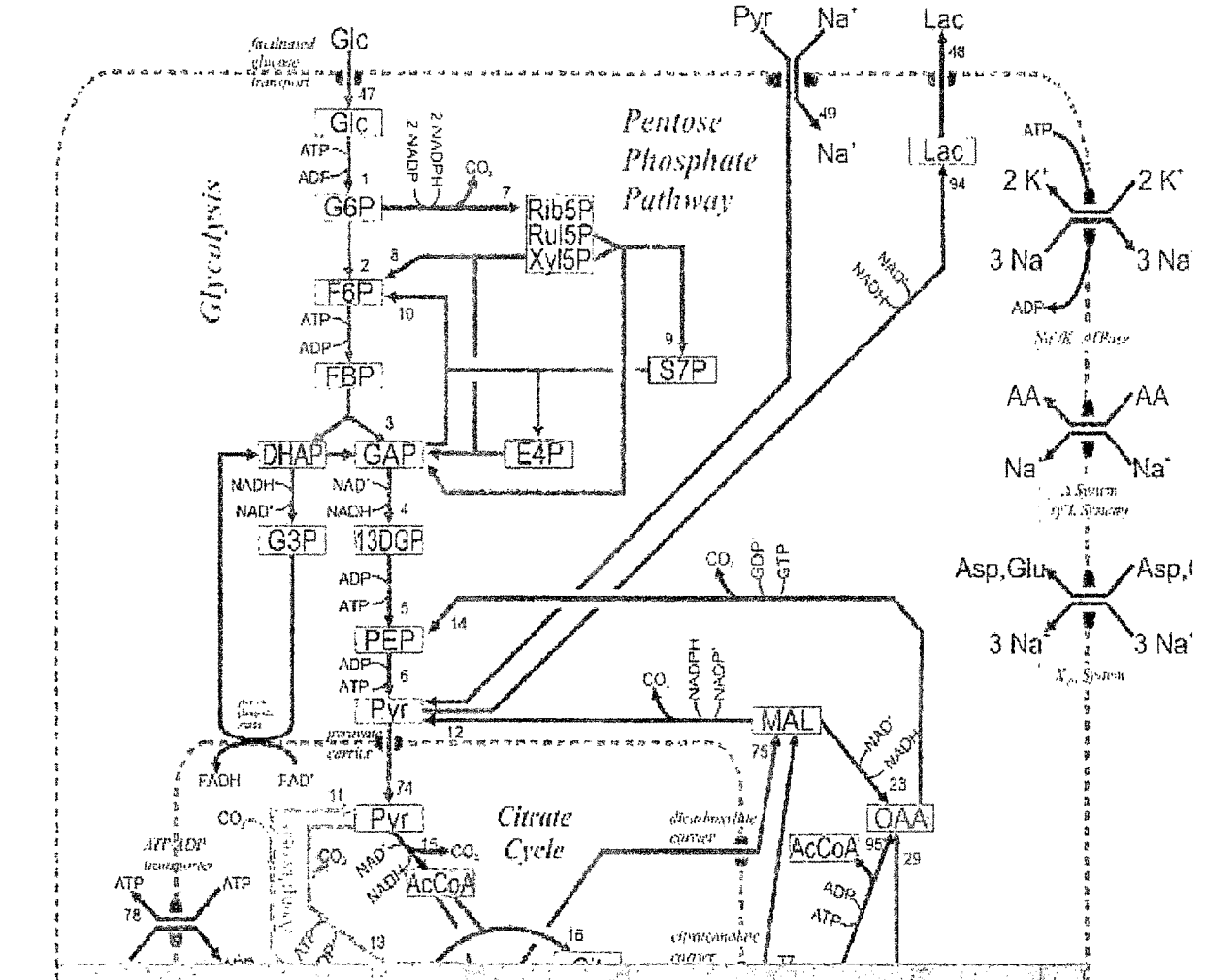

CHAPTER 9

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

9.1: INTRODUCTION

Citric acid (2-hydroxy-propane-1,2,3 tricarboxylic acid) is a universal product of metabolism and it is commonly found in all plants and animals. The first industrial process for citric acid production was carried out using *Aspergillus niger* in 1919. Many more microorganisms belonging to this family are known to secrete citrate eg. *A. niger, A. awamori, A. nidulans, A. fonsecaeus, A. luchensis, A. phoenicus, A.wentric, A.saitoi, A. flavus, Botyritis, Eupenicillium sp., Mucor periforum, Peniclillium janthinellum, Trichoderma viride and Ustulina vulgaris* (Papagianni, 2007). *A. niger* growing on high sugar and mineral salts, low pH (2.5-3.5) accumulated high citrate. Apart from these molds a large number of yeasts viz *Candida, Hasenula, Pischia, Torula, Saccharomyces* and *Yarrowia*, produce citrate but *C. tropicallis, C. catenula, C. guilliermondii* and *C. intermedia* are commercially viable. One of the common drawbacks using yeast was that they produced high isocitrate as a byproduct. Hence, mutants with low aconitase activity were selected for citrate production.

