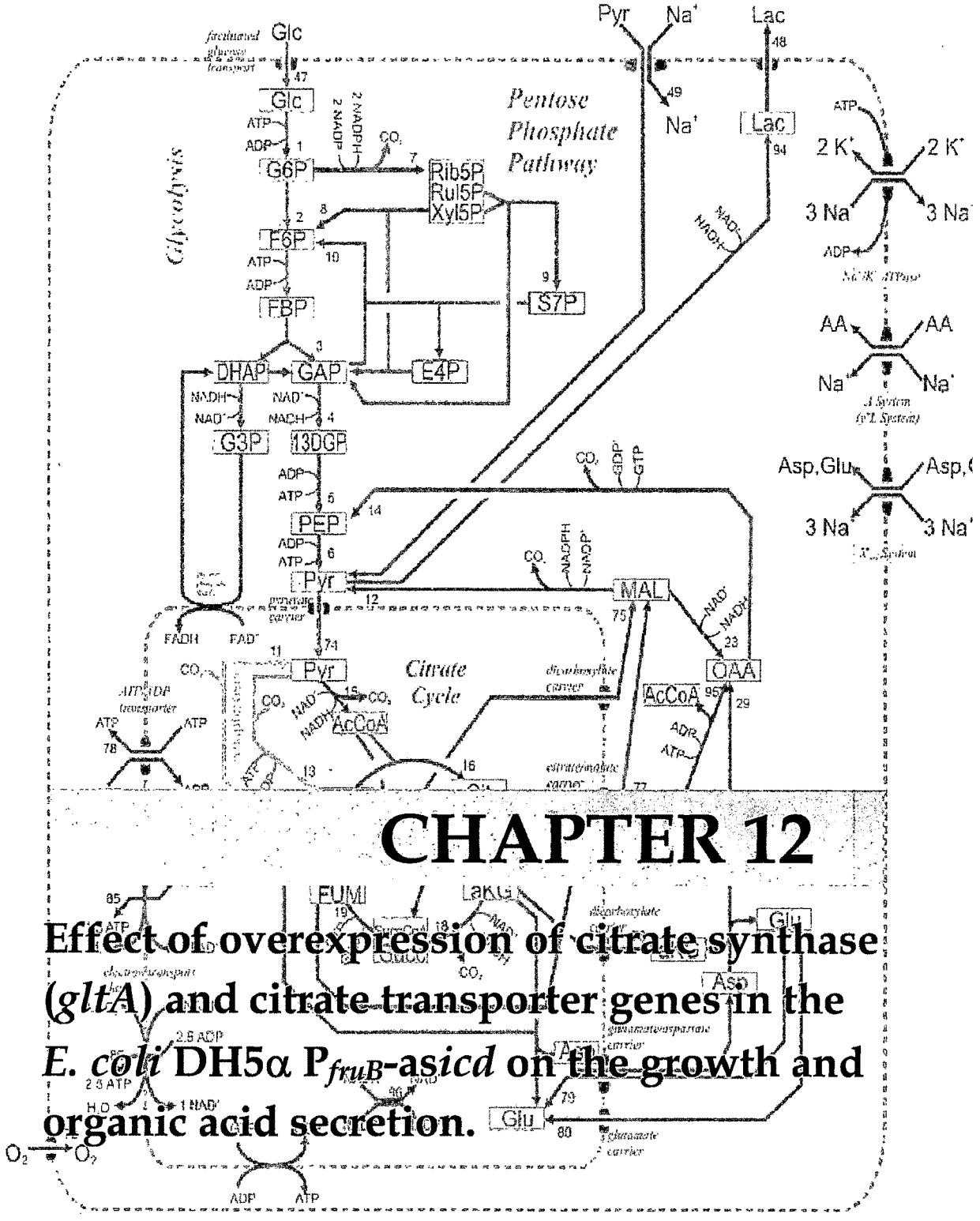

CHAPTER 12

Effect of overexpression of citrate synthase (*gltA*) and citrate transporter genes in the *E. coli* DH5α P_{fruB}-as*icd* on the growth and organic acid secretion.

12.1: Rationale of work.

E. coli icd mutants (rich media) and as-*icd* expressed in *E. coli* strains showed increased CS activity and high citrate accumulation. This work suggested that CS could play a significant role in citrate metabolism. Hence, the present study investigates the overexpression of *gltA* gene coupled with as-*icd* expression under P_{fruB} promoter in *E. coli* DH5α expressing citrate transporter.

