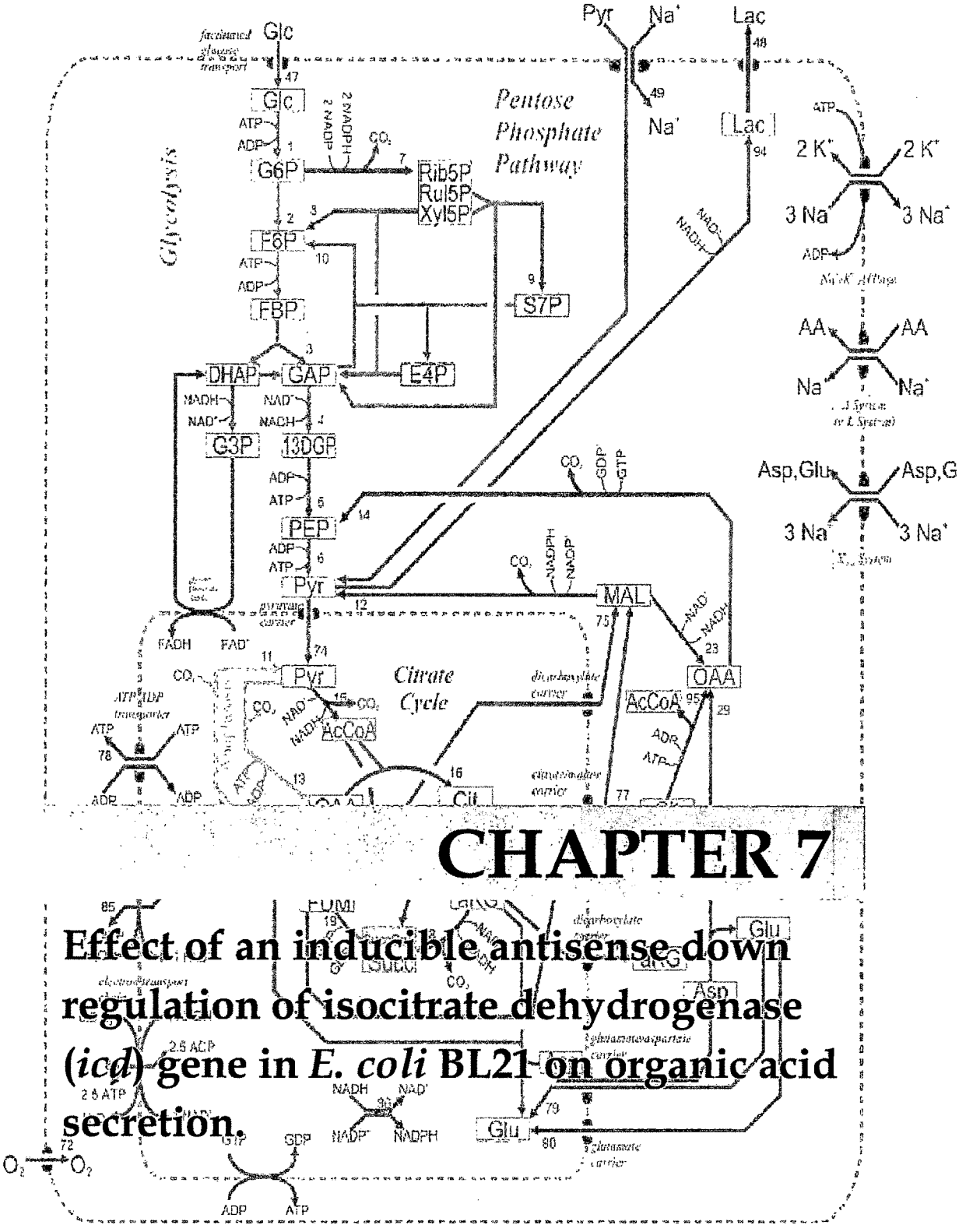


CHAPTER 7

Effect of an inducible antisense down regulation of isocitrate dehydrogenase (*icd*) gene in *E. coli* BL21 on organic acid secretion.

7.1: Rationale for the present study.

Alteration in the Cra regulation in *E. coli* Bl21 was suggested to be one of the reasons that P_{fruB} promoter was not able to regulate the as-*icd* expression in *E. coli* BL21 (**Chapter 6**). Therefore the present study investigates the effect of as-*icd* expression under a strong inducible promoter (P_{tac}).