Very few bacterial species like *Corynebacterium, Arthrobacter, Brevibacterium, Bacillus sp., Bradyrhizobium japonicum*, and *Citrobacter koseri* are known to secrete or accumulate citrate (Carson et al., 1992; Rohr et al., 1996; Gyaneshwar et al., 1998; Khan et al., 2006). Despite having a long list of microorganisms producing citrate *A. niger* still is the most preferred industrial strain for its efficient ability to produce citrate. Even with this long history of citrate production very little has been looked into the biochemical basis of the process during fermentation. *A. niger* has been extensively investigated at the physiological and biochemical conditions that allow citrate accumulation (Berovic and Legisa, 2007; Legisa and Mattey, 2007; Papagianni, 2007).

9.1.1: Biochemical basis of citric acid accumulation in fungi, yeast and bacteria

Optimization of the bioprocess for commercial citrate production has been carried out using *A. niger* grown on molasses, sucrose or glucose (Xu et al., 1989; Ali et al., 2002; El-Holi and Al-Delaimy, 2003; Haq et al., 2004). *Candida* (including *Yarrowia lipolytica*) were optimized utilizing various carbon sources including n-alkanes, glucose, raw glycerol, ethanol and galactose (Anastassiadis and Rehm, 2005; Rymowicz et al., 2006).

Factors that play a role in enhancing citrate overflow in *A. niger* include the type and concentrations of the carbon source, nitrogen and phosphate limitation, pH, aeration, concentration of trace elements and morphology of the producer (**Table 9.1**) (Mlakar and Legisa, 2006). Apart from the media composition, citrate formation in this mold is regulated by - (i) fast uptake of glucose based on simple diffusion; (ii) Unrestricted metabolic flow through glycolysis, making precursors for synthesis of TCA cycle intermediates readily available; and (iii) Uncoupled NADH re-oxidation resulting in lower levels of ATP and therefore decreased anabolic reactions.

Parameter	Requirement for citric acid accumulation
Carbon source concentration	Must be higher than 50 g l^{-1}
Carbon source type	Must enable rapid catabolism
Nitrogen source	Consumption must lead to decrease in pH
Phosphate concentration	Must be suboptimal
Aeration	Must be in excess
Trace metal ions	Must be limiting, especially Mn^{2+}
pH	Must be below pH 3

Table 9.1: Environmental parameters influencing citric acid accumulation by *A. niger* (Röhr et al. 1996).

Phosphofructokinase-1 (PFK-1) is the key enzyme responsible for regulating the glycolytic flux in *A. niger* and it is susceptible to activation by fructose-2,6-bisphosphate (F-2,6-bP), AMP and NH_4^+ and inhibition by PEP, citrate and ATP (Röhr et al., 1996; Kubicek and Kominek, 1996). Efficient citric acid accumulation in *A. niger* in the presence of excess sugar is attributed to increased intracellular levels of F-2,6-bP which relieves the ATP inhibition of PFK-1 leading to increased and uncontrolled glycolytic rate, which is a prerequisite for citric acid accumulation (Kubicek-Pranz et al., 1990; Mlakar and Legisa, 2006).

Citrate overproduction in *A. niger* is regulated by NH_4^+ levels in the medium and its uptake is correlated with glucose transport but the mechanism is unclear (Papagianni et al., 2005). During the early phase of citric acid fermentation by *A. niger* on glucose, uptaken NH_4^+ combines with glucose to form glucosamine. Glucosamine-6-phosphate deaminase has been suggested to be responsible for glucosamine accumulation as a consequence of direct competition with PFK-1 for the common substrate fructose-6-phosphate (F-6-P). Glucosamine accumulation reduces the glycolytic flux by the removal of F-6-P leading to slower citrate accumulation rate (Solar et al., 2008). When NH_4^+ is depleted from the medium, one of the substrates for glucosamine deaminase becomes limiting and hence F- 6-P can be catabolized by PFK-1 leading to enhanced glycolytic flux. Nitrogen depletion in the fermentation medium, and increase in intracellular NH_4^+ concentration caused by proteolysis, triggered citrate production and secretion induction in yeasts (Anastassiadis and Rehm, 2005). Further enhancement in citric acid production is achieved by media optimization such as iron depletion

9.1.2: Genetic manipulations for citric acid overproduction.

Mathematical modeling of citric acid pathway in *A. niger* predicted that increase in the glucose influx could increase the citric acid productivity by 45% (Guebel and Torres Darias, 2001). Increase in the mitochondrial phosphate carrier activity was proposed to enhance citric acid production in *A. niger*. Papagianni (2007) has summarized various modeling strategies (kinetic, metabolic, stoichiometric, mechanistic, deterministic and morphologically structured) for enhancing citrate production.

Overexpression of *pfk*, pyruvate kinase (*pk*) and citrate synthase (*cs*) genes in *A. niger* was not successful in increasing the citrate production (Ruijter et al. 1997; 2000). On the other hand, *Yarrowia lipolytica* improved citric acid production with isocitrate lyase (*icl*) gene overexpression on glycerol, glucose and sucrose (Forster et al., 2007a). Recombinant *Y. lipolytica* expressing invertase and *icl* genes produced high yields of citric acid from sucrose, with a concomitant reduction in the formation of the by-product, isocitrate (Forster et al., 2007b). *E. coli* K and B isocitrate dehydrogenase (*icd*) mutants (Lakshmi and Helling, 1976; Aoshima et al., 2003; Kabir and Shimizu, 2004) and *B. subtilis icd* mutant (Matsuno

et al., 1999) reported high intracellular accumulation of citrate. *E. coli icd* mutants also demonstrated 2 fold increased CS activity (Lakshmi and Helling, 1976; Aoshima et al., 2003). However, role of CS in citrate accumulation is unclear.

