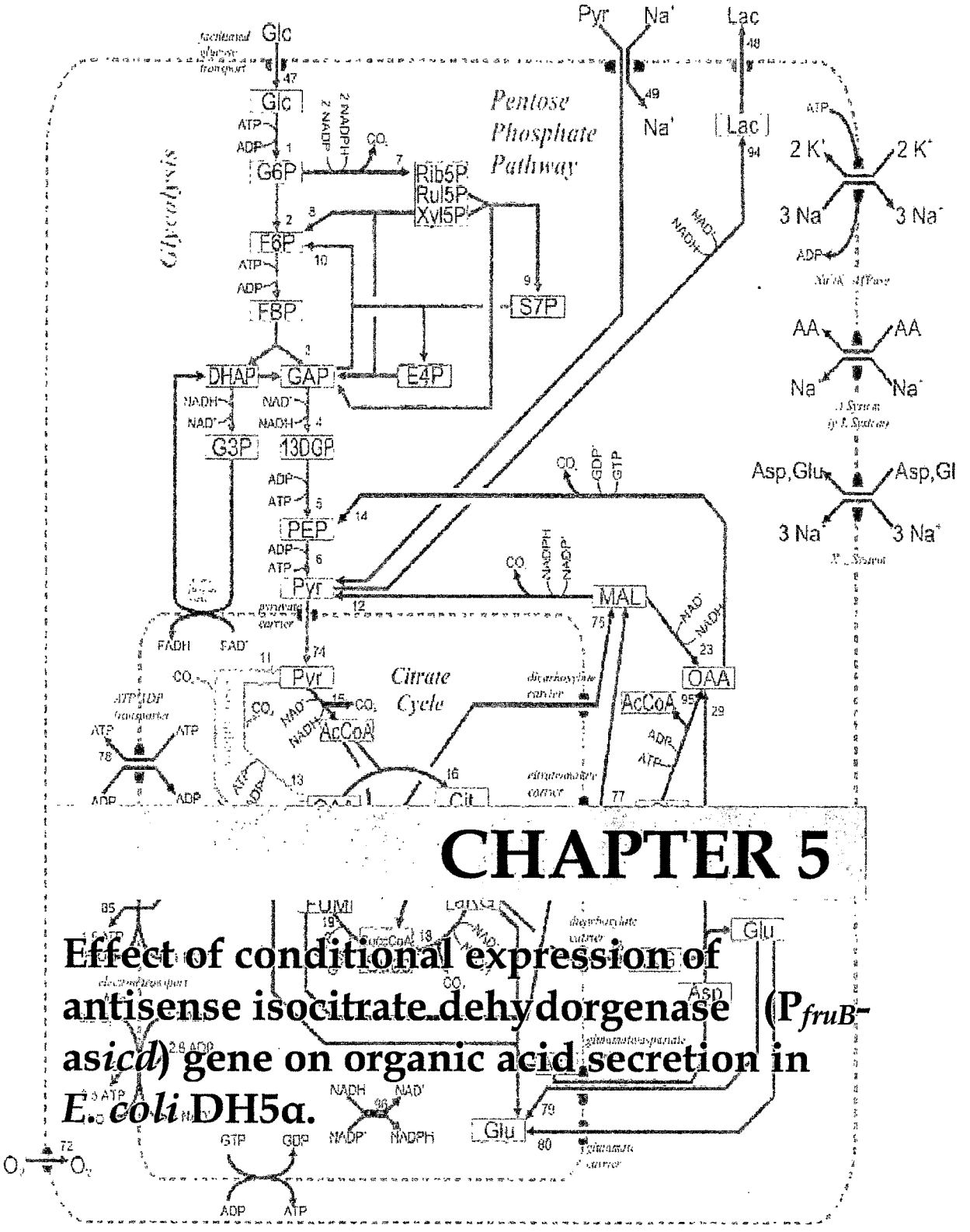

CHAPTER 5

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

5.1: Rationale of the work

Chapter 4 demonstrated that TrP medium and high glucose levels had diminished the effect of antisense expression as result of acetate overflow. Hence, the present study describes the effect of *icd* gene down regulation mediated by a plasmid carrying an antisense for the *icd* gene under a P_{fruB} promoter on the metabolism of *E. coli* DH5α when grown on lesser levels of glucose (50mM) and 100mM glycerol.

5.2: WORK PLAN

5.2.1: 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 5.1: List of the bacterial strains used for the present study. The details of the plasmids and the concentration of the antibiotics used for growth on both rich and minimal media are given in the table 2.2 and 2.3.

5.2.2: Growth and physiological parameters addressed for *E. coli* DH5α transformants.

All the physiological studies were carried out in M9 minimal medium with micronutrients and either glucose (50mM) and glycerol (100mM) as carbon source. IPTG (0.1mM) was used for induction of citrate transporter gene (pVSD1). 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 2.5.3). Enzyme assays were performed as per protocol mentioned in (section 2.6); ICDH, G-6-PDH, CS and ICL at stationary phase.

5.3 : RESULTS

5.3.1: Effect of *E. coli* DH5α pVS2k3 in M9 minimal medium with 50mM glucose.