7.2: WORK PLAN

7.2.1: Bacterial strains used for the present study.

E. coli strains	Genotype	References
BL21	*F*, *ompT*,*hsdS*$_B$ (r_{B-}, m^{B+}), *dcm*, *gal*	Sambrook and Russell, 2001
BL21pTTQ18	P_{tac} Ap^r	Present study
BL21 pJE6	as-*icd* cloned under P_{tac}, ap^r	Present study
BL21 pVSD1	citrate transporter cloned under P_{tac}, Chl^r	Present study
BL21 pTTQ18:pVSD1	Ap^r, Chl^r	Present study
BL21pJE6:pVSD1	Ap^r, Chl^r	Present study
DH5α	F-ϕ80Δ*lac*ZΔM15Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 λ-*thi*$^{-1}$ *gyr*A96 *rel*A1.	Sambrook and Russell, 2001
DH5α pJE4	as-*icd* cloned under P_{lac}, Ap^r	Present study
DH5α pJE6	as-*icd* cloned under P_{tac}, Ap^r	Present study

Table 7.1: List of the *E. coli* strains used for the present 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.

7.2.2: Construction of P_{tac}as-*icd* .

PCR amplification of *E. coli* DH5α genomic DNA was carried out using EC*icd*L1 and EC*icd*R1 primers (referred in 3.2.1). The fragments corresponding to 122 bp region of the *icd* gene were obtained. The, *icd* amplicon had 103 bp regions of *icd* with 11 bp corresponding to *fruB* and 8bp extra with a *Bam*H1 recognition site on either ends. The amplified product was cloned into pTZ57R using InsT/Aclone™ PCR Product Cloning Kit, MBI Fermentas (pJE4) and transformed into *E. coli* DH5α. The presence of the appropriate plasmid was checked by PCR using EC*icd*L1 and EC*icd* R1 primers. Digestion of the plasmid with *Bam*H1 released an insert of 125bp corresponding to as-*icd*. The cloning into T-vector results in a shift in reading frame hence the antisense could not be expressed from the P_{tac} promoter therefore, as- *icd* gene fragment was subcloned into pTTQ18 vector.

Both the vectors pTTQ18 and pJE4 were digested with *Bam*H1 and the 125 bp fragment released from pJE4 corresponding to the *icd* gene was purified using the method referred in section 2.3.5. and ligated into pTTQ18 followed by transformation into *E. coli* DH5α. Presence of the insert was checked by PCR using EC*icd*L1 and EC*icd* R1 primers and by restriction digestion with *Bam*H1. The orientation of the *icd* gene with respect to P_{tac} promoter was confirmed through PCR using 5'GGTGATCAAGCTGTTGACAATTAATCATCGG3' Primer BF1 and EC*icd* R1.

7.2.3: Developing *E. coli* BL21 expression P_{tac}as-*icd* carrying citrate transporter.

pJE6 and the plasmid control pTTQ18 were transformed into *E. coli* BL21 following the protocol referred in section 2.3.2. *E. coli* BL21 was co-transformed with pJE6 and pVSD1 to give double transformant carrying both P_{tac} as-*icd* and citrate transporter.

7.2.4: Physiological parameters addressed

The *E. coli* BL21 transformants were grown on M9 minimal media with micronutrients and 100mM glucose as carbon source. IPTG (0.1 mM) was used for

induction of expression of citrate transporter gene in plasmid pVSD1 (section 2.2.2). 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.

7.3: RESULTS

7.3.1: Construction of P_{tac}as-*icd*

The construction of the as-*icd* under *tac* promoter was carried out as described in the section 5.2.2 of the chapter. First the 122bp fragment corresponding to *icd* gene was cloned into T-vector and the clones were selected through blue white selection and ampicilin resistance. The clones were screened for insert release by BamH1 and PCR. Although the clones were in orientation with the *lac* promoter of the T-vector (the plasmid was referred as pJE4) but cloning into the vector brought about a frame shift in the open reading frame hence there was no functional expression of the as-*icd* hence *icd* fragment was further subcloned into pTTQ18 under P_{tac} promoter. The transformants were selected on ampicilin plates and confirmed for insert release. The orientation of the gene with the promoter was confirmed by PCR and the 200bp fragment (**Fig. 7.1**) was obtained corresponding to P_{tac} as-*icd* these clones were selected out for further studies. The plasmid thus derived was named pJE6.

