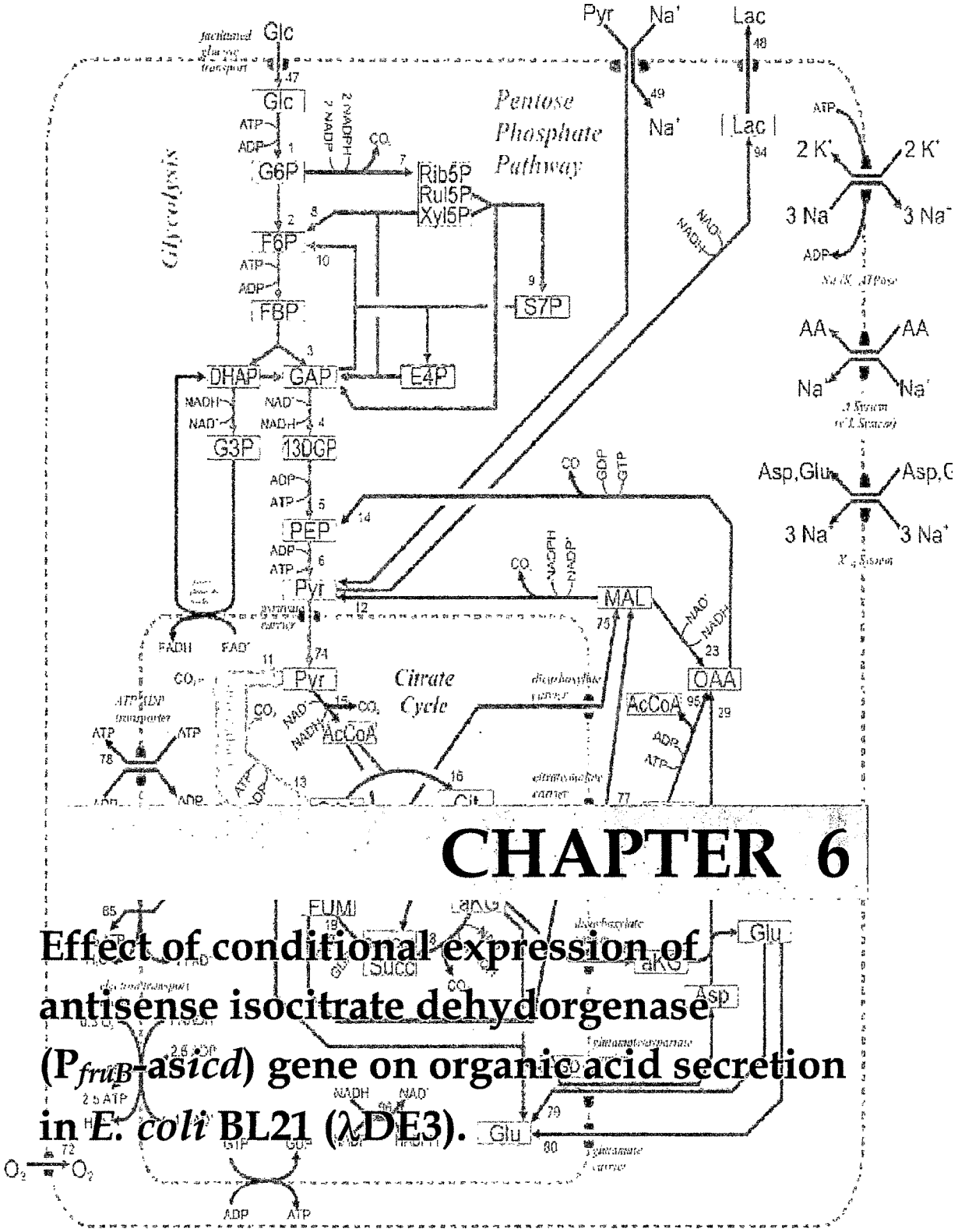

CHAPTER 6

Effect of conditional expression of antisense isocitrate dehydorgenase (P_{frdB}-as*icd*) gene on organic acid secretion in *E. coli* BL21 (λDE3).