12.2: PLAN OF WORK

12.2.1: Bacterial strains used in the present study.

E. coli strains	Genotype	References
DH5α pTZ57R	P_{lac}, Ap^r	Ch 1
DH5α pVS2k3	as-*icd* under P_{fruB}, Ap^r	Ch1
DH5α pTZ57Rg	pTZ57R carrying *aac* gene Gm^r, Ap^r	Present study
DH5α pVS2k3g	as-*icd* under P_{fruB}, *aac* gene Gm^r, Ap^r	Present study
DH5α pJE1	pTTQ18, P_{tac}, Km^r, Ap^r	Ch 9
DH5α pJE2	*gltA* gene cloned under P_{tac}, Km^r, Ap^r	Ch 9
DH5α pJE1:pVSD1	P_{tac}, citrate transporter, Km^r, Chl^r, Ap^r	Present study
DH5α pJE2:pVSD1	*gltA* gene, citrate transporter, Km^r, Chl^r, Ap^r	Present study
DH5α pTZ57Rg :pJE1:pVSD1	P_{tac}, citrate transporter ,Km^r, Chl^r, Gm^r, Ap^r	Present study
DH5α pVS2k3g:pJE2:pVSD1	P_{fruB} as-*icd*, *gltA* gene, citrate transporter, Km^r, Chl^r, Gm^r, Ap^r	Present study

Table 12.1: List of bacteria strains used in the study. The details of the plasmids and the concentrations of the antibiotics used for both the rich media and the minimal media are listed in table 2.2 and 2.3.

12.2.2: Incorporation of gentamycin resistance (*aac*) gene in pVS2k3 and pTZ57R.

Plasmids pGM160, pTZ57R and pVS2k3 were digested with *Hind*III; pGM160 released a 1,674bp corresponding *gen*^{r} fragment and pTZ57R and pVS2k3 were linearized both the vectors and the insert were gel purified and set up for ligation. The ligation mixture was transformed into *E. coli* DH5α and the transformants were selected on gentamycin plates. The plasmids were isolated and confirmed for insert release by restriction digestion with *Hind*III. The plasmids were designated as pTZ57Rg (control plasmid) and pVS2k3g (test). All the molecular biology techniques like plasmid preparation, gel purification, transformation and electrophoresis are described in section 2.3.