9.1.3: Biochemistry of Citrate Synthase

Citrate synthase (*glt*A) is a ubiquitous enzyme that catalyzes the first committed step of Tricarboxylic acid (TCA) cycle involving condensation of oxaloacetate (OAA) and acetyl-CoA to form citrate. It plays a key role in directing the carbon flux towards TCA for energy production (aerobic) or to generation of various biosynthetic precursors (anaerobic) thus making it an indispensable enzyme (Park et al., 1994). *B. subtilis* and *E. coli* have two citrate synthase genes localized in the chromosome (Patton et al., 1993; Jin and Sonenshein, 1994) while in *Rhizobium tropici* one is chromosomally localized and another is found in the symbiotic plasmid (Hernández- Lucas et al., 1995). *Pseudomonas aeruginosa* PAC514 possesses two isozymes of CS, CSI and CSII (Solomon and Weitzman, 1983; Mitchell and Weitzman, 1986; Mitchell et al 1995). The two citrate synthase genes (*cit*A and *cit*Z) of *B. subtilis* are differentially expressed in response to the metabolic conditions. *cit*A did not show any significant role in regulating growth, CS activity or sporulation while *cit*Z gene is important for sporulation suggesting that it played an important role in regulating the metabolism (Jin and Sonenshein, 1996). In case of *R. tropici,* two citrate synthase genes were required for nodulation (Lucas et al., 1995). In *P. aeruginosa* PAC514, CSI was found during exponential phase and CSII was found during stationary phase and both were demonstrated to be integral part of a multienzyme complex of TCA cycle enzymes catalyzing the consecutive reactions from fumarate to 2-oxoglutarate (Mitchell, 1996).

9.1.4: Effects of *gltA* gene overexpression in *E. coli* and other microorganisms

E. coli gltA mutants failed to grow on glucose and required supplementation of glutamate or other TCA cycle intermediates (Gruer et al., 1997; Vandedrinck et al., 2001; De Maeseneire et al., 2006). Overexpression or underexpression of *gltA* gene in *E. coli* showed no effect on growth on glucose while on acetate as sole carbon source it influenced the growth rate (Walsh and Koshland, 1985a; Vandedrinck et al., 2001). *E. coli* demonstrated an increase in maximum cell dry weight by 23 % and reduced acetate

secretion with *gltA* gene overexpression (De Maeseneire et al., 2006). Overexpression of citrate synthase gene in *A. niger* did not alter citric acid production (Guebel and Darias, 2001). Overexpression of mitochondrial citrate synthase in *Arabidopsis thaliana* and carrot enhanced citrate secretion which improved growth on a phosphorus-limited soil and Al-phosphate medium, respectively (Koyama et al., 1999; 2000). Similarly in tobacco (*Nicotiana tabacum*; CSb lines) overexpression of citrate synthase gene from *P. aeruginosa* improved P uptake and Al tolerance by secreting citrate (de la Fuente et al., 1997; López-Bucio et al., 2000) but this strategy was not reproducible for citric acid mediated enhancement in P uptake and Al-tolerance (Delhaize et al., 2001). On the other hand, overexpression of mitochondrial citrate synthase gene alone and in combination with mitochondrial malate dehydrogenase (MDHI) increased citrate accumulation by 2-3 fold thus enhanced the Al-tolerance in *S. cerevisiae* and canola (*Brassica napus cv Westar*) (Anoop et al, 2003). Similarly high CS activity coupled with poor aconitase (ACN) and NAD-isocitrate dehydrogenase (NAD-ICD) enhanced citrate accumulation in *Pseudomonas fluorescens* in response to aluminum toxicity when grown on malate. (Mailloux et al., 2008).

9.1.5: Rationale of the present study

Earlier work demonstrates that *E. coli* MA 1935, *icd* mutant, does not accumulate high amount of citrate on minimal media as observed in case of rich media (Aoshima et al., 2003). On rich media, the strain also exhibited high CS activity which correlated with the high citrate accumulation. Hence the present work investigates the role of *gltA* overexpression in citrate accumulation in *E. coli* MA1935.

9.2: WORK PLAN

9.2.1: Bacterial strains used for the present study

E. coli strains	Genotype	References
DH5α AB7	*gltA* gene cloned under P_{tac} promoter in pUCpM18 plasmid (*kan*^{r}) resistance gene, Tc^r, Ap^r	Present study
DH5α pJE1	P_{tac} promoter, (*kan*^{r}) resistance gene, Km^r, Ap^r	Present study
DH5α pJE2	*gltA* gene cloned under P_{tac} promoter, (*kan*^{r}) resistance gene. Km^r, ap^r	Buch, 2008
MA1935 pJE1:pVSD1	P_{tac} promoter, (*kan*^{r}) resistance gene, citrate transporter. Chl^r, Km^r, Ap^r	Present study
MA1935 pJE2: pVSD1	*gltA* gene cloned under P_{tac} promoter, (*kan*^{r}) resistance gene, citrate transporter. Chl^r, Km^r, Ap^r	Present study
W620	*gltA* mutant strain exhibiting glutamate auxotrophy, *Str*^{r}	*E. coli* genetic stock center
W620 pJE1	*gltA* mutant harboring pJE1 Str^r ,Km^r, Ap^r	Present study
W620 pJE2	*gltA* mutant harboring pJE2 $Str,^r$ Km^r, Ap^r	Present study

Table 9.2: *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.

