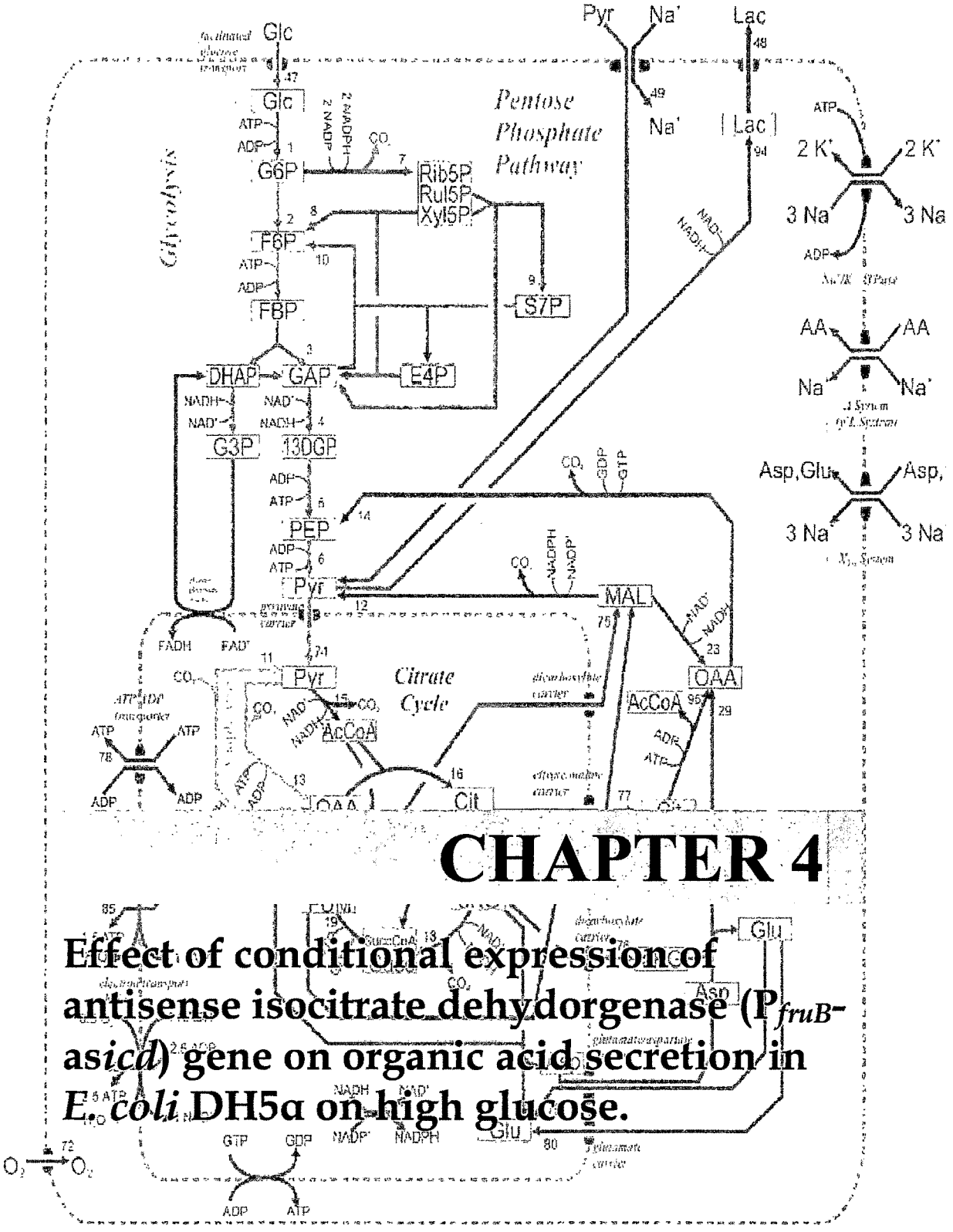

CHAPTER 4

Effect of conditional expression of antisense isocitrate dehydrogenase (P*fruB*-as*icd*) gene on organic acid secretion in *E. coli* DH5α on high glucose.

4.1: Rationale of the work

Chapter 3 indicated that the TrP minimal medium exerts stress on *E. coli* DH5α with respect to its growth and metabolism. In order to investigate the effect of antisense mediated *icd* gene down regulation under non-stress conditions, the present study reports the growth and metabolism of *E. coli* DH5α transformants on M9 minimal medium with high glucose.