E. coli DH5α had a specific growth rate of (0.26 ± 0.02 h^{-1}) and a glucose consumption rate of (7 ± 1.5 g/g/h). The presence of the control plasmid altered specific growth rate and glucose consumption rate. Antisense expression showed no change in the growth rate (0.37 ± 0.08 h^{-1}) when compared to the corresponding plasmid control, *E. coli* DH5α pTZ57R (0.32 ± 0.08 h^{-1}), but it significantly improved the glucose consumption rate (5.7 ± 1.25 g/g/h) (**Table 5.2, Fig. 5.1(i) and (ii)**).

E. coli strains	Growth rate (μ)h^{-1}	Specific glucose consumption rate (Q_{glc}) (g glc.g^{-1}dcw.h^{-1})	Total glucose consumed (mM)
DH5α	0.26 ± 0.02	7 ± 1.5	48 ± 2.5
DH5α pTZ57R	0.32 ± 0.08	3.15 ± 1	42 ± 5
DH5α pVS2k3	**0.37 ± 0.08$^{ns\ (a)}$**	**5.7 ± 1.25 ****	**35 ± 1.5**
DH5α pVSD1	0.42 ± 0.05	7.15 ± 3	23 ± 0.98
DH5α pTZ57R:pVSD1	0.54 ± 0.17	3.3 ± 1	24 ± 1.23
DH5α pVS2k3:pVSD1	**0.39 ± 0.21** (a)**	**0.7 ± 0.2*****	**28 ± 3**

Table 5.2: Physiological parameters demonstrating the effect of P_{fruB}-asicd expression in *E. coli* DH5α. The results are expressed as Mean ± SD of 8-10 independent observations. Specific growth rate (μ(h^{-1})) and specific glucose consumption rate (Q_{Glc}) were determined from mid log phase of each experiment. Total glucose utilized was determined at the end of growth curve. (a) significance calculated with respect to the respective plasmid control. * $p<0.05$, ** $p<0.01$, ***$p< 0.001$, ns=non-significant.

The presence of the plasmid carrying citrate transporter affected growth rate (0.42 ± 0.05 h^{-1}) and specific glucose consumption rate (7.15 ± 3 g/g/h). In case of the double transformants the antisense expression showed a significant decrease in both the growth

rate (0.39 ± 0.21 h^{-1}) and the glucose consumption rate (1.7 ± 0.2 g/g/h) when compared to the respective plasmid control (**Fig. 5.1 (iii) and (iv)**).

5.3.2 : Effect of *icd* gene down regulation on ICDH and other enzymes of the central metabolic pathway.

E. coli DH5α pVS2k3 with or without citrate transporter showed 3-4 fold (0.09 ± 0.05 U) decrease on ICDH activity as compared to the respective plasmid control (0.30 ± 0.02 U) (**Fig. 5.2 (i) and (ii)**). Within the controls there was no change in the ICDH activity.

E. coli DH5α pVS2k3 showed a significant decrease in G-6-PDH activity whereas CS activity (0.079 ± 0.008 U) was increased as compared to the plasmid control (0.04 ± 0.002 U) bearing strain. Similar results were found in *E. coli* DH5α pVS2k3: pVSDI. Expression of the citrate transporter slightly increased CS activity (0.048 ± 0.002 U) when compared to the *E. coli* DH5α (**Fig. 5.3 (i) and (ii)**). The ICL activity was not detected on glucose medium in all cultures irrespective of the antisense expression.

5.3.3 : Effect of *icd* gene down regulation on organic acid accumulation and secretion.

Antisense RNA mediated down regulation of *icd* gene was well reflected in the increased citrate accumulation (1.32 ± 0.013 mM) as compared to plasmid control strain (0. 65 ± 0.05 mM) (**Table 5.3**). Similar results were obtained in *E. coli* DH5α pVS2k3: pVSD1 (1.32 ± 0.01 mM). A relative high citrate accumulation was also obtained in *E. coli* DH5α pVSD1 (1.27 ± 0.18 mM) when compared to the other plasmid bearing *E. coli* DH5α and *E. coli* DH5α devoid of any plasmid. The extracellular medium from the culture grown on 100mM glucose contained acetate as a major acid and it was negligible from the cultures grown on 50mM glucose. *E. coli* DH5α pVS2k3 (1.95 ± 0.65 mM) had slight increase in acetate levels compared to its plasmid control bearing *E. coli* DH5α (1.04 ± 0.25 mM). However, expression of citrate transporter did not change in the levels of acetate secreted as compared to the controls. *E. coli* DH5α pVS2k3:pVSD1 secreted low levels of acetate (0.5 ± 0.15 mM) but no acetate was seen in the respective plasmid control.