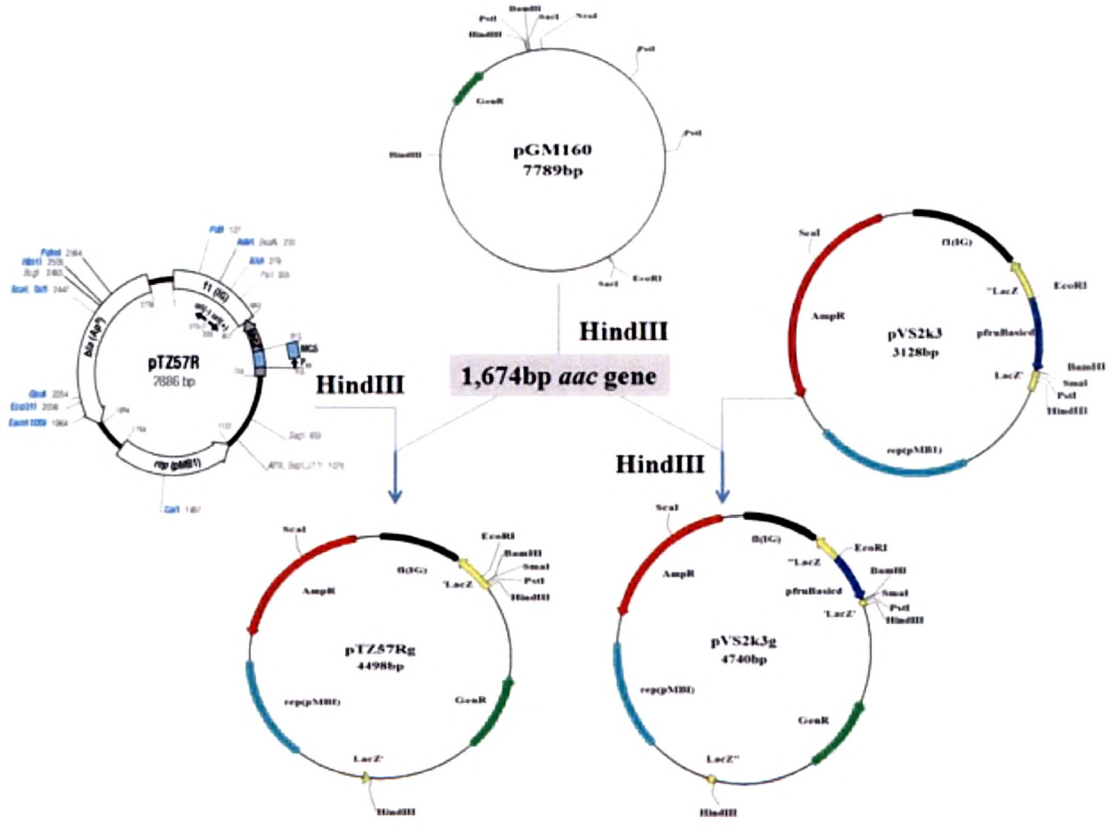

Fig. 12.1: Schematic representation for construction of pTZ57Rg and pVS2k3g.

12.2.3: Incorporation of pJE2 (*cs* gene cloned under P_{tac}) and pVS2k3g (P_{fruB}-*asicd*) in *E. coli* DH5α with citrate transporter.

Plasmids (pJE1, pJE2, pTZ57Rg and pVS2k3g) were isolated from *E. coli* DH5α and then transformed into *E. coli* DH5α : pVSD1. Transformation of pJE1 and pJE2 into *E. coli* DH5α :pVSD1 gave the double transformants while transforming *E. coli* DH5α pJE1: pVSD1 and *E. coli* DH5α pJE2: pVSD1 with pTZ57Rg and pVS2k3g, respectively, gave the triple transformants. All transformants were selected on the respective antibiotic plates (antibiotic concentration as mentioned in table 2.3.

12.2.4: Determination of physiological parameters.

The *E. coli* DH5α transformants were grown on M9 minimal medium with micronutrients and 50mM glucose as carbon source. 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, G-6-PDH, CS and ICL at stationary phase.

12.3 RESULTS

12.3.1: Construction of pVS2k3g and developing *E. coli* DH5α overexpressing both *gltA* and as-*icd* in presence of citrate transporter.

Plasmids pTZ57Rg and pVS2k3g were confirmed through restriction digestion pattern showing the 1,674bp insert release using *Hind*III (**Fig. 12.1**). The double transformants expressing *gltA* gene and citrate transporter and the triple transformant expressing both *gltA* and as-*icd* along with citrate transporter were designated as *E .coli* DH5α pJE1:pVSD1, *E .coli* DH5α pJE2:pVSD1, *E .coli* DH5α pTZ57Rg:pJE1:pVSD1 and *E .coli* DH5α pVS2k3g:pJE2:pVSD1 respectively.

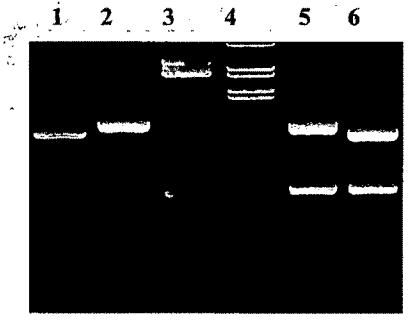

Fig. 12.2: Restriction digestion map of pTZ57Rg and pVS2k3g. Lane 1: pTZ57R HindIII (2,886bp), Lane 2: pVS2k3 HindIII (3,128bp), Lane 3: pGM160 HindIII (6,115bp and 1,674bp), Lane 4: BstEII molecular weight marker, Lane 5: pVS2k3g HindIII (3,128bp and 1,674bp), Lane 6: pTZ57Rg HindIII (2,886bp and 1,674bp).

12.3.2: Effect of *gltA* gene overexpression coupled with down regulation of ICDH on the physiological parameters in *E. coli* DH5α with citrate transporter.

Overexpression of *gltA* gene in *E. coli* DH5α with citrate transporter showed no change in the growth rate, glucose utilization rate and the glucose utilized but it significantly improved the biomass (**Table 12.2, Fig. 12.3(i) and (ii)**).