9.2.2: Cloning of *gltA* gene under P_{tac} promoter.

gltA gene of *E. coli* from pAB7 plasmid was subcloned in pTTQ18, a pUC based high copy number plasmid with *LacZ*α gene, under P_{tac} promoter. Both the plasmids were digested with *Eco*RI / *Hind*III enzymes. pAB7 gave two fragments 5,100bp (vector backbone) and 2,967bp corresponding to *gltA* gene along with kanamycin marker (*npt*) gene while pTTQ18 was linearized. Both the vector and insert were gel purified using the method discussed in section 2.3.5 and were ligated. Ligation mixture was transformed into *E. coli*

DH5α and the clones were selected on kanamycin plates. The *gltA carrying* plasmid was designated as pJE2 (**Fig. 9.1**).

To construct the control vector pAB7 and pTTQ18 were subjected to BamH1/HindIII double digestion. pAB7 released a 1,637bp fragment corresponding to kanamycin resistance (*npt*) gene. Both the vector and insert were gel purified ligated. Ligation mixture was transformed into *E. coli* DH5α and the clones were selected on kanamycin plates. The plasmid was designated as pJE1 (**Fig. 9.1**).

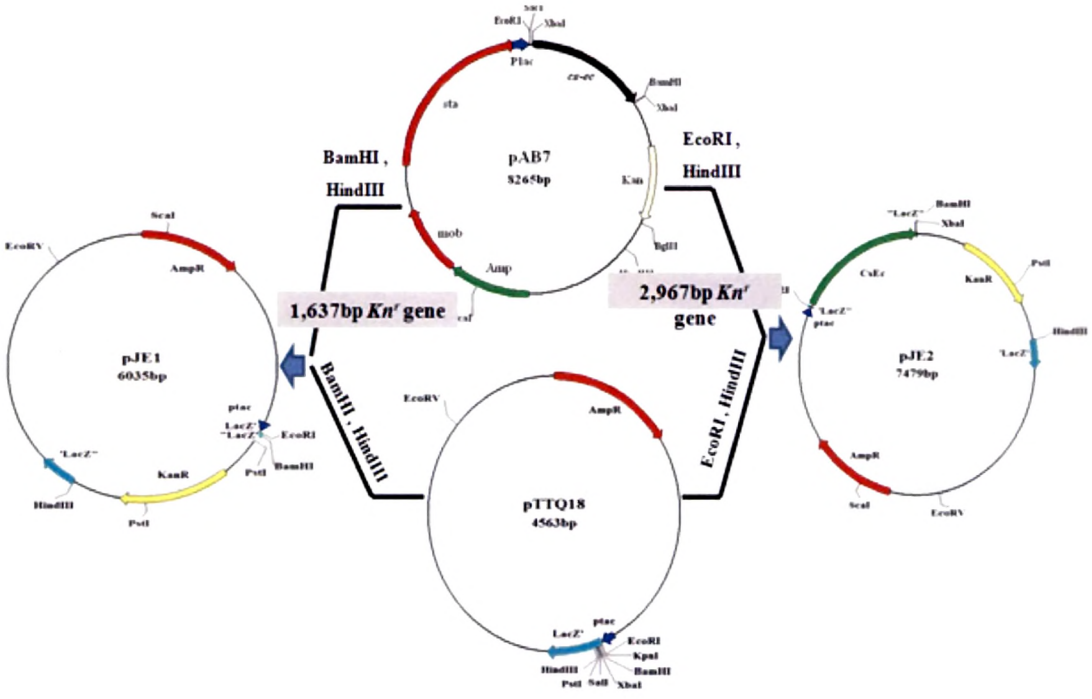

Fig. 9.1: Schematic representation of construction of vector carrying *E. coli gltA* gene under *tac* promoter.

All the molecular biology techniques like plasmid preparation, transformation, gel elution and purification, restriction digestion, DNA ligation and gel electrophoresis were performed based on the protocol described in section 2.3.

9.2.3: Functional confirmation of *gltA* expressed from pJE2 and Incorporating pJE1 and pJE2 into *E. coli* MA1935 *icd* mutant expressing citrate transporter.

E. coli W620 *gltA* mutant required glutamate supplementation when grown on glucose media. pJE1 and pJE2 plasmids were transformed into *gltA* mutant. The transformants were selected on streptomycin and kanamycin (concentrations mentioned in table 2.3) plates. Plasmids were isolated and confirmed by restriction digestion and were subjected to complementation studies (section 2.4).

E. coli MA1935 carrying pVSD1 was also transformed with pJE1 and pJE2 plasmids, respectively, to obtain *E. coli* MA1935 pJE1:pVSD1 (control) and *E. coli* MA1935 pJE2:pVSD1 (test) strains.

9.2.4: Monitoring on growth and physiological parameters of *E. coli* MA1935 *icd* mutant overexpressing *gltA* gene.