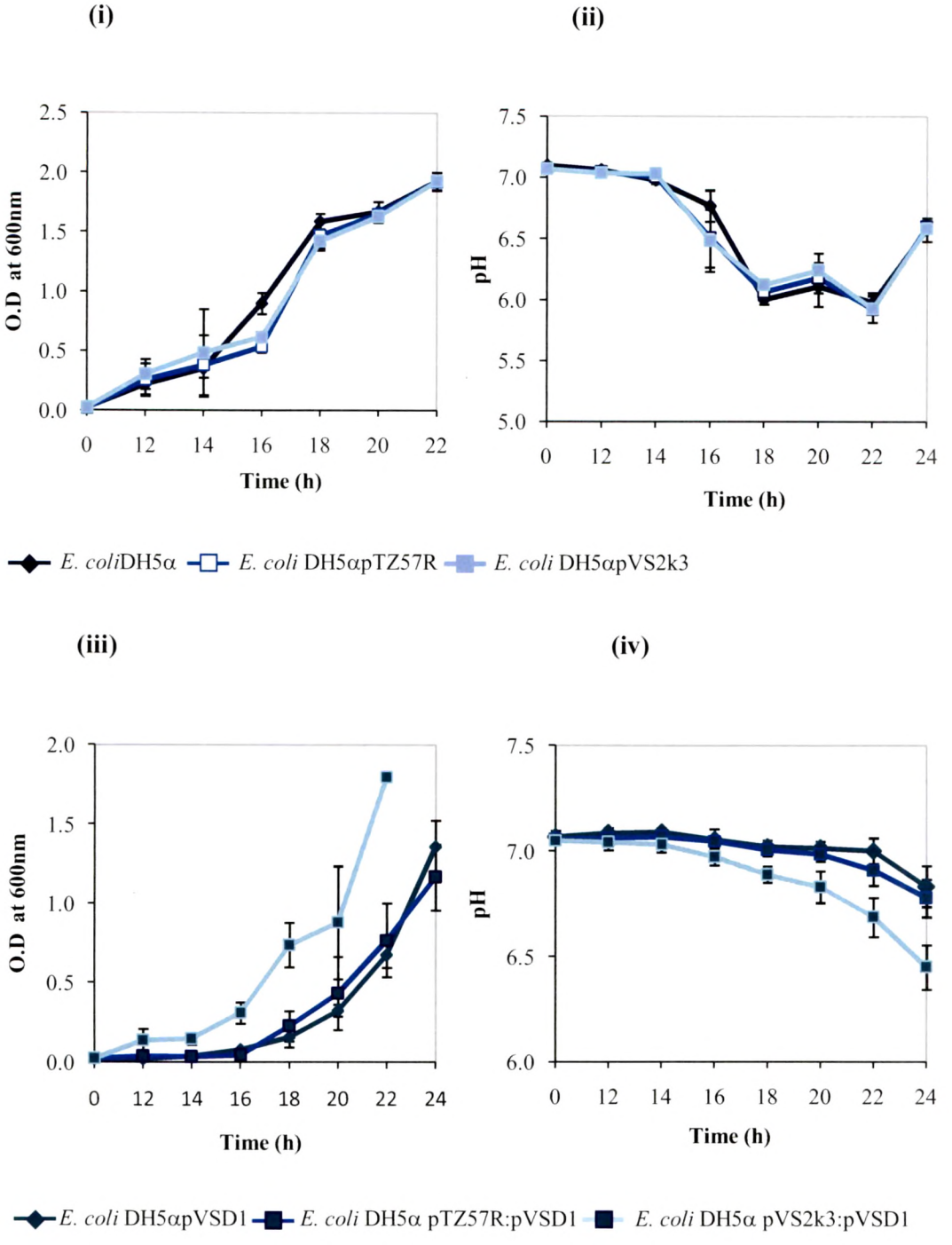

Fig. 5.1: Growth characteristics of *E. coli* pVS2k3 on M9 minimal medium with 50mM glucose in presence and absence of citrate transporter. (i) & (ii) $O.D_{600nm}$ and pH without citrate transporter (iii) & (iv) $O.D_{600nm}$ and pH with citrate transporter. All values plotted here are mean± SD of n=5-7 observations.