7.3.2: *E. coli* BL21 expressing P_{tac}as-*icd* and citrate transporter

The plasmid pJE6 and pTTQ18 were transformed into *E. coli* BL21 and the transformants were selected on ampicilin plates. The plasmids were later confirmed through restriction digestion before proceeding with the experiments (**Fig. 7.2**). The double transformants were designated as *E. coli* BL21pTTQ18:pVSD1 and *E. coli* BL21pJE6:pVSD1.

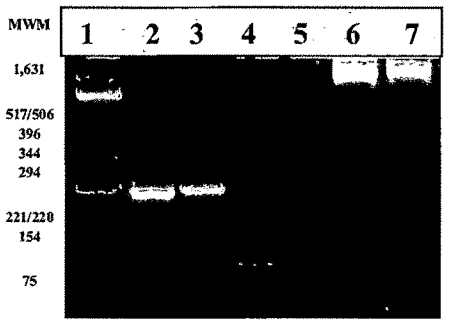


Fig 7.1: PCR and Restriction digestion pattern for pJE4. Lane 1: pBR322 HinfI digest, Lane 2 and 3: P_{tac}-as-*icd* (500bp), Lane 4: pTZ57R (PCR), Lane 5: pJE4 (PCR) (125bp), Lane 6 and 7: pJE4 BamH1 (125bp)

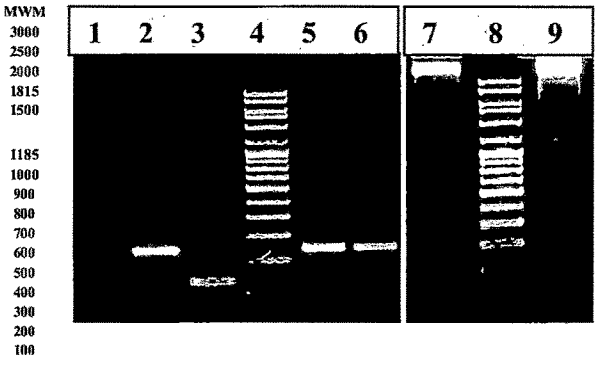


Fig 7.2: PCR and restriction digestion pattern for pJE6. Lane 1: pTTQ18 (control), Lane 2: P_{tac} -as-*icd* (225bp), Lane 3- as-*icd* (125bp), Lane 4: Low range DNA ruler plus, Lane 5 and 6: P_{tac}-as-*icd* (225bp), Lane 7: pTTQ18 BamH1 (4,563bp), Lane 8: Low range DNA ruler plus Lane 9: pJE6 pJE4 BamH1 (4,563bp, 125bp)

7.3.3: Effect of pJE6 on the physiological parameters of *E. coli* BL21.

Expression of as-*icd* showed no deleterious effect on the growth profile of BL21. The growth rate did not alter in response to the antisense expression. *E. coli* BL21 showed a growth rate of 0.54 ± 0.04 h^{-1} and presence of the control plasmid pTTQ18 and pJE6 also reported a growth rate of 0.51 ± 0.07 h^{-1}. There was significant decrease in the glucose consumption rate and biomass with as-*icd* expression as compared to the control plasmid bearing *E. coli* BL21. *E. coli* BL21pTTQ18 had a glucose consumption rate of 5.8 ± 0.33 g/g/hand that in *E. coli* BL21 pJE6 was 4.2 ± 0.43 g/g/h (**Table 7.2, Fig. 7.3 (i) and (ii)**).

E. coli BL21 pVSD1 carrying the citrate transporter observed a significant decrease in the growth rate and glucose consumption rate (0.32 ± 0.03, 3.6 ± 0.61) as compared to the wild type *E. coli* BL21. This change was also observed in the double transformants, there was no significant change in the growth rate and glucose consumption in case of *E. coli*

BL21 pJE6:pVSD1 (0.36 ± 0.04 h^{-1}, 3.9 ± 0.36 g/g/h) when compared to the respective control (0.24± 0.01 h^{-1}, 3.6 ± 0.61 g/g/h) **(Fig. 7.3 (iii) and (iv))**.

