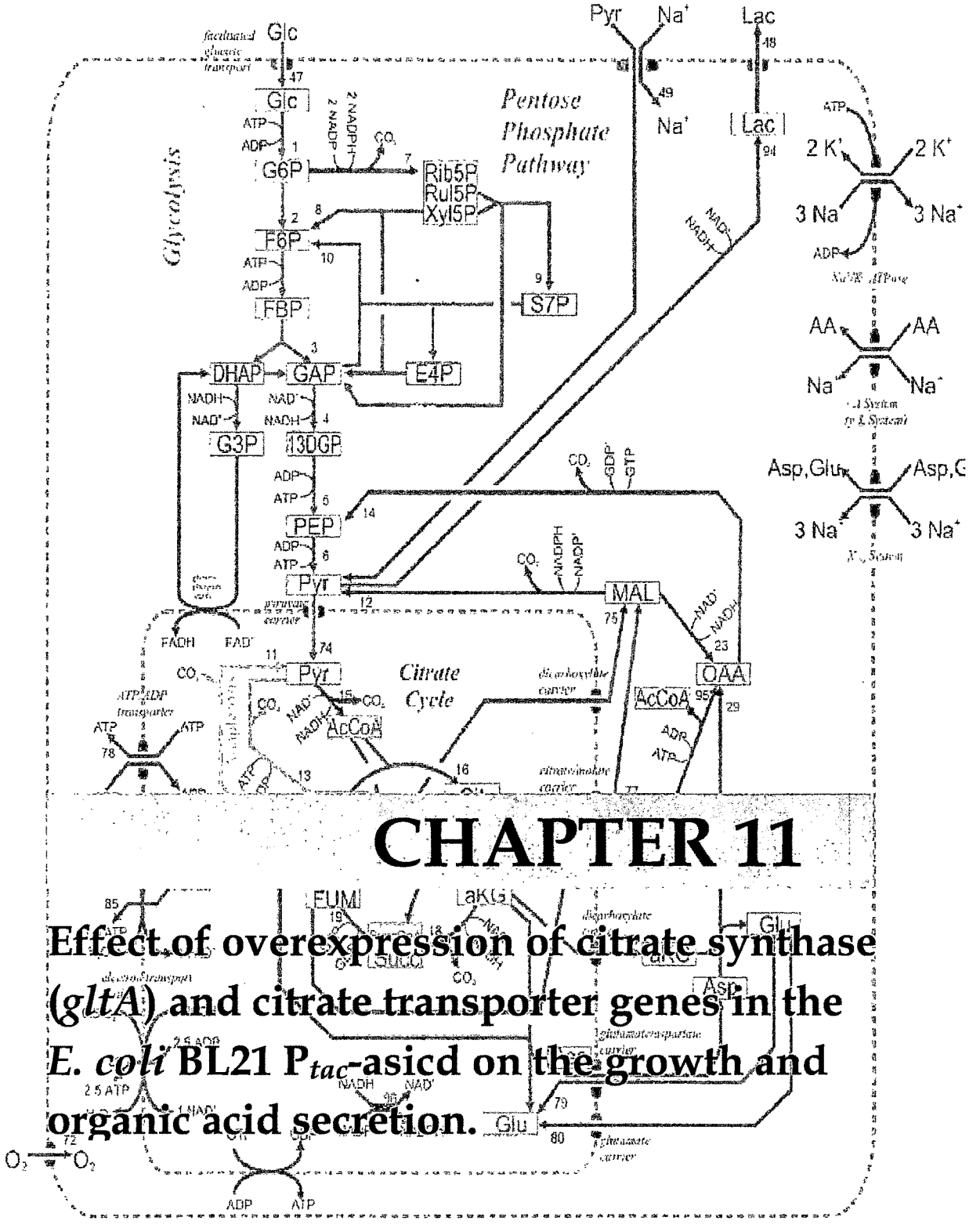


CHAPTER 11

Effect of overexpression of citrate synthase (*gltA*) and citrate transporter genes in the *E. coli* BL21 P_{tac}-asicd on the growth and organic acid secretion.

11.1: Rationale of the present study.