4.2: WORK PLAN

4.2.1: Bacterial strains used for the present study.

E. coli strains	Genotype	Reference
E. coli 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 pTZ57R	T-vector, Ap^r	MBI fermentas
DH5α pVS2k3	P_{fruB}, of *fruBKA* operon , antisense isocitrate dehydrogense (as-*icd*) for *E. coli* , Ap^r	Present study
DH5α pVSD1	P_{lac}, H^+ -Citrate transporter from *Klebsiela pneumoniae*, Chl^r	Patel, 2001
DH5α pTZ57R:pVSD1	Ap^r, Chl^r	Present study
DH5α pVS2k3:pVSD1	Ap^r, Chl^r	Present study

Table 4.1: List of the bacterial strains used for the present study. The details of the plasmid and the concentration of the antibiotics used for growth on both rich and minimal media are given in the table 2.2 and table 2.3.

4.2.3: Growth parameters addressing *E. coli* DH5α expressing P_{fruB} as-*icd*.

E. coli DH5α transformants were grown on M9 minimal medium with micronutrients and glucose (100mM) as carbon source. IPTG (0.1mM) 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 $_{600nm}$, and organic acid in the medium. ICDH assay performed at stationary phase as per protocol mentioned in (section 2.6.2.4).

4.3: RESULTS

4.3.1: Effect of pVS2k3 expression on the growth and ICDH activity of *E. coli* DH5α both in the presence and absence of citrate transporter.

E. coli DH5α pVS2k3 had no positive effect on the growth as compared to the respective controls, *E. coli* DH5α and *E. coli* DH5α pTZ57R. The growth was faster and improved on M9 minimal medium as compared to 100mM TrP minimal medium. Acidification (pH<5) was observed in all the cultures within 48h. Presence of citrate transporter improved growth compared to the respective plasmid control *E. coli* DH5α pTZ57R:pVSD1 which showed an initial lag phase that was longer than the single or double transformants (**Fig. 4.1 (i), (ii), Fig. 4.1 (i) and (ii)**).

E. coli DH5α pVS2k3 with or without citrate transporter showed 3-4 fold (0.09 ± 0.05U) decrease on ICDH activity as compared to the respective plasmid control (0.30 ± 0.02U). Within the controls there was no change in the ICDH activity (**Fig. 4.3 (i) and (ii)**).

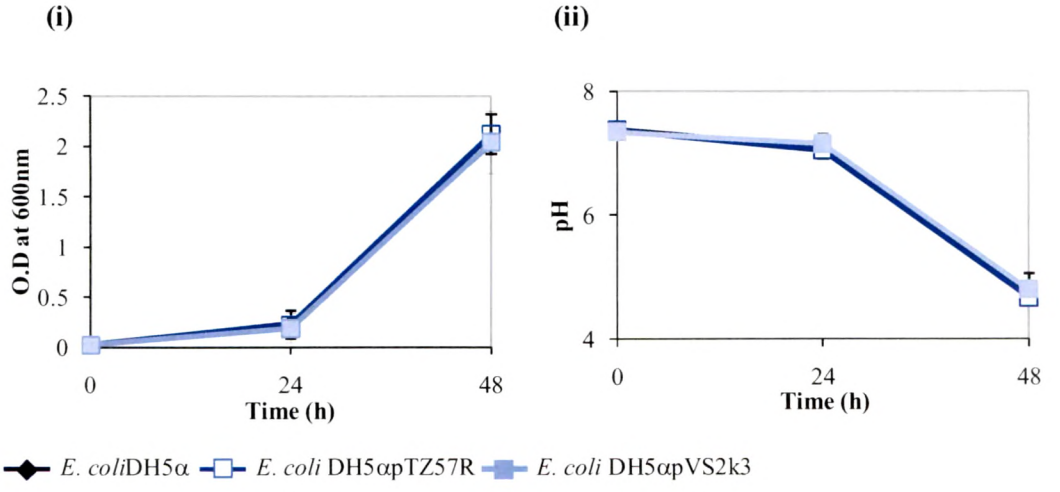

Fig 4.1: Growth characteristics of *E. coli* pVS2k3 on M9 minimal medium with 100mM glucose in absence of citrate transporter. (i) & (ii) O.D$_{600nm}$ and pH without citrate transporter All values plotted here represent mean ± SD of n=5 observation.