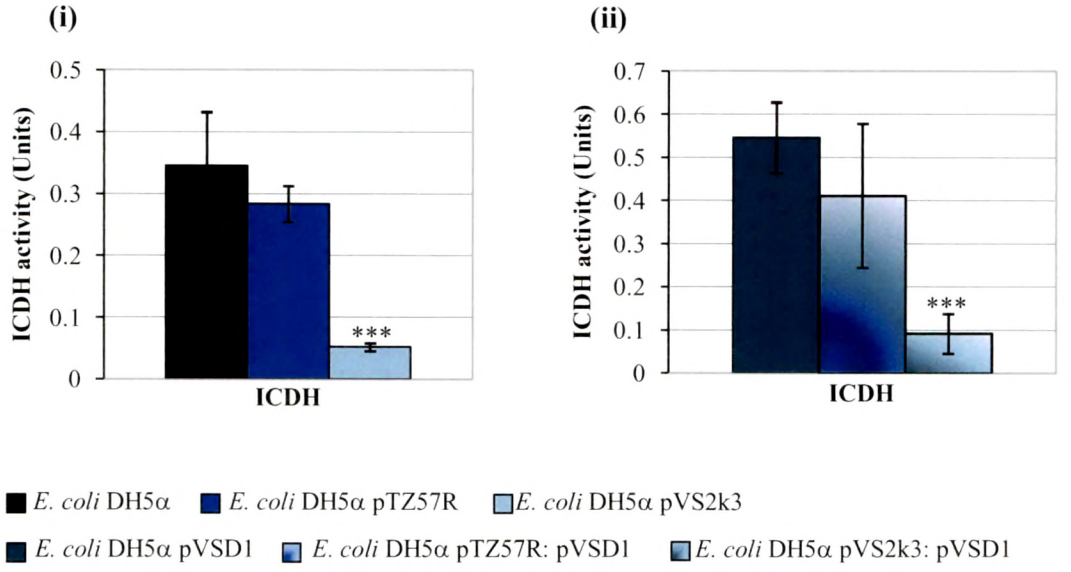

Fig. 5.2: Effect of *icd* gene downregulation on ICDH activity on M9 minimal media with 50mM glucose. (i) without citrate transporter (ii) with citrate transporter. All the values represented here are Mean ± SD of n= 5-9 independent observations. Units- μmoles/mg. protein/min. *** p< 0.001.

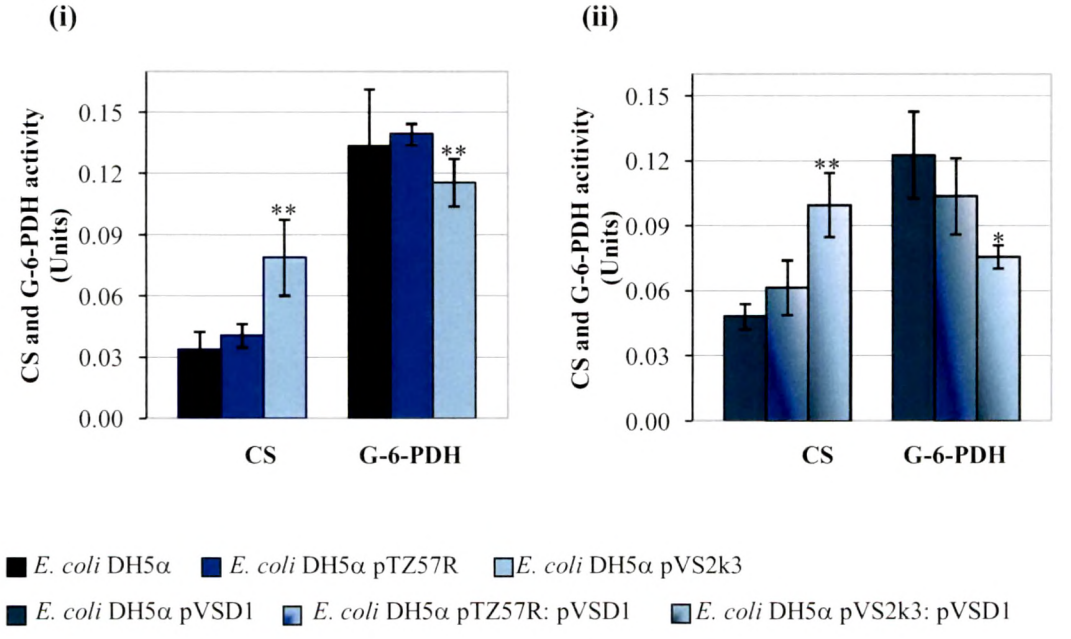

Fig. 5.3: Effect of *icd* gene down regulation on citrate synthase and glucose 6-phosphate dehydrogenase activity on M9 minimal media with 50mM glucose. (i) without citrate transporter (ii) with citrate transporter. All the values represented here are Mean ± SD of n= 5-9 independent observations. Units- μmoles/mg. protein/min. * p< 0.05, **p< 0.01, ***p< 0.001.

E. coli strains	Intracellular citrate (mM)	Extracellular Acetate (mM)
DH5α	0.65 ± 0.06	ND
DH5α pTZ57R	0.69 ± 0.1	1.04 ± 0.25
DH5α pVS2k3	**1.32 ± 0.13****	**1.95 ± 0.65**
DH5α pVSD1	1.27 ± 0.18 *	ND
DH5α pTZ57R: pVSD1	0.50 ± 0.04	ND
DH5α pVS2k3 : pVSD1	**1.32 ± 0.10*****	**0.5 ± 0.15**

Table 5.3: Organic acid production of *E. coli* DH5α expressing as-*icd* 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. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns=non-significant.

5.3.4 : Effect of *E. coli* DH5α pVS2k3 in M9 minimal medium with 100mM glycerol.

All cultures grew relatively slower on glycerol as compared to that on glucose. *E. coli* DH5α on glycerol (0.13 ± 0.015) showed a slower growth rate as compared to that on glucose (0.26 ± 0.02). Expression of antisense *icd* (pVS2k3) did not alter the growth rate (0.17 ± 0.007) when compared to the plasmid control (pTZ57R) (0.12 ± 0.13). Moreover on glycerol on no acidification of the medium was seen with the *E. coli* cultures (**Table 5.4, Fig. 5.4 (i) and (ii)**).