E. coli icd mutants (rich medium) and down regulation of ICDH activity using as-*icd* expressed under P_{tac} /P_{fruB} promoters in *E. coli* BL21 and *E. coli* DH5α respectively resulted in increased CS activity which led to high citrate accumulation. Hence, the present study investigates the effect of overexpression of *gltA* gene coupled with as-*icd* expression under P_{tac} promoter in *E. coli* BL21 expressing citrate transporter.

11.2: WORK PLAN

11.2.1: Bacterial strains used in the present study.

E. coli strains	Genotype	References
DH5α pTZ57Rg	pTZ5R carrying gentamycin resistance (*aac*) marker Gm^r, Ap^r	Chapter 12
DH5α pTTQ18	P_{tac}, Ap^r	Present study
DH5α pJE6	as-*icd* under P_{tac}, Ap^r	Present study
DH5α pJE7	pTTQ18 carrying *aac* gene Gm^r, Ap^r	Present study
DH5α pJE8	as-*icd* under P_{tac}, *aac* gene Gm^r, Ap^r	Present study
BL21pJE1:pVSD1	P_{tac}, citrate transporter under P_{lac}, Km^r, Chl^r, Ap^r	Present study
BL21 pJE2:pVSD1	*gltA* gene cloned under P_{tac}, citrate transporter, Km^r, Chl^r, Ap^r	Present study
BL21 pJE7:pJE1:pVSD1	P_{tac}, citrate transporter under P_{lac} Km^r, Chl^r, Gm^r, Ap^r	Present study
BL21pJE8:pJE2 : pVSD1	as-*icd* under P_{tac}, *gltA* gene cloned under P_{tac}, citrate transporter, Km^r, Chl^r, Gm^r, Ap^r	Present study

Table 11.2: List of bacteria strains used in the study. The details of the plasmids and the concentrations of the antibiotics used for both the rich medium and the minimal medium are listed in table 2.2 and 2.3.

11.2.2: Incorporation of gentamycin marker gene into pJE6.

Incorporation of gentamycin (*aac*) gene marker into pJE6 and pTTQ18; plasmids pTZ57Rg, pTTQ18 and pJE6 were digested with HindIII; pTZ57Rg released a 1674bp corresponding Gm^r fragment and pTTQ18 and pJE6 were linearized both the vectors and the insert was gel purified and set up for ligation. The ligation mixture was transformed into *E. coli* DH5α and the transformants were selected on gentamycin plates.