The *E. coli* MA1935 transformants were grown on M9 minimal medium with micronutrients and 50mM glucose and 100mM glycerol as carbon source. IPTG (0.1 mM) 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.

9.3: RESULTS

9.3.1: Construction and functional characterization of *gltA* gene under P_{tac} promoter

Based on the above mentioned strategies (section 9.2.2, **Fig. 9.1**) *gltA* gene was cloned under the *tac* promoter of high copy number pTTQ18 plasmid with Km^r gene. pJE1 and pJE2 plasmids were confirmed by restriction digestion pattern using different restriction enzymes (**Fig. 9.2**). The plasmids were further confirmed for their functionality through mutant complementation. *E. coli gltA* mutant strain harboring pJE1 and pJE2 were grown on M9 minimal media with glucose in presence and absence of glutamate. *E. coli* W620 harboring pJE2 grew on glucose in the absence of glutamate as compared to the control

harboring pJE1 (**Fig. 9.3**). *E. coli* DH5α pJE2 showed CS activity of 0.160 ± 0.05 U compared to *E. coli* DH5α pJE1 0.09 ± 0.02 U, ~1.8 fold increase in CS activity. The double transformants used in the present study were designated as *E. coli* MA1935 pJE1: pVSD1 and *E. coli* MA1935 pJE2: pVSD1.

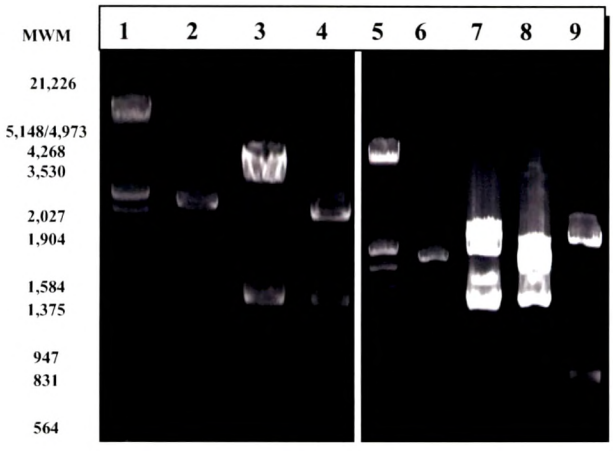

Fig. 9.2: Restriction digestion pattern of pJE1 and pJE2. Lane 1,5: Molecular weight marker EcoRI/HindIII double digest, Lane 2: pTTQ18 BamHI/HindIII (4,563bp), Lane 3: pAB7 BamHI/HindIII (6,608bp, 1,637bp), Lane 4: pJE1 BamHI/HindIII (4,563bp, 1,637bp), Lane Lane 6: pTTQ18 EcoRI /HindIII (4,563bp), Lane 7: pAB7 EcoRI/HindIII (5,100bp, 2,967bp), Lane 8: pJE2 EcoRI /HindIII (4,563bp, 2,967bp), Lane 9: pJE2 BamHI/HindIII (5,893bp, 1,637bp).

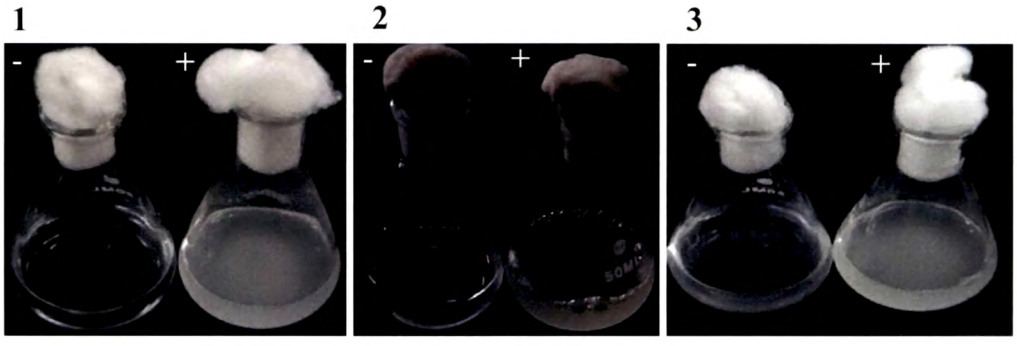

Fig 9.3: Complementation of *E. coli* W620 mutant phenotype by pJE2 plasmids. 1: *E. coli* W620-deletion mutant of *gltA* gene **2**: *E. coli* W620 with pJE1 plasmid; **3**: *E. coli* W620 with pJE2 plasmid induced with 0.1mM IPTG. Growth is monitored on M9 medium with 0.2% glucose –/+ at the corners of each image indicates absence and presence of 340μg/ml glutamate in the medium, respectively.

9.3.2: Physiological parameters of *E. coli* MA1935 *gltA* gene carrying citrate transporter.