***E. coli* strains**	**Growth rate μ (h^{-1})**	**Specific glucose consumption rate (Qglc) (g glc.g^{-1}.h^{-1})**	**Total glucose consumed (mM)**	**Biomass Y_{dcw}/glc (g dcw.g^{-1}.h^{-1})**
BL21	0.54 ± 0.04	6.7 ± 0.72	56.23 ± 2.2	.0.22 ± 0.08
BL21 pTTQ18	0.51 ± 0.07	5.8 ± 0.33	66.04 ± 1.8	0.14 ± 0.05
BL21 pJE6	**0.51 ± 0.06^{ns}**	**4.2 ± 0.43***	**47.76 ± 2.7****	**0.07 ± 0.01 ****
BL21 pVSD1	0.32 ± 0.03	3.6 ± 0.61	38.26 ± 0.8	0.09 ± 0.02
BL21 pTTQ18:pVSD1	0.24± 0.01	4.0± 0.26	56.62 ± 3.1	0.13 ± 0.06
BL21 pJE6:pVSD1	**0.36 ± 0.04***	**3.9 ± 0.36^{ns}**	**54.55 ± 3.8^{ns}**	**0.08 ± 0.02 ****

Table 7.2: Physiological parameters demonstrating the effect of P_{tac}-as*icd* expression in *E. coli* BL21. The results are expressed as Mean ±SD of 8-10 independent observations. Specific growth rate (μ(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.

7.3.4: Effect of pJE6 on Isocitrate dehydrogenase activity of *E. coli* BL21.

Antisense expression under an inducible promoter (P_{tac}) was able to bring about 3-4 fold (0.36 ± 0.06) down regulation in the ICDH activity both in *E. coli* BL21 (1.01 ± 0.09) and the respective plasmid control (1.33 ± 0.18) **(Fig. 7.4 (i))**. Similarly in the double transformants *E. coli* BL21 pJE6:pVSD1 showed a ~ 4 fold decrease (0.22 ± 0.024) in the ICDH activity as compared to the control (0.9 ± 0.076) **(Fig. 7.4 (ii))**.