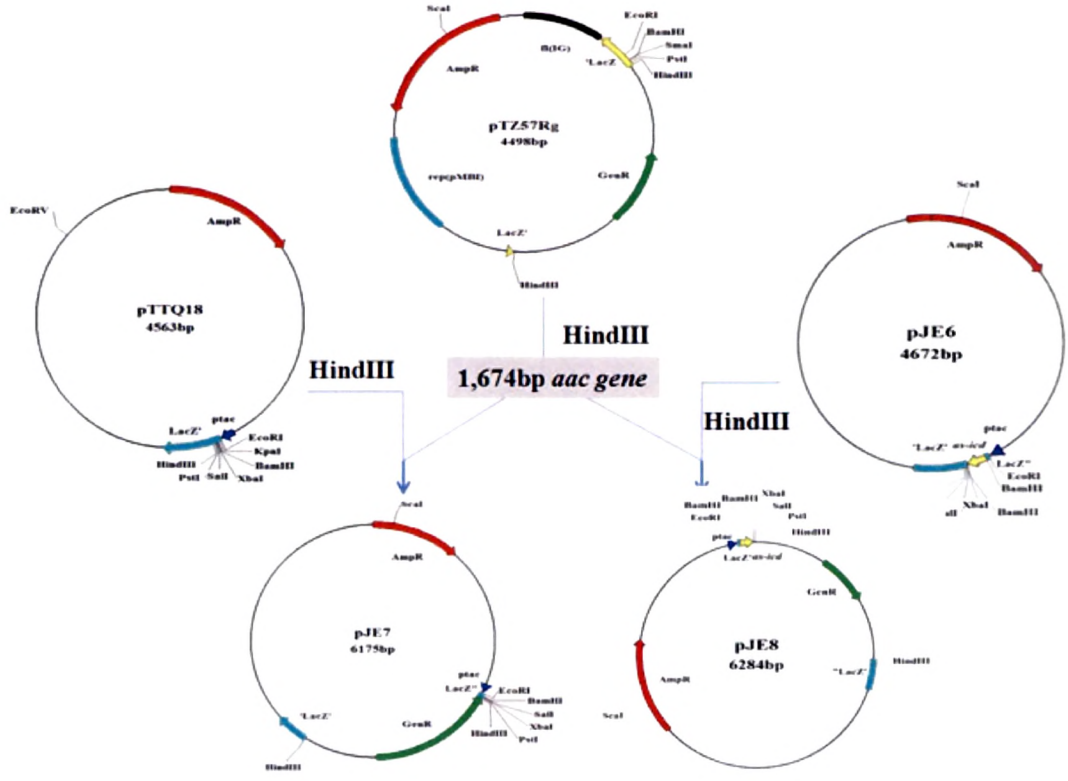


Fig. 11.1: Schematic representation for construction of pJE7 and pJE6. *aac* gene corresponds to Gm^r

The plasmids were isolated and confirmed for insert release by restriction digestion with HindIII. The plasmids were designated as pJE7 (control plasmid) and pJE8 (test). All the molecular biology techniques like plasmid preparation, gel purification, transformation and electrophoresis are described in section 2.3 of chapter 2.

11.2.3: Incorporation of pJE1, pJE2 and pJE8 in *E. coli* BL21 carrying citrate transporter.

Plasmids (pJE1, pJE2 , pJE7 and pJE8) were isolated from *E. coli* DH5α and then transformed into *E. coli* BL21pVSD1. Transformation of pJE1 and pJE2 into *E. coli* BL21pVSD1 gave the double transformants while transforming *E. coli* BL21 pJE1: pVSD1 and *E. coli* BL21 pJE2: pVSD1 with pJE7 and pJE8, respectively, gave the triple transformants. All the transformants were selected on the respective antibiotic plates (antibiotic concentration as mentioned in Table 2.3 of chapter 2.

11.2.4: Growth and physiological parameters.

The *E. coli* BL21 transformants were grown on M9 minimal medium with micronutrients and 100mM 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.

11.3: RESULTS

11.3.1: Construction of pJE8 and developing the *E. coli* BL21pVSD1 strains expressing both *gltA* and *as-icd*.

Plasmids pJE7 and pJE8 were confirmed through restriction digestion pattern showing the 1674bp insert release using HindIII (**Fig. 11.12 (i) and (ii)**). The double transformants expressing *gltA* and citrate transporter genes were designated as *E. coli* BL21 pJE1: pVSD1 and *E. coli* BL21 pJE2: pVSD1 and the triple transformants expressing both *gltA* and as-*icd* along with citrate transporter were designated as *E. coli* BL21 pJE1: pJE7: pVSD1 and *E. coli* BL21 pJE2: pJE8:pVSD1.

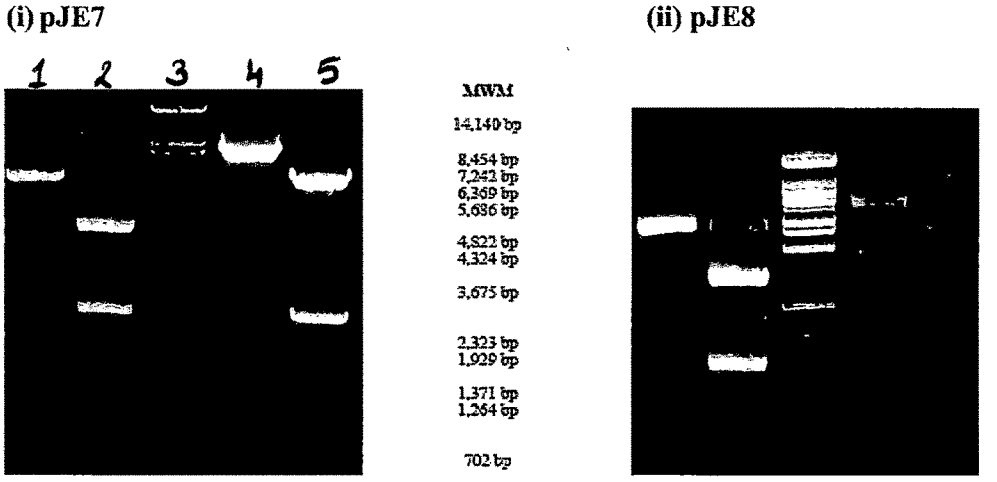


Fig. 11.2: Restriction digestion pattern for pJE7 and pJE8.