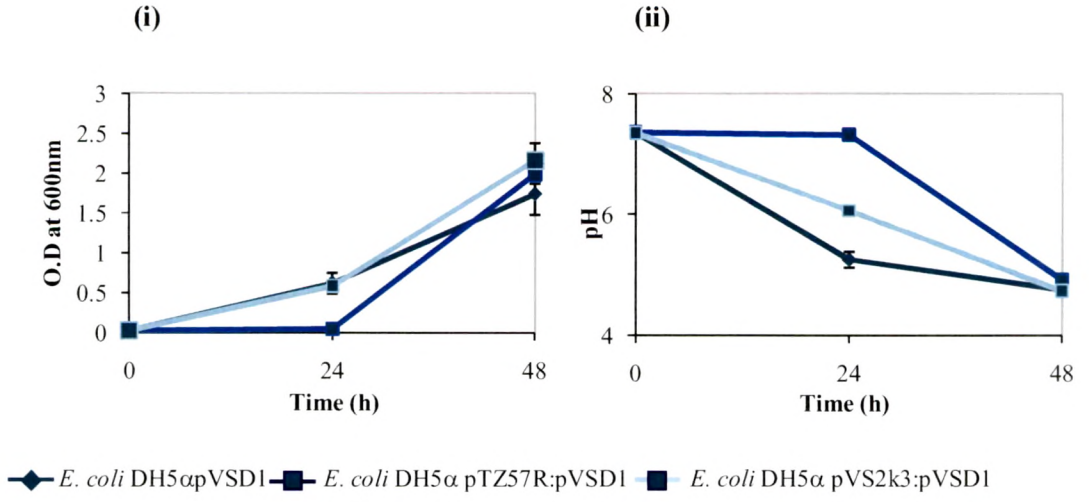

Fig 4.2: Growth characteristics of *E. coli* pVS2k3 on M9 minimal medium with 100mM glucose in presence of citrate transporter. (i) & (ii) $O.D_{600nm}$ and pH with citrate transporter. All values plotted here represent mean ± SD of n=5 observation.

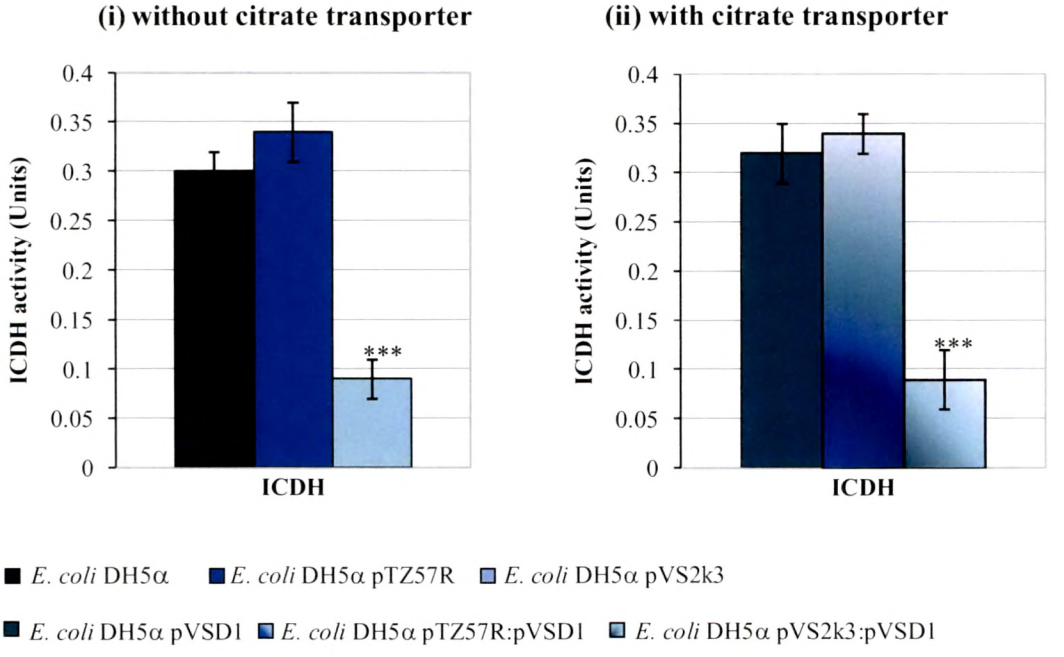

Fig. 4.3: Effect of P_{fruB} as-*icd* on Isocitrate dehydrogenase activity on M9 minimal medium with 100mM glucose. (i) ICDH activity without citrate transporter (ii) ICDH activity with citrate transporter. All the values plotted here are Mean ± SD for n= 4-6 observations. Units- µmoles/mg. protein/min. *** $p<0.001$.