E. coli DH5α pTZ57R : pVSD1 showed a initial lag phase longer as compared to *E. coli* DH5α pVS2k3: pVSD1 but there was no change in the growth rate due to the antisense expression. Expression of the citrate transporter (*E. coli* DH5α pVSD1) (0.09 ± 0.02 h^{-1}) alone altered the growth rate as compared to the plasmid free strain. No acidification was observed even after 120 h. There seemed no variation in the amount of glycerol consumed in any of the *E. coli* strains (**Fig. 5.4 (i) and (ii)**).

E. coli strains	Growth rate (μ) h^{-1}	Total glycerol consumed (mM)
E. coli DH5α	0.13 ± 0.015	78 ± 5.29
DH5α pTZ57R	0.12 ± 0.13	66 ± 2.9
DH5α pVS2k3	**0.17 ± 0.007 ns**	**66 ± 2.7 ns**
DH5α pVSD1	0.09 ± 0.02**	67 ± 0.6
DH5α pTZ57R:pVSD1	0.10 ± 0.02	68 ± 5.29
DH5α pVS2k3:pVSD1	**0.12 ± 0.004 ns**	**68 ± 5.29 ns**

Table 5.4: Physiological parameters of *E. coli* DH5α pVS2k3 on M9 minimal media with glycerol (100mM) as carbon source. The results are expressed as Mean ±SD of 8-10 independent observations. specific growth rate (μ (h^{-1})) and was determined from mid log phase of each experiment. Total glycerol utilized was determined at the end of growth curve. ** $p<0.01$, ns=non-significant.

5.3.5: Effect of *E. coli* DH5α pVS2k3 on the ICDH, citrate synthase and glucose-6-phosphate dehydrogenase activity on M9 minimal medium with 100mM glycerol.

On glycerol the ICDH activity was (0.3 ± 0.04 U) for *E. coli* DH5α. No down-regulation of the ICDH activity was observed in *E. coli* DH5α pVS2k3 (0.34 ± 0.03 U). Similar was the case for *E. coli* DH5α pVS2k3: pVSD1 (0.32 ± 0.04 U). This suggests that there was no significant change in the ICDH activity with antisense expression on glycerol (**Fig. 5.5 (i) and (ii)**).

G-6-PDH activity did not vary for *E. coli* DH5α expressing as-*icd* (0.087 ± 0.004 U) as compared to *E. coli* DH5α pTZ57R (0.096 ± 0.003 U). Presence of plasmid resulted in high G-6-PDH activity as compared to the plasmid free strain. as-*icd* expression did not alter CS activity on glycerol (**Fig. 5.6 (i)**).

E. coli DH5α carrying citrate transporter both as a single and double transformants showed high G-6-PDH compared to plasmid free strain. *E. coli* DH5α pTZ57R:pVSD1 (0.102 ± 0.002 U) had much higher G-6-PDH compared to *E. coli* DH5α pVS2k3:pVSD1 (0.082 ± 0.002 U). *E. coli* DH5α pVSD1 (0.087 ± 0.004 U) showed no significant change in the CS activity when compared the plasmid free strain.

E. coli DH5α pVS2k3:pVSD1 (0.09 ± 0.004 U) had significant increase in the CS activity when compared to *E. coli* DH5α pTZ57R: pVSD1 (**Fig. 5.6 (ii)**).

5.3.6: Effect of *E. coli* DH5α pVS2k3 on organic acid secretion in M9 minimal medium with 100mM glycerol.

Intracellular citrate levels in *E. coli* DH5α (3.12 ± 0.18 mM) were significantly higher on glycerol as compared to that on glucose (**Table 5.5**). A significant decrease in citrate levels was observed in the plasmid bearing *E. coli* strains (2.24 ± 0.14 mM, 2.66 ± 0.22 mM) but not significant when compared amongst the respective plasmid controls. The double transformants with citrate transporter had lower citrate levels compared to the parent strain. Extracellular levels of acetate were found to be almost negligible in *E. coli* DH5α on glycerol. Although low levels of acetate was observed in case of *E. coli* DH5α carrying citrate transporter (single and double transformants). No extracellular citrate was detected *E. coli* DH5α expressing as-*icd* either in presence or absence of citrate transporter.

E. coli strains	Intracellular citrate (mM)	Extracellular Acetate (mM)
DH5α	3.12 ± 0.14	5.7 ± 0.4
DH5α pTZ57R	2.24 ± 0.18 **[(a)]	1.46 ± 0.14 ***[(a)]
DH5α pVS2k3	**2.66 ± 0.22 [ns(b)]**	**1.9 ± 0.1 [ns(b)]**
DH5α pVSD1	1.01 0.08 ***[(a)]	ND
DH5α pTZ57R: pVSD1	1.37 ± 0.18	1.14 ± 0.10
DH5α pVS2k3 : pVSD1	**1.41 ± 0.35 [ns (b)]**	**1.6 ± 0.18 [ns (b)]**

Table 5.5: Organic acid production of *E. coli* DH5α expressing as-*icd* 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 100 mM glycerol as carbon source. All the values are represented as Mean ± SD of n=4-8 observations. [(a)] shows the significance with respect to *E. coli* DH5α devoid of any plasmid and [(b)] shows the significance with respect to the plasmid control ** p<0.01, *** p<0.001, ns=non-significant.