(i) **pJE7:** Lane 1: pTTQ18 HindIII (4,563bp), Lane 2: pTZ57Rg HindIII (2,886bp, 1,674bp), Lane 3: BstEII molecular marker, Lane 4: pJE7 EcoRI (6,175bp), Lane 5: pTTQ18 HindIII (4,563bp, 1,674bp).

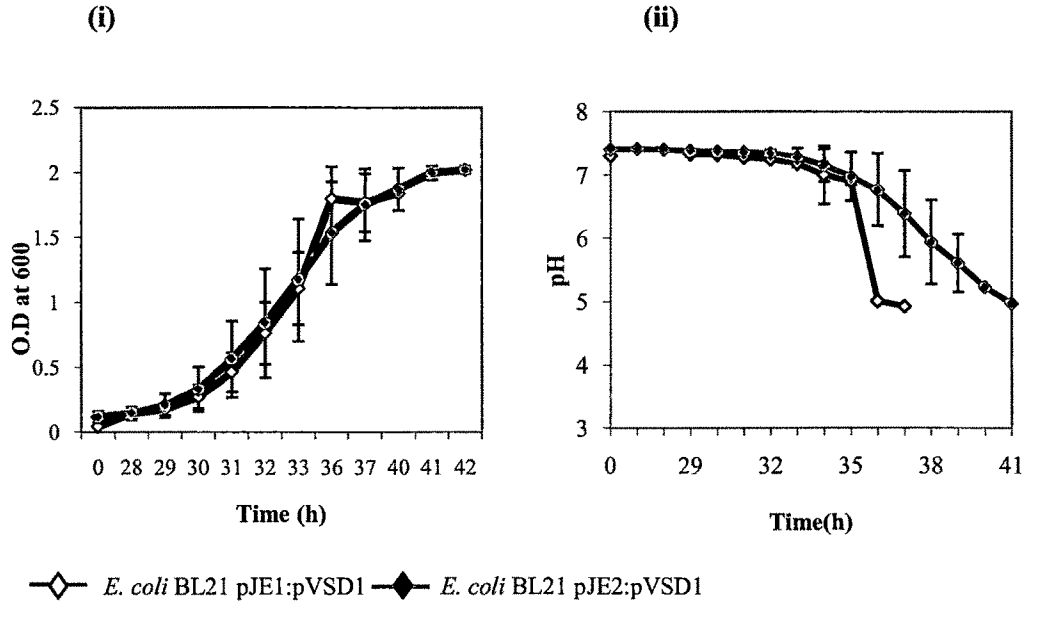
(ii) **pJE8:** Lane 1: pJE6 HindIII (4,663bp), Lane 2: pTZ57Rg HindIII (2,886bp, 1,674bp), Lane 3: BstEII molecular marker, Lane 4: pJE8 EcoRI (6,275bp), Lane 5: pJE8 HindIII (4,663bp, 1,674bp).

11.3.2: Monitoring the effect of *gltA* overexpression on growth and other physiological parameters in *E. coli* BL21pVSD1 both in presence and absence of *as-icd*.

Overexpression of *gltA* gene under P_{tac} promoter in *E. coli* BL21 expressing citrate transporter showed no significant change with respect to growth rate, specific glucose consumption rate, glucose consumed but significantly increased the biomass (**Table 11.2, Fig. 11.3 (i) and (ii)**). Co-expression of *gltA*with P_{tac} as-*icd* in *E. coli* BL21 expressing citrate transporter increased growth rate but decreased the glucose consumption rate and biomass (**Table 11.2, Fig. 11.3 (iii) and (iv)**).