E. coli MA1935 expressing *gltA* gene of *E. coli,* on glucose showed no change in the growth rate but significantly increased the glucose consumption rate and reduced the amount

of glucose consumed with respect to the control. Significant increase in biomass was also observed in *E. coli* MA1935 *icd* mutant expressing *gltA* gene **(Table 9.3, Fig. 9.3 (i) and (ii)).**

On glycerol, *E. coli* MA1935 pJE2:pVSD1 and *E. coli* MA1935 pJE1: pVSD1 showed a comparatively poor growth to that on glucose but overexpression of *gltA* improved glycerol uptake and biomass **(Table 9.4, Fig. 9.3 (iii) and (iv)).**

E. coli strains	Growth rate μ (h^{-1})	Specific glucose consumption rate (Q glc) ($gglc.g^{-1}h^{-1}$)	Total glucose utilized (mM)	Biomass $Y_{dcw/glc}$ ($gdcw.g^{-1}.h$)
MA1935 pJE1:pVSD1	0.34 ± 0.02	1.93± 0.73	15.16 ± 2.2	0.07 ± 0.01
MA1935pJE2:pVSD1	0.31 ±0.09	3.85 ±0.96	6.2 ± 1.9	0.18 ± 0.02
	ns	***	**	***

Table 9.3: Physiological variables and metabolic data from *E. coli* MA1935 *gltA* transformants grown on M9 minimal medium with 50mM glucose. The results are expressed as Mean ±SD of 8-10 independent observations. Specific growth rate ($\mu(h^{-1})$), 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 pJE1:pVSD1	0.14 ± 0.003	22.77 ± 5.5
MA1935pJE2:pVSD1	0.17 ± 0.002***	56.8 ± 2.5***

Table 9.4: Physiological variables and metabolic data from *E. coli* MA1935 *gltA* transformants 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 ($\mu(h^{-1})$) was 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.

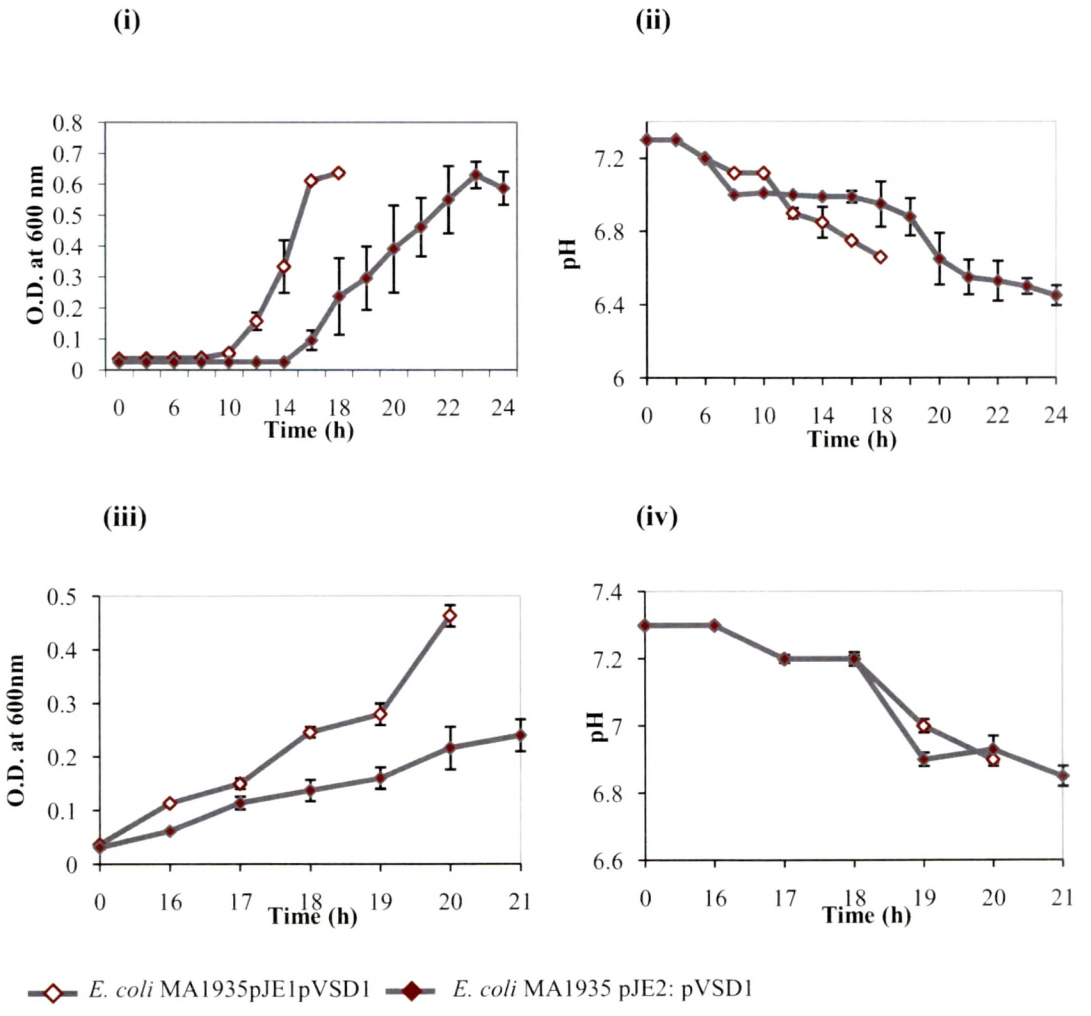

Fig 9.4: Growth characteristics of *E. coli* MA1935 *gltA* gene on M9 minimal medium with 50 mM glucose and 100mM glycerol. (i) & (ii) $O.D_{600nm}$ and pH on glucose, (iii) & (iv) $O.D_{600nm}$ and pH on glycerol. All values plotted are represented as Mean ± SD for n= 4 to 7 observations.