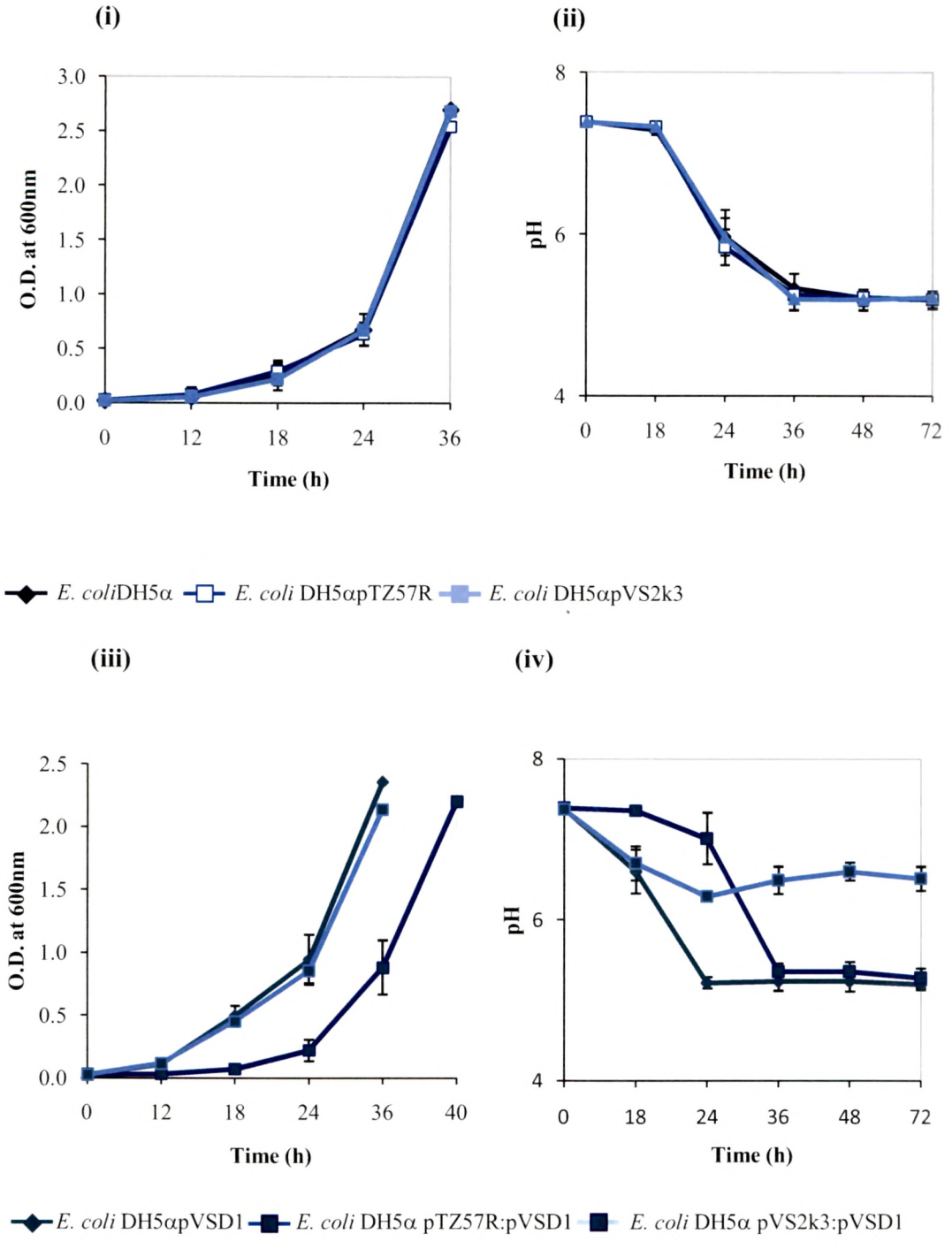

Fig. 5.4: Growth characteristics of *E. coli* pVS2k3 on M9 minimal medium with 100mM glycerol in presence and absence of citrate transporter. (i) & (ii) $O.D_{600nm}$ and pH without citrate transporter (iii) & (iv) $O.D_{600nm}$ and pH with citrate transporter. All values plotted here are represented as Mean ± SD of n=5-8 observations.