***E. coli* strains**	**Growth rate μ (h^{-1})**	**Specific glucose consumption rate (Qglc) ($g.glc.g^{-1}$ $dcw.h^{-1}$)**	**Total glucose consumed (mM)**	**Biomass ($Y_{dcw/glc}$) ($g.dcw.g^{-1}glc.h^{-1}$)**
BL21 pJE1:pVSD1	0.43 ±0.09	5.96 ± 2.3	43.00 ± 2.3	0.12 ± 0.01
BL21 pJE2:pVSD1	**0.43 ±0.12 ns**	**5.30 ±1.36 ns**	**49.14 ± 4.5 ns**	**0.21 ± 0.01****
BL21 pJE7:pJE1:pVSD1	0.17 ± 0.02	7.11 ± 0.90	47.67 ± 2.3	0.18 ± 0.01
BL21 pJE8:pJE2:pVSD1	**0.30 ± 0.01 ****	**4.6 ± 1.06 ****	**50.34 ± 3.5 ns**	**0.11 ± 0.008 ****

Table 11.2: Physiological parameters demonstrating the effect of P_{tac}-a*sicd* expression and *gltA* overexpression in *E. coli* BL21 on M9 minimal medium with 100mM 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.



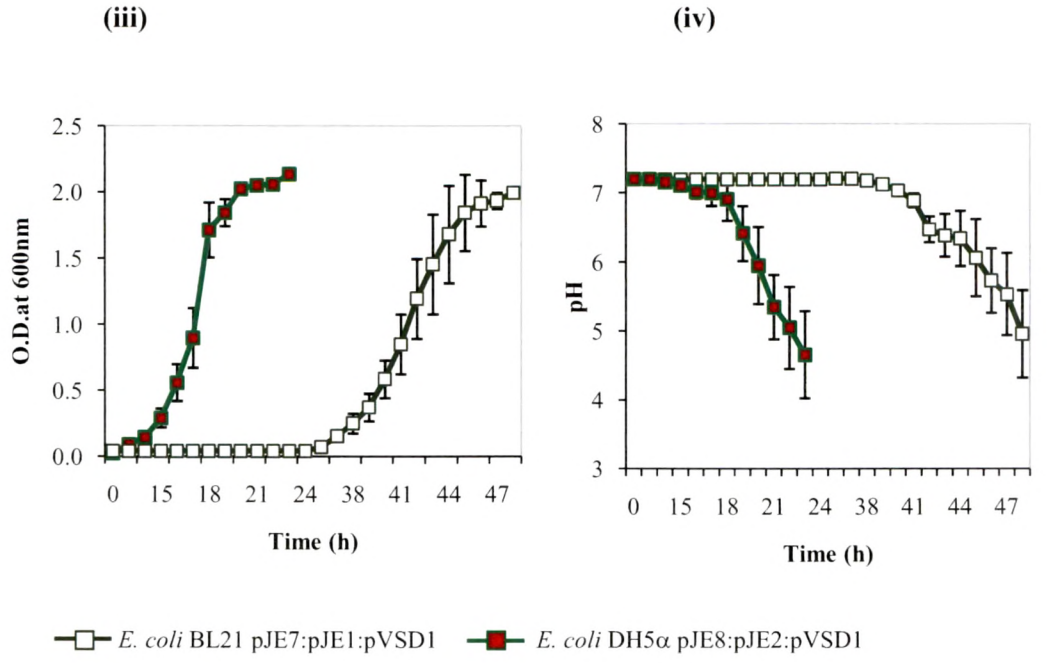


Fig. 11.3: Effect of *gltA* overexpression coupled with ICDH down regulation in *E. coli* BL21 on growth and acidification. (i) & (ii) $O.D_{600nm}$ and pH for *E. coli* BL21 overexpressing *gltA* and (iii) & (iv) $O.D_{600nm}$ and pH for *E. coli* BL21 overexpressing *gltA* and as-*icd* All values plotted are represented as Mean ± SD for n= 4 to 7 observations.

11.3.3: Monitoring the effect of *gltA* overexpression on ICDH, G-6-PDH and ICL in *E. coli* BL21pVSD1 both in presence and absence of *as-icd.*

Overexpression of *gltA* gene in absence of as-*icd* in *E. coli* BL21 expressing citrate transporter increased CS activity by 1.8 fold but did not alter ICDH, G-6-PDH and ICL activities (**Fig. 11.4**). Co-expression of *gltA* gene in presence of as-*icd* in *E. coli* BL21 expressing citrate transporter increased CS activity by 2.5 fold along with 2 fold reduction in ICDH. No alteration was found in G-6-PDH and ICL activities (**Fig. 11.5**).