***E. coli* strains**	**Growth rate μ (h^{-1})**	**Specific glucose consumption rate (Qglc) (gglc.g $dcw^{-1}.h^{-1}$)**	**Total glucose consumed (mM)**	**Biomass ($Y_{dcw/glc}$) (gdcw.$g^{-1}.h^{-1}$)**
DH5αpJE1:pVSD1	0.27 ± 0.01	7.21 ± 0.33	50 ± 0.86	0.11 ± 0.01
DH5α pJE2:pVSD1	**0.26 ± 0.01**	**6.42 ± 0.16**	**49 ± 1.4**	**0.14 ± 0.01**
	ns	ns	ns	*
DH5α pTZ57Rg :pJE1:pVSD1	0.26 ± 0.01	5.38 ± 0.24	32 ± 0.61	0.10 ± 0.01
DH5α pVS2k3g:pJE2:pVSD1	**0.39 ± 0.01**	**4.24 ± 0.35**	**47 ± 1.32**	**0.22 ± 0.01**
	***	*	***	***

Table 12.2: Physiological parameters demonstrating the effect of P_{fruB}-a*sicd* expression and *gltA* overexpression in *E. coli* DH5α 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.

Overexpression of *gltA* gene coupled with ICDH down regulation (P_{fruB}-as*icd*) in *E. coli* DH5α with citrate transporter showed a significant increase in growth rate, glucose consumption rate, glucose utilized and biomass (**Table 12.2, Fig. 12.3(iii) and (iv)**).