9.3.3: Effect of *gltA* gene overxpression on the CS, G-6-PDH and ICL activities in *E. coli* MA1935.

The double transformant of *E. coli* MA1935 expressing *gltA* gene showed a ~ 1.8 fold increase in CS activity and ~ 1.3 fold increase in G-6-PDH activity compared to the

control. No alterations were seen in PPC and ICL activities in response to *gltA* overexpression (**Fig. 9.5**). Similar results observed in *E. coli* MA1935 pJE2:pVSD1 when grown on glycerol.

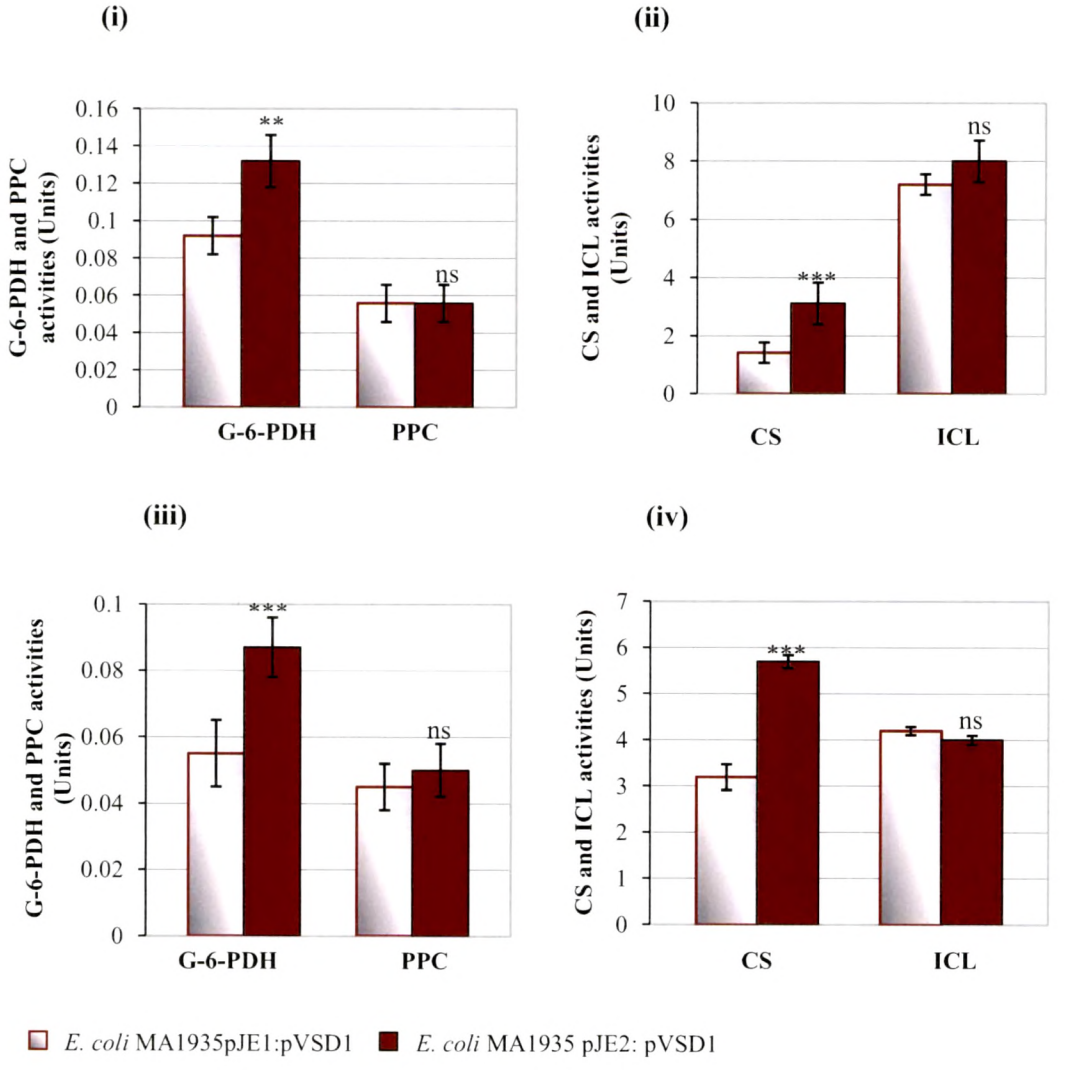

Fig. 9.5: Effect *gltA* overexpression in *E. coli* BL21(λDE3) *icd* mutant on enzyme activities in presence and absence of citrate transporter on M9 minimal medium with 50 mM glucose and 100mM glycerol. (i) and (ii) enzyme activities on glucose (50mM) (iii) and (iv) enzyme activities on glycerol (100mM). 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. **p<0.01, ***p<0.001, ns-non significant.

9.3.4: Effect of *gltA* gene overexpression on the organic acid profile in *E. coli* MA1935.