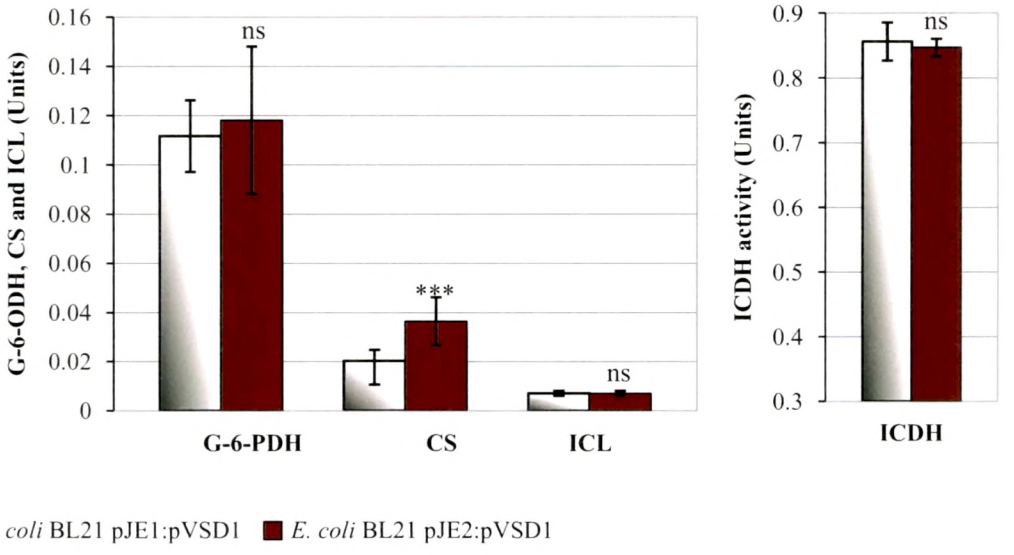


Fig. 11.4: Effect of *gltA* overexpression on enzyme activities in *E. coli* BL21 pVSD1 on M9 minimal medium with 50 mM glucose. Units for the specific activity are given as µmoles/mg. protein/min. All the values are represented as Mean ± SD for n= 5-8 observations. Units- µmoles/mg. protein/min. ***p<0.001, ns-non significant.

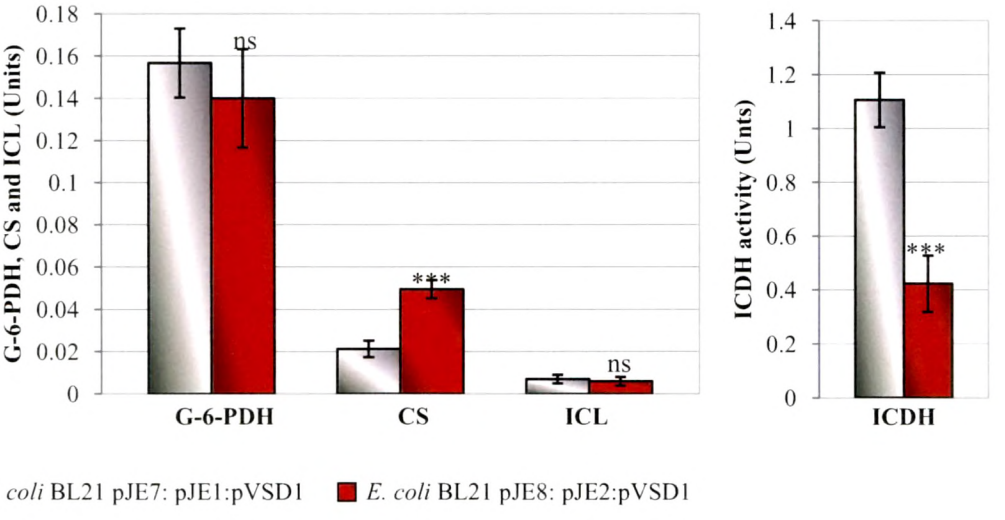


Fig. 11.5: Effect of *gltA* overexpression and ICDH down regulation on enzyme activities in *E. coli* BL21 pVSD1 on M9 minimal medium with 50 mM glucose. Units for the specific activity are given as µmoles/mg. protein/min. All the values are represented as Mean ± SD for n= 5-8 observations. Units- µmoles/mg. protein/min. ***p<0.001, ns-non significant.