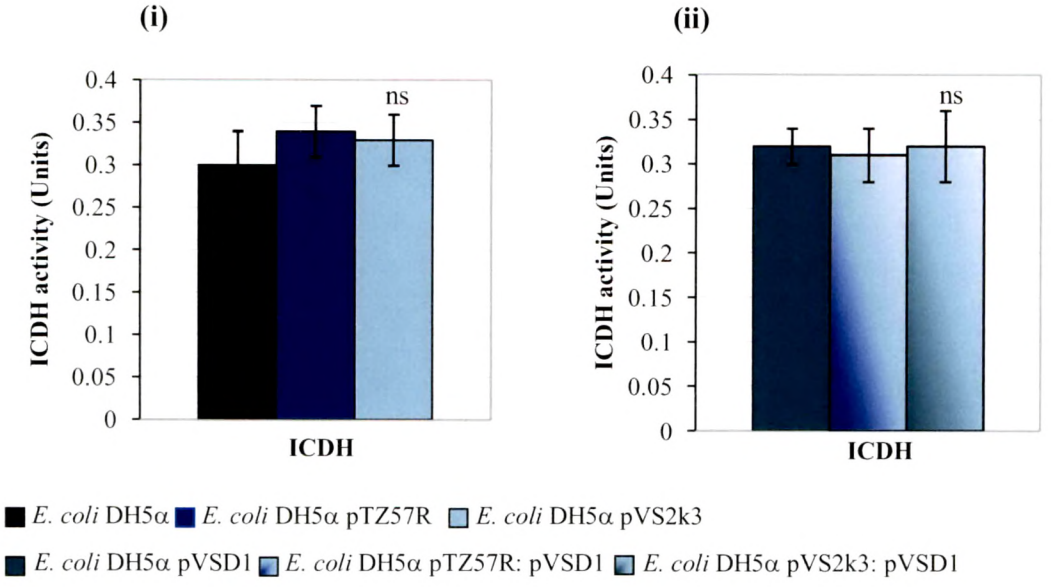

Fig. 5.5: Effect of *icd* gene downregulation on ICDH activity on M9 minimal media with 100mM glycerol. (i) without citrate transporter (ii) with citrate transporter. All values plotted here are represented as Mean ± SD of n= 4-10 observations. Units - μmoles/mg. protein/min. ns=non-significant.

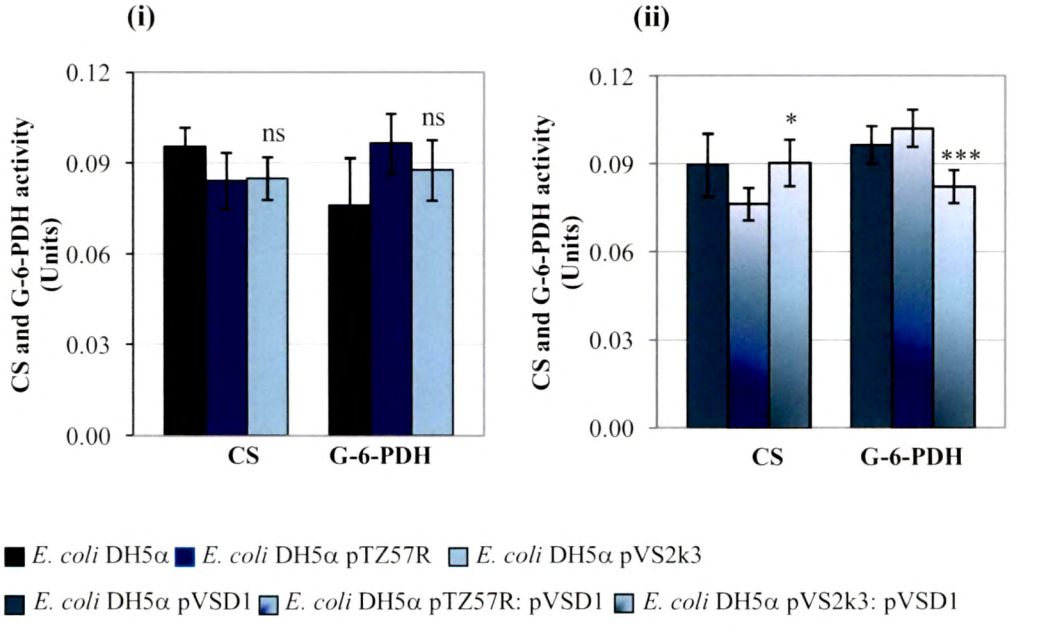

Fig 5.6: Effect of *icd* gene downregulation on citrate synthase and glucose 6-phosphate dehydrogenase activity on M9 minimal media with 100mM glycerol. (i) without citrate transporter (ii) with citrate transporter. All values plotted here are represented as Mean ± SD of n= 5-8 observations. Units- μmoles/mg. protein/min. * $p<0.05$, *** $p<0.001$, ns=non-significant.

5.4: DISSCUSSION

Antisense RNA has been used to down-regulate the expression of bacterial genes to overcome the problems encountered with null mutants (Kurreck, 2003, Kernodle et al, 1997). Constitutively expressed antisense constructs have limitations due to lethality and slow growth. Conditional antisense RNA expressing systems are considered to be more effective as they do not inhibit growth. A tetracycline (*tet*) regulated antisense RNA expressing system was developed to allow selective genes of the chromosome to be switched on and off and be expressed at certain levels thus proving a potential tool to generate a quantitative data of the gene product (Dezhong and Yinduo, 2002). Parish and Stoker (1997) demonstrated that conditional regulation of antisense RNA expression under an inducible promoter in mycobacteria facilitated the elucidation of the role of essential genes.