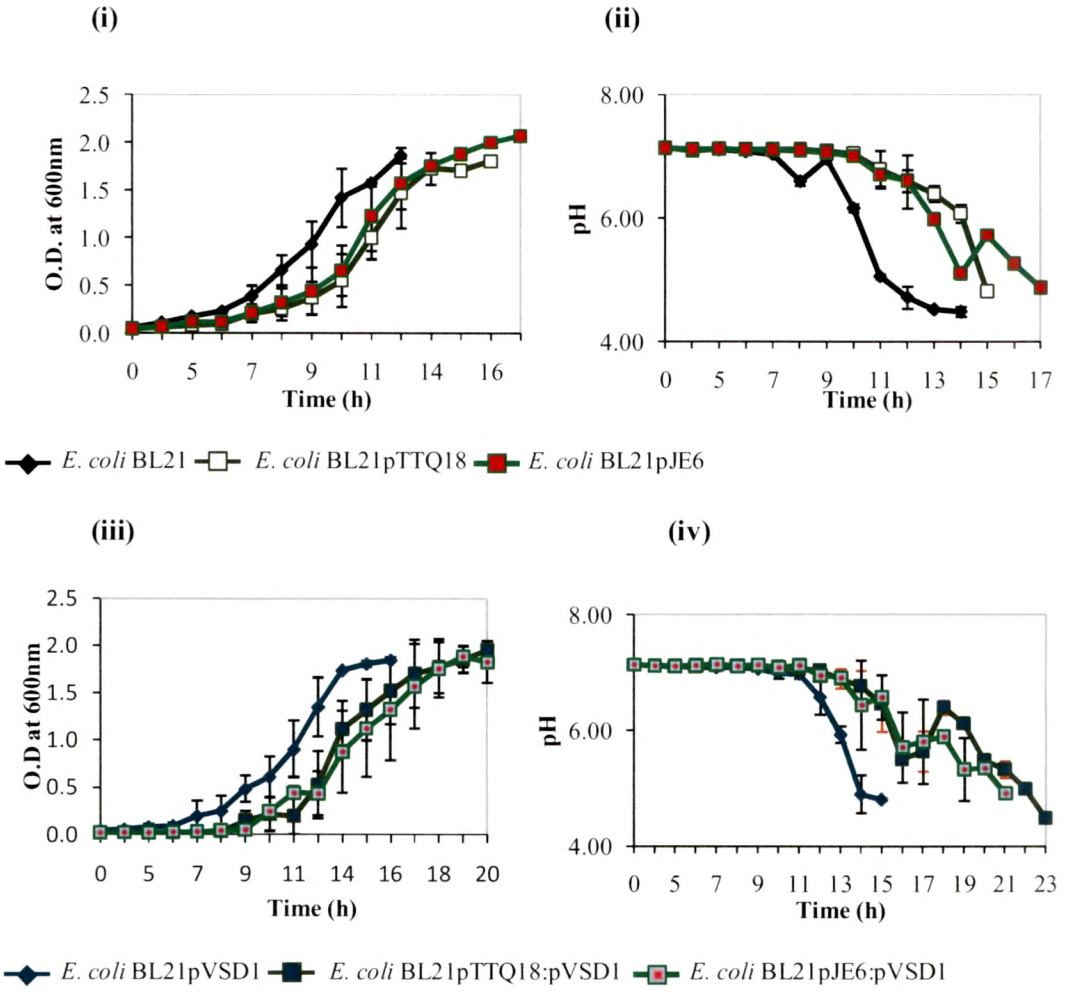
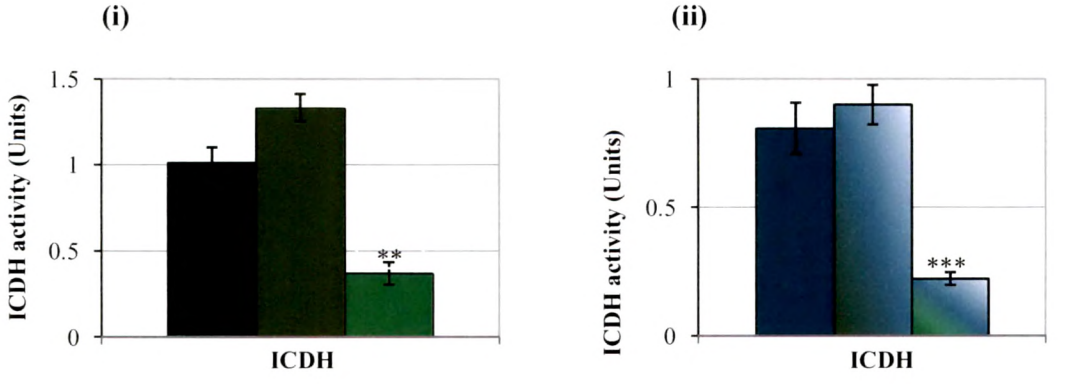


Fig 7.3: Growth characteristics of *E. coli* BL21 pJE6 and *E. coli* BL21 pJE6: pVSD1on M9 minimal medium with 100mM glucose. (i) & (ii) $O.D_{600nm}$ and pH on glucose All values plotted are represented as Mean ± SD for n= 4 to 7 observations.