11.3.4: Monitoring the effect of *gltA* overexpression on organic acid secretion in *E. coli* BL21pVSD1 both in presence and absence of *as-icd*.

Overexpression of *gltA* gene in *E. coli* BL21 expressing citrate transporter resulted in ~2.5 fold increase in citrate accumulation (intracellular) and ~ 2 fold increase in acetate secretion on glucose medium (**Table 11.3**). Co-expression of *gltA* gene with ICDH downregualtion using as-*icd* in *E. coli* BL21 expressing citrate transporter showed ~3.25 fold increase in intracellular citrate accumulation but no significant change was observed in acetate secretion (**Table 11.3**).

E. coli strains	Intracellular citrate (mM)		Acetate (mM)	
	(mM)	Yield(g/g)	(mM)	Yield (g/g)
BL21 pJE1:pVSD1	0.85 ± 0.07	0.002 ± 0.00	34 ± 1.7	0.013 ± 0.001
BL21 pJE2:pVSD1	**2.11 ± 0.09** ***	**0.045 ± 0.01** ***	**65 ± 1.2** ***	**0.021 ± 0.007** ***
BL21 pJE7:pJE1:pVSD1	1.52 ± 0.15	0.003 ± 0.00	37 ± 3.7	0.014 ± 0.004
BL21 pJE8:pJE2:pVSD1	**4.78 ± 0.34** ***	**0.101 ± 0.02** ***	**31 ± 1.3** ns	**0.010 ± 0.005** ns

Table 11.3: Organic acid production of *E. coli* BL21 expressing both as-*icd* and *gltA* gene under P_{tac} promoter. The table depicts the citrate (intracellular) and acetate (extracellular) levels in the late stationary phase culatures of *E. coli* BL21 transformants (plasmid control and test) grown on M9 minimal medium with 100 mM glucose as carbon source. All the values are represented as Mean ± SD of n=4-8 observations. *** $p<0.001$, ns=non-significant.

11.4 DISCUSSION

The present study demonstrates that overexpression of *cs* gene upto ~1.8 fold in *E. coli* BL21 expressing citrate transporter showed no effect on 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* where, as low as 2 fold

overexpression did not alter growth and glucose consumption rate but 50 fold high activity resulted in 50% reduction in glucose utilization (Walsh and Koshland, 1985; De Maeseneire et al., 2006). Increased CS activity supported high citrate accumulation although the results were contrary to the earlier reports of *gltA* overexpression in *E. coli* K strain (Walsh and Koshland, 1985; De Maeseneire et al., 2006). High acetate secretion in respect to *gltA* overexpression was also contrary to earlier reports where *gltA* overexpression in *E. coli* K strain demonstrated reduced acetate secretion (De Maeseneire et al., 2006).

Overexpression of *gltA* coupled with down regulation of ICDH (2 fold) activity supported high growth rate, poor glucose consumption rate and reduced biomass. *E. coli icd* mutants have demonstrated CS activity but showed poor growth coupled with reduced glucose consumption rate (Lakshmi and Helling, 1976; Aoshima et al., 2003; Kabir and Shimzu, 2004). Hence, the present work supports the earlier results with respect to poor glucose consumption rate. High CS activity also marked high citrate accumulation which was in agreement with the earlier reports of *E. coli icd* mutants showing high citrate accumulation (Lakshmi and Helling, 1976; Aoshima et al., 2003; Kabir and Shimzu, 2004). No extracellular citrate was observed supporting an inefficient citrate transport system.

Overexpression of *gltA* gene both in the absence and presence of as-*icd* did not alter other enzyme activities *viz* G-6-PDH, ICL and ICDH. Thus, the present study supports that CS activity controls the TCA flux towards citrate formation and also varies with host metabolism.