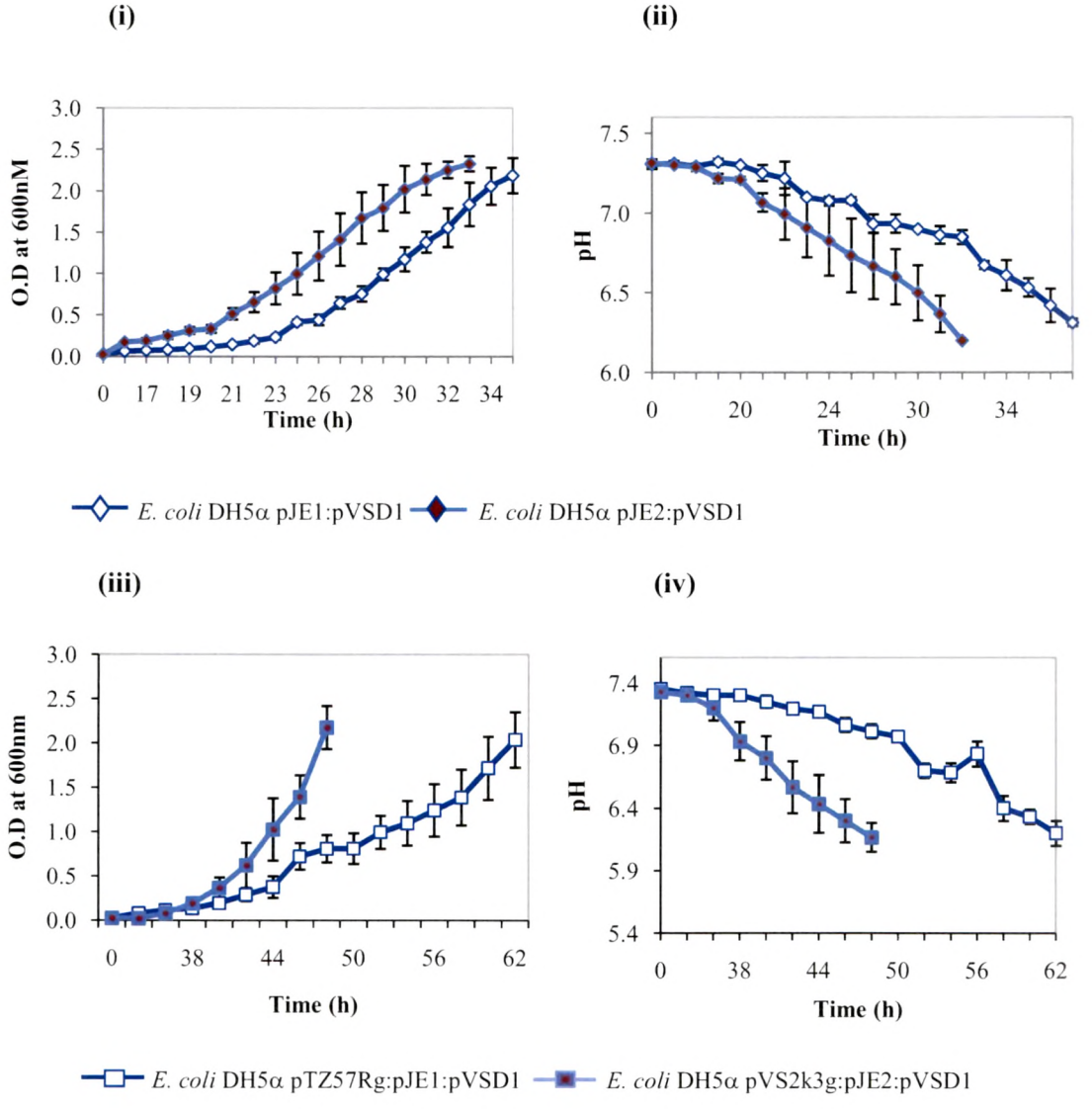

Fig. 12.3: Effect of *gltA* overexpression coupled with ICDH down regulation in *E. coli* DH5α on growth and acidification. (i) & (ii) $O.D_{600nm}$ and pH for *E. coli* DH5α overexpressing *gltA* and (iii) & (iv) $O.D_{600nm}$ and pH for *E. coli* DH5α overexpressing *gltA* and as-*icd* All values plotted are represented as Mean ± SD for n= 4 to 7 observations.

12.3.3: Effect of *gltA* gene overexpression coupled with down regulation of ICDH on enzyme activities and organic acid secretion in *E. coli* DH5α with citrate transporter.

Overexpression of *gltA* gene by ~2 fold in *E. coli* DH5α with citrate transporter significantly decreased G-6-PDH activity while no alteration in ICDH activity (**Fig. 12.4**). ~2.5 fold, increase in CS activity was observed in *E. coli* DH5α pVSD1 expressing as-*icd* under P_{fruB} promoter ((**Fig. 12.5**). As-*icd* expression demonstrated ~2 fold lower ICDH activity with no alteration in G-6-PDH activity. No ICL activity was observed in either of the *E. coli* cultures on glucose. Overexpression of *gltA* gene also increased intracellular citrate levels by ~1.5 fold and decreased acetate secretion by ~2.25 fold. ~2.5 fold high CS activity coupled with ~2 fold reduction in ICDH activity increased intracellular citrate levels and acetate secretion by ~2 fold (**Table: 12.3**). No extracellular citrate was detected.

E. coli strains	Intracellular citrate		Acetate	
	(mM)	Yield (g/g)	(mM)	Yield (g/g)
DH5α pJE1:pVSD1	0. 87 ± 0.01	0.018 ± 0.002	5.3 ± 1.5	0.002 ±0.00
DH5α pJE2:pVSD1	**1.30 ± 0.01**	**0.027 ± 0.001**	**2.1 ± 0. 95**	**0.001 ±0.00**
	**	***	***	***
DH5α pTZ57Rg :pJE1:pVSD1	1.02 ± 0.01	0.034 ± 0.01	1.8 ± 0.92	0.001± 0.00
DH5α pVS2k3g:pJE2:pVSD1	**2.14 ± 0.014**	**0.048 ± 0.01**	**3.85 ± 0.56**	**0.002 ±0.00**
	***	***	***	***

Table 12.3: Organic acid production of *E. coli* DH5α expressing both as-*icd* and *gltA* gene under P_{fruB} promoter. The table depicts the citrate (intracellular) and acetate (extracellular) levels in the late stationary phase cultures of *E. coli* DH5α transformants (plasmid control and test) grown on M9 minimal media with 50mM glucose as carbon source. All the values are represented as Mean ± SD of n=4-8 observations. *** p<0.001, ns=non-significant.

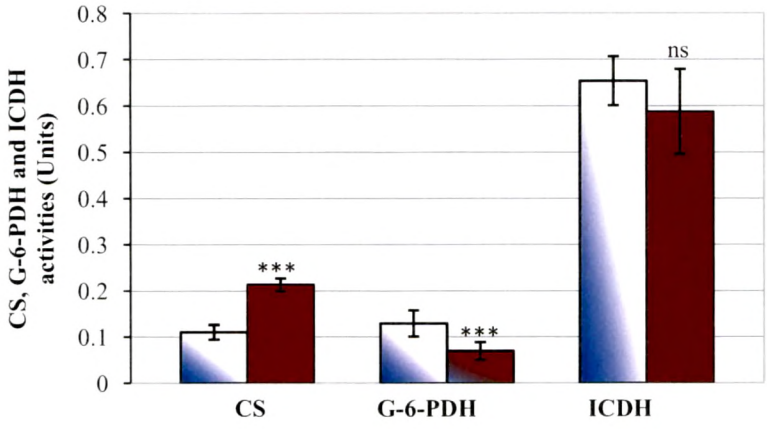

Fig. 12.4: Effect of *gltA* overexpression on enzyme activities in *E. coli* DH5α pVSD1 on M9 minimal medium with 50 mM glucose. Units for the specific activity are given as μmoles/mg. protein/min. All the values are represented as Mean ± SD for n= 5-8 observations. ***p<0.001, ns-non significant.