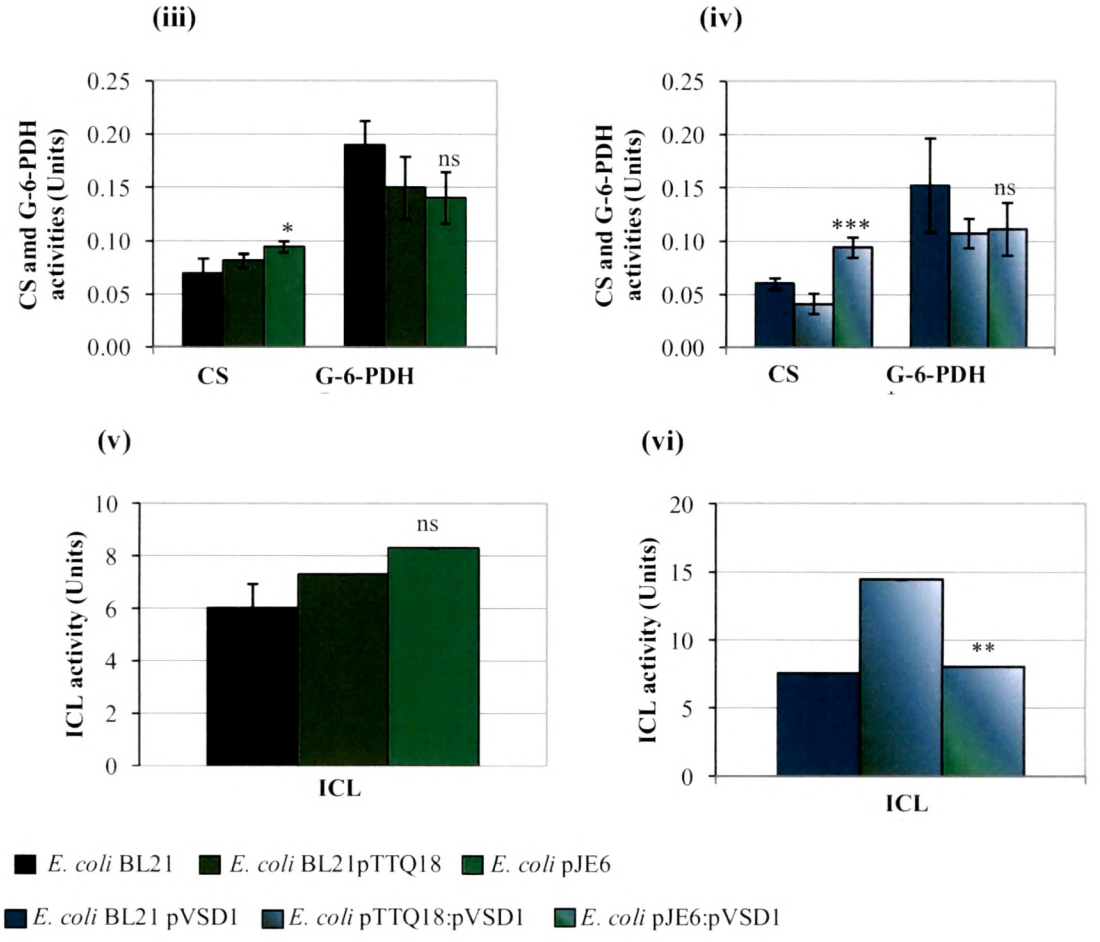


Fig 7.4: Effect of as-*icd* expression under an inducible promoter on the various enzymes of the central metabolic pathway. (i) & (ii) ICDH activity with and without citrate transpoter (iii) & (iv) CS and G-6-PDH activity with and without citrate transporter (v) & (vi) ICL activity with and without citrate transporter. Units for the specific activity are given as µmoles/mg. protein/min except for ICL its nmoles/mg. protein/min. All the values are represented as Mean ± SD for n= 5-8 observations. *p< 0.05, **p<0.01,***p<0.001and ns- non significant.

7.3.5: Effect of pJE6 on the CS G-6-PDH and ICL activity of *E. coli* BL21.

Down regulation of *icd* gene showed a significant increase in the CS activity in *E. coli* BL21: pJE6 (0.09 ± 0.006 U) as compared to the control (0.08 ± 0.006 U). Similar results were observed for *E. coli* BL21:pJE6:pVSD1 (0.09 ± 0.009 U) compared to its

respective plasmid control (**Fig. 7.4 (iii) and (iv)**). Significant decrease in the G-6-PDH activity was observed in all *E. coli* BL21 strains irrespective of the antisense expression (**Fig. 7.5 (iii) and (iv)**). As-*icd* expression had no effect on ICL activity. Similar results were found for *E. coli* BL21 expressing as-*icd* along with citrate transporter (**Fig. 7.4 (v) and (vi)**).

7.3.6: Effect of *icd* gene down regulation on the organic acid production in *E. coli* BL21

Intracellular citrate levels significantly increased in *E. coli* Bl21 expressing antisense under and inducible promoter compared to the control while a reduction in acetate levels was observed in the same case (**Table 7.3**). Similar results with respect to intracellular citrate were seen in case of *E. coli* Bl21 expressing antisense under and inducible promoter in presence of citrate transporter.