4.3.2: Effect of *E. coli* DH5α pVS2k3 on organic acid secretion.

Organic acid excretion profile was monitored for the extracellular sample collected in the stationary phase. Acetate was the major acid secreted on glucose and glycerol. *E. coli* DH5α showed a relative higher acetate secretion (34.36 ± 1.65 mM) as compared to the TrP medium with 100mM glucose (24.07 ± 0.65 mM). *E. coli* DH5α pVS2k3 (45.16 ± 21.25 mM) and respective plasmid control (53.26 ± 31.56 mM) bearing strain showed a no change in the levels of acetate secreted compared to *E. coli* DH5α devoid of any plasmid. In presence of the citrate transporter very high acetate secretion (92.53 ± 1.95 mM) was observed which was similar to that of *E. coli* DH5α pVS2k3:pVSD1 (expressing antisense) (85.05 ± 17.43 mM). The corresponding plasmid control showed relative lower acetate secretion (**Table 4.2**).

E. coli strains	Acetate (mM)
E. coli DH5α	34.36 ± 1.65
DH5α pTZ57R	53.26 ± 31.56
DH5α pVS2k3	**45.16 ± 21.25^{ns}**
DH5α pVSD1	92.53 ± 1.95
DH5α pTZ57R:pVSD1	42.16 ± 11.52
DH5α pVS2k3:pVSD1	**85.05 ± 17.43****

Table 4.2: Acetate secretion in *E. coli* DH5α expressing P_{fruB} as-*icd*, on M9 minimal medium with 100mM glucose. All the values expressed here are Mean ± SD for n= 4-6 observations. ** $p \leq 0.05$, ns-non significant.

4.4 : DISCUSSION

Functional genomics of *E. coli* on minimal medium showed that out of the 409 genes for carbon and energy metabolism 31 genes were expressed to higher levels than that on nutrient rich Luria broth (Tao et al., 1999). These included genes involved in D-lactate

utilization (*dld*), acetate formation (*poxB*), stationary phase sigma factor (*rpoS*), acetate utilization (*aceA*, *aceB*, *gltA*, *icd*, and *mdh*), and coupling of glucose and acetate cometabolism (*uspA*). Such a high expression of genes regulating carbon metabolism on minimal medium supported its use over rich medium. Growth on minimal medium also demonstrated increased expression of various global regulators regulating cell processes for stress tolerance. High glucose concentration in minimal medium resulted in high acetate secretion this protected the cell from the stress of living in a self-formed acidic environment. Studies on M9 minimal medium demonstrated improved growth of the *E. coli* transformants compared to 100mM TrP minimal medium suggesting the TrP medium exerted some stress on the growth. The expression of antisense was better on TrP as it showed 5-6 fold decrease in ICDH activity as compared to 3-4 fold on M9 minimal medium with glucose but had poor growth which remained unexplainable hence M9 minimal medium appear to be a better choice for studying the physiological parameters. Hence, further studies were carried out with M9 minimal medium.

High levels of acetate secreted on the minimal medium compared to the TrP medium may be attributed to increased expression of pyruvate oxidase (*pox*B) on minimal medium high levels on high glucose (Tao et al., 1999). This enzyme is responsible for the conversion of pyruvate to acetate and is mostly expressed during the shift from the late log to stationary phase. RpoS regulates *pox*B expression (Chang et al., 1994) and *rpo*S expression was also found to be high in the late logarithmic phase hence RpoS might play a critical role in acetate metabolism.

Acetate secretion under aerobic condition can also be attributed to the low flux through TCA (low respiration) in presence of excess carbon source (Chang et al., 1999; Holms, 2001). Hence 100mM glucose used for the present study was excess. Moreover, acetate outflow also hinders recombinant protein expression (Kleman and Strohl, 1994). Hence, 50mM glucose was selected for studying the physiological parameters and the effect of pVS2k3 expression on metabolism.