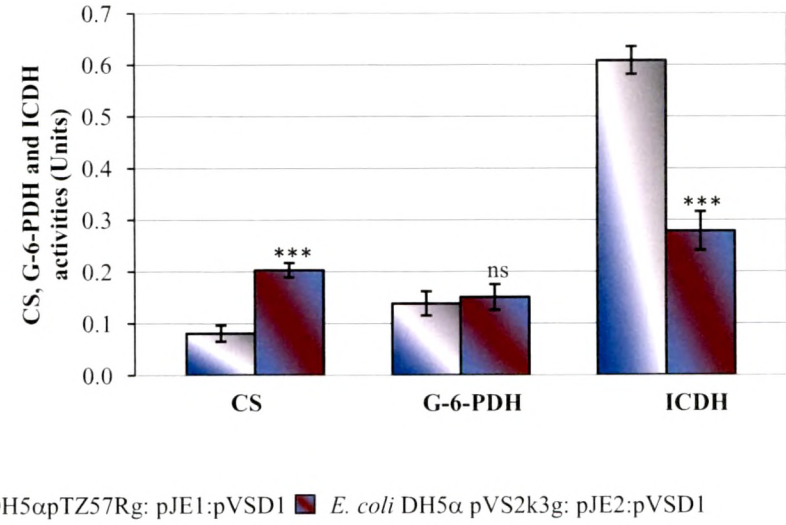

Fig. 12.5: Effect of *gltA* overexpression and ICDH down regulation on enzyme activities in *E. coli* DH5α pVSD1 on M9 minimal medium with 50 mM glucose. Units for the specific activity are given as μmoles/mg. protein/min. All the values are represented as Mean ± SD for n= 5-8 observations. ***p<0.001, ns-non significant.

12.4: DISCUSSION

The present study showed that overexpression of *gltA* gene upto ~2 fold in *E. coli* DH5α expressing citrate transporter had not influenced glucose consumption rate, glucose consumed and growth but it improved biomass by 2 fold. This result was in agreement with earlier reports of *gltA* overexpression in *E. coli* by 2 fold had not altered growth and glucose consumption rate but showed increase in cell dry weight (De Maeseneire et al., 2006). However, ~50 fold increase in CS activity decreased glucose utilization by 50% (Walsh and Koshland, 1985). Increased CS activity decreased acetate levels by ~2.25 fold which is in agreement with the earlier reports (Walsh and Koshland, 1985; De Maeseneire et al., 2006). In contrast to earlier reports intracellular citrate levels increased by ~1.5 fold.

Overexpression of *gltA* coupled with down regulation of ICDH (~2 fold) activity resulted in ~2.5 fold higher CS activity coupled with higher growth rate, poor glucose consumption rate, increased biomass and increase glucose utilization. Although *E. coli icd* mutants have demonstrated high CS activity, growth was poor and had reduced glucose consumption rate (Lakshmi and Helling, 1976; Aoshima et al., 2003; Kabir and Shimzu, 2004). The present work is partially in agreement with the earlier work with respect to poor glucose consumption rate. Citrate accumulation in response to increased CS activity was in agreement with the earlier reports of *E. coli icd* mutants accumulated high citrate (Lakshmi and Helling, 1976; Aoshima et al., 2003; Kabir and Shimzu, 2004). In contrast to *E. coli icd* mutants the present study showed ~ 2 fold increased acetate secretion. No extracellular citrate was observed supporting an inefficient citrate transport system.

Overexpression of *gltA* gene in the presence of as-*icd* did not alter the activities of G-6-PDH and ICL but in absence of as-*icd* decreased G-6-PDH activity. G-6-PDH has been considered as a marker enzyme for the growth of the organism (Wolf et al., 1979). Although, plasmid en-coded protein expression is known to reduce G-6-PDH activity (Flores et al., 2004) it remains unclear whether increased CS activity led to increased G-6-PDH activity or it was an effect of metabolic load. Thus, the present study supports, that CS activity could be the rate limiting enzyme regulating the TCA towards citrate biosynthesis.