Present study demonstrates the development of a conditionally regulated antisense RNA expression against the *icd* gene of *E. coli*. The strategy exploits the natural mechanism of Cra protein-mediated control of *fruB* promoter which connects carbon source availability through intracellular F-1-P and F-1,6-P concentrations to gene expression. *E. coli icd* mutants were unable to grow on glucose as a sole carbon source and required glutamate supplementation for synthesis of various amino acids. These mutants demonstrated poor growth rate and low glucose consumption rate. The present study exhibits that the use of conditional downregulation of *icd* gene using antisense, could not only overcome the need for glutamate supplementation but had an improved growth and glucose consumption rate compared to the mutant. This could be attributed to the partial block of the ICDH activity (3-4 fold) which could meet the requirement for glutamate.

P_{fruB} as-*icd* expression showed higher CS activity with respect to its control. This result is in agreement with the earlier results related to *E. coli icd* mutants (Lakshmi and Helling, 1976; Kabir and Shimizu, 2004). Increase in CS activity in icd mutants was proposed to enhance NAD(P)H levels which were generated by ICDH in wild type. In

contrast to earlier reports (Park et al., 1994), the present study demonstrates the increase in growth along with high CS activity in the conditional as*icd*.

Characteristic properties	*E. coli icd* mutant[1,2]	*E. coli* DH5α pVS2k3[3]
Growth rate	slow	Same as control
Glucose consumption rate	low	high
Glutamate supplementation	required	Not required
ICDH activity	ND	Partial (30-40%)
G-6-PDH	high	low
CS	high	high
ICL	high	ND
citrate (intracellular)	11mM	1.34 mM
Acetate	Low	high

Table 3.6: Characteristic differences between an *E. coli icd* mutant and *E. coli* expressing as-*icd* gene under *fruB* promoter.[1,2] (Lakshmi and Helling 1976; Kabir and Shimizu, 2004), [3] Present study.

Compared to the *E. coli icd* mutants, G-6-PDH activity was found to be lower in *E. coli* expressing as-*icd* with respect to its control. This could be attributed to the partial block that suffices the requirements of NADPH. ICL activity was not observed in *E. coli* on glucose irrespective of antisense expression which is well supported by the reports that on glucose there is no ICL activity in *E. coli* K-12 derivative (Phue et al., 2005).

P_{fruB} as-*icd* expression significantly increased intracellular citrate levels was similar to the *icd* mutant. High acetate levels were observed in case of *E. coli* expressing antisense compared to low acetate levels monitored in the *icd* mutant (Kabir and Shimizu, 2004). *E. coli* has a reportedly low TCA flux and the partial block in the ICDH activity probably leads to further decrease in the TCA flux hence the large amount of pyruvate accumulated could be directed to acetate formation. Absence of citrate in the extracellular medium is due to the absence of a citrate efflux mechanism in *E. coli* (Lara and Stokes, 1952).

All *E. coli* strains including P_{fruB} *asicd* had reduced growth rate on glycerol which is in agreement with earlier study (Holms, 2001). No significant change in the ICDH activity *E. coli* P_{fruB} *asicd* suggested that the P_{fruB} promoter is active as on glycerol low levels of fructose-1-phosphate represses P_{fruB} promoter. This is in agreement with earlier reports that *fruB* promoter responded to the fructose in the medium but failed to express on galactose (Leveau and Lindow, 2000). On glycerol all *E. coli* cultures showed higher CS activity and increased intracellular citrate levels compared to that on glucose All the *E. coli* cultures secreted low levels of acetate which is in agreement with the flux analysis carried out by Holms (2001). *E. coli* P_{fruB} *asicd* containing *Klebsiella pneumoniae* H^+-citrate transporter showed poor growth and glucose consumption rate compared to that of the control. Presence of citrate transporter did not influence enzyme activities (CS, G-6-PDH, ICL and ICDH), citrate accumulation and acetate secretion. But no citric acid was secreted.

Plasmids have also reported to alter metabolism (growth rate, glucose consumption rate, enzymes of TCA cycle) in response to the metabolic load (Neubauer et al, 2002; Wang et al., 2006; De Gelder, 2007). In the present study pTZ57R (high copy number plasmid) caused an overall reduction in the glucose consumption rate and poor growth rate. Although expression of the citrate transporter (pVSD1) alone had no effect on the growth rate and glucose consumption rate, it reduced glucose consumption and increased CS activity; while *E. coli* pTZ57R:pVSD1 showed increased growth rate and poor glucose consumption rate compared to the single transformants and the plasmid free strain.

Expression of as-*icd* under P_{fruB} promoter demonstrated a constitutive expression of antisense in presence of glucose and fructose irrespective of the growth phase of the organism (Chapter 3). Hence such a conditional expression is advantageous over the use of other inducible promoters which depend on the concentration of the inducer and the the growth phase (high expression monitored between log to mid log phase (Wu et al., 2007, Chao et al., 2003). P_{fruB} has directed efficient gene expression even under very low concentration of fructose (Leveau and Lindow, 2000).

The present study suggests that exploiting the conditional down regulation of genes could be a promising tool in studying regulation of certain genes especially those that play an important role in metabolism like isocitrate dehydrogenase. The conditional antisense RNA strategy employed in this study is novel in that it avoids the use of external inducers for the down- regulation of target genes and depends upon the nature of the available carbon source.