E. coli strains	Intracellular citrate (mM)	Acetate (mM)
Bl21	1.206 ±0.04	27.67 ± 0.4202
Bl21 :pTTQ18	1.049 ± 0.03	36.90 ± 1.518
BL21 :pJE6	**2.025 ± 0.29 ***	**29.64 ± 1.506***
BL21 : pVSD1	1.264 ± 0.06	14.05 ± 1.460
BL21:pTTQ18: pVSD1	1.210 ± 0.06	14.76 ± 1.110
BL21:pJE6: pVSD1	**2.708 ± 0.23 *** **	**16.65 ± 0.5371^{ns}**

Table 7.3: Organic acid production of *E. coli* BL21 expressing as-*icd* under P_{tac} promoter. The table depicts the citrate (intracellular) and acetate (extracellular) levels in the late stationary phase cultures of *E. coli* BL21 transformants (plasmid control and test) grown on M9 minimal media with 100 mM glucose as carbon source. All the values are represented as Mean ± SD of n=4-8 observations. * $p<0.05$, *** $p<0.001$, ns=non-significant.

An overall reduction in acetate levels were seen in the double transformants. No extracellular citrate was observed in either of the cases. Similar results with respect to intracellular citrate were seen in case of *E. coli* Bl21 expressing antisense under and

inducible promoter in presence of citrate transporter. An overall reduction in acetate levels were seen in the double transformants. No extracellular citrate was observed in either of the cases.

7.4: DISCUSSION

E. coli BL21(λDE3) *icd* mutant showed poor growth and required glutamate supplementations to grow on glucose as carbon source (Aoshima et al., 2003). Present study demonstrates that expression of as-*icd* under IPTG inducible promoter not only improved the growth rate but also did not require glutamate suggesting that the partial block in ICDH activity (3-4 fold) meets the requirement of glutamate through isocitrate. Down regulation of ICDH demonstrated poor glucose consumption rate and decreased biomass in as-*icd* expressing *E. coli* BL21. This result is in agreement with earlier work where *E. coli icd* mutants observed a poor glucose consumption rate (Kabir and Shimzu, 2004).

High CS activity observed in *E. coli* BL21 expressing as-*icd*, were in agreement with high CS activity of *E. coli icd* mutants (Aoshima et al., 2003; Kabir and Shimzu, 2004). No alterations in G-6-PDH and ICL activities were observed in *E. coli* BL21 expressing as-*icd*. *E. coli* K strain *icd* mutant had observed high G-6-PDH and ICL activities (Kabir and Shimzu, 2004) while *E. coli* BL21(λDE3) *icd* mutant reported low ICL activity in stationary phase compared to wild type (Aoshima et al., 2003) and no reports were obtained with respect to G-6-PDH activity. *E. coli* BL21expressing as-*icd* showed no alterations in ICL activity as compared to the decrease ICL activity observed in the *icd* mutant.

Expression of as-*icd* in *E. coli* BL21 observed high intracellular citrate accumulation and lower acetate secretion compared to the plasmid control. These results were in agreement with the earlier reports on *E. coli* BL21(λDE3) *icd* mutant ((Aoshima et al., 2003). No extracellular citrate was observed in *E. coli* BL21 expressing as-*icd* supporting

the absence of citrate transporter (Lara and Stokes, 1952). Co-expression of citrate transporter and as-*icd* showed decreased growth rate without altering glucose consumption rate, enzyme activities (ICDH, CS, G-6-PDH and ICL) and citrate accumulation as compared to *E. coli* BL21 expressing as-*icd*. No change was observed with respect to acetate secretion as compared to *E. coli* BL21 expressing as-*icd* in absence of citrate transporter.

High copy plasmid expressing as-*icd* did not seem to alter metabolism of *E. coli* BL21 as compared to the medium copy plasmid expressing citrate transporter (pVSD1). pVSD1 incorporation resulted in poor growth and low glucose consumption rate thus resulting in lower acetate levels suggesting that it imparted metabolic load. These results were in agreement that incorporation of plasmids lead to alterations in growth and glucose consumption rate (Wang et al., 2006).

The present study supports that alterations in Cra lead to the failure of expressing as-*icd* in *E. coli* BL21. It further suggests that down regulation of ICDH along with high CS activity enhanced citrate accumulation in *E. coli* BL21; these results were in agreement with conditional antisense down regulation in *E. coli* DH5α (chapter 3,4 and5).