On glucose, *E. coli* MA1935 overexpressing *gltA* showed ~ 3.25 fold increase in the intracellular citrate level compared to the control **(Table 9.5)**. Almost 60 fold increase in the extracellular citrate was observed in presence of the citrate transporter in the same strain. Overexpression of *gltA* gene also significantly increased acetate secretion compared to that of the control. *E. coli* MA1935 pJE2:pVSD1 showed 4.7 ± 1.2 mM acetate which was absent in the control. On glycerol, *E. coli* MA1935 pJE2:pVSD1 had accumulated more intracellular citrate than that observed in case of glucose medium **(Table 9.5)**.

E. coli strains	Intracellular citrate		Extracellular citrate	
	(mM)	Yield (g/g)	(µM)	Yield (g/g)
On Glucose 50mM				
MA1935 pJE1:pVSD1	3.12 ± 0.43	0.23 ± 0.05	ND	ND
MA1935 pJE2:pVSD1	10.14 ± 1.5	0.32 ± 0.3	60 ± 5	0.0018 ± 0.02
	***	***	***	***
On Glycerol 100mM				
MA1935 pJE1:pVSD1	1.4 ± 0.08	1.8 ± 0.2	ND	ND
MA1935 pJE2:pVSD1	15.1 ± 1.8	8.6 ± 0.95	85 ± 8	0.24 ± 0.04
	***	***	***	***

Table 9.5: Intracellular citrate accumulation in *E. coli* MA1935 expressing *gltA* gene under P_{tac} promoter. 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.

	50 mM glucose	
***E. coli* strains**	Extracellular Acetate	
	mM	**Yield (g/l)**
MA1935 JE1:pVSD1	ND	ND
MA1935pJE2: pVSD1	4.7 ± 1.2***	0.027 ± 0.003***
	100 mM glycerol	
MA1935 JE1:pVSD1	ND	ND
MA1935pJE2: pVSD1	1.8 ± 0.03***	0.06 ± 0.007***

Table 9.6: Acetate secretion in *E. coli* MA1935 overexpressing *gltA* gene 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.

9.4 : DISCUSSION

Overexpression of *gltA* gene (~ 1.8 fold) in *E. coli* MA1935 *icd* mutant on glucose had no effect on growth but increased the glucose consumption rate by ~2 fold and reduced glucose consumption by ~ 40 %. These results were contradictory to the results where up to 50 fold increase in CS activity did not the alter glucose utilization but reduced the glucose consumption rate by 50% (Walsh and Koshland, 1985a, De Maeseneire et al., 2006). ~1.8 fold increase in CS activity in the *icd* mutant resulted in ~ 3.25 fold increase in the intracellular accumulation of citrate. This was in agreement with *E. coli icd* mutant with ~3.8 fold increase in CS activity increased citrate accumulation by ~4 fold at stationary phase on rich medium (Lakshmi and Helling, 1976; Aoshima et al., 2003). This also supports that the medium composition plays a significant role in regulating the central carbon metabolism (Tao et al., 2005). The *icd* mutation resulted in poor growth and biomass probably due to increase in energy demand which further multiplied in response to protein expression induced by IPTG but this effect is not significant on rich medium. Accumulation

of citrate was detrimental to growth of *E. coli icd* mutant with *gltA* overexpression on rich medium but not in minimal medium. *gltA* overexpression did not alter other enzyme activities like that of PPC and ICL in minimal medium but it significantly increased G-6-PDH activity. These results were in agreement with earlier reports on *E. coli icd* mutant with increased CS activity demonstrating high G-6-PDH activity to meet the NADPH requirement (Kabir and Shimizu, 2004).

Approximately 60 fold increase in extracellular was observed in presence of the citrate transporter. Considering the high amount of intracellular citrate the extracellular levels were relatively low suggesting that the citrate transporter (H^+citrate) was inefficient compared to the other organisms like *Pseudomonas*, *Citrobacter koseri* and *A. niger* which demonstrated high citrate secretion (Mailloux et al., 2005; Gyaneshwar et al, 1998; Burgstaller, 2006). An efficient transport system for citrate secretion was considered to rate limiting in case of yeast (Anastassiadis and Rehm, 2005) while in addition to citrate transport, transport of sugar and ammonia into the cell was important for citrate production in *A. niger* (Papagianni, 2007). *gltA* overexpression resulted in high acetate (~ 5 fold) out flow compared to the control suggesting that block in ICDH down regulates TCA flux in conjunction with *gltA* overexpression directing pyruvate towards acetate overflow.

On glycerol, *gltA* overexpression (~ 2.2 fold) showed similar results with respect to G-6-PDH, PPC, ICL and citrate accumulation. The amount of citrate accumulated and secreted were higher than that on glucose media. This was in agreement with high flux through TCA on glycerol (Holms, 2001).

The present study demonstrates that CS activity plays a key role in citrate accumulation in the background of *icd* mutation as against *gltA* overexpression upto ~50 fold in *E. coli* did not result in citrate accumulation. In *A. niger*, large number of enzyme modulation and nutrients play significant role in citrate production (Ruijter et al., 2000; Guebel and Torres, 2001) while in *Pseudomodas* and *Bradyrhizobium japonicum* citrate secretion is dependent on Al- toxicity (Delhaize et al., 2001; Lopez-Bucio et al., 2000) or Fe deficiency (Carson et al., 1992). Thus, the present study demonstrates that down regulation

of ICDH coupled with *gltA* overexpression is required for efficient citrate accumulation but an efficient citrate efflux mechanism is needed for its secretion.