6.1: INTRODUCTION

E. coli has been one of the most preferred model organisms for production of various recombinant proteins. Most common problem encountered when *E. coli* is grown on excess glucose is acetate secretion. Excess glucose was suggested to exceed the capacity of the carbon flux of TCA through glycolysis and hence results in acetate accumulation (Majewski and Domach, 1990). High concentrations of acetate (above 40mM) have shown to affect growth and recombinant protein synthesis (Kleman and Strohl, 1994). Multiple strategies have been developed to decrease acetate accumulation; these include controlling glucose addition to the fed batch cultures and generating mutants reducing acetate formation (Aristodou et al., 1994; Chou et al., 1994; Han et al., 1992; Resienberg and Guthke, 1999).

E. coli BL21 a derivative of *E. coli* B strain has reported to produce less amount of acetate as compared to *E. coli* JM109, a K-12 derivative (Shiloach et al., 1996). This ability was hypothesized to be a result of mutation in one or two enzymes that play a key role in production of acetate (phosphotransacetylase and acetate kinase) (Kleman and Stohl, 1994). Although both the strains of *E. coli* have shown to produce acetate at a similar specific production rate the amount of acetate secreted is different which has been attributed to functioning of an alternative acetate control mechanism in *E. coli* BL21.

6.1.1: Acetate utilization in *E. coli*

Acetate can be formed from acetyl CoA via phosphotransacetylase (Pta) and acetate kinase (AckA) and from pyruvate via pyruvate oxidase (PoxB) (Abdel-Hamid et al., 2001; El-Mansi and Holms, 1989). Acetate can be converted back to acetyl CoA by acetyl CoA synthetase and via the reversible Pta-AckA pathway. Acetyl CoA can be metabolized through the TCA and glyoxalate shunt (El-Mansi and Holms, 1989; Oh et al., 2002). Glyoxalate shunt pathway is regulated by *ace*BAK operon that includes *ace*K (isocitrate phosphatase/kinase), *ace*A (isocitrate lyase) and *ace*B (malate synthase) (Chung et al., 1988). ICDH plays a key role in the regulation of the shunt; the enzyme itself undergoes

posttranslational modifications (phosphorylation and dephosphorylation) mediated by *ace*K (ICDH phosphatase/kinase) (Thorsness and Koshland, 1987). The expression of glyoxylate pathway is under the negative control of isocitrate lyase repressor (*icl*R) repressor of the operon and the activator of fatty acid biosynthesis operon (*fad*R) (Gui et al., 1996; Sunnaborg et al., 1990). This pathway is also activated by FruR, also called Cra, and integration host factors (IHF) (Ramsaier et al., 1995; Resnik et al., 1996). Acetyl CoA could be utilized efficiently via glyoxalate pathway in *E. coli* BL21 as isocitrate lyase activity is high even on glucose. C^{13}NMR and Mass spectrometric studies showed that 22% of citrate is catabolized via glyoxalate shunt in *E. coli* Bl21 (Noronha et al., 2000). In addition, gluconeogenesis plays a significant role in acetate utilization and it is constitutive in *E. coli* BL21 (Yang et al 2003).

6.1.2: Metabolic differences between *E. coli* K and *E. coli* B strains

E. coli BL21 has a remarkably high TCA flux compared to *E. coli* JM109 and supports high growth rate. Fluxes of TCA and phosphoenolpyruvate carboxylase shunt (PPC shunt) in *E. coli* BL21 are of equal magnitude while in *E. coli* JM109, one third of the TCA cycle operates through PPC (Noronha et al., 2000). Gene expression profile of *E. coli* BL21 on high glucose demonstrated an active glyoxalate shunt, TCA cycle, gluconeogenesis pathway, glycogen biosynthesis, acetate conversion to acetyl CoA, oxaloacetate conversion to phosphoenolpyruvate and fatty acid degradation while *E. coli* JM109 had lower activity of all pathways excepting the conversion of pyruvate to acetate through PoxB and malate conversion to pyruvate via NAD-dependent malate dehydrogenase. This suggested that *E. coli* JM109 was more sensitive to glucose concentration than *E. coli* BL21 (Phue et al., 2005).

Pyruvate accumulation in *E. coli* JM109 on high glucose takes three routes that include an inactivation of phosphoenol pyruvate synthase (*pps*) (pyruvate to phosphoenol pyruvate), low pyruvate dehydrogenase (pyruvate to acetyl CoA) activity and a high malate dehydrogenase (*sfc*A) (malate to pyruvate). All these cumulatively point towards possible outflow of metabolite (acetate) due to inefficient regulation of pyruvate levels. Moreover it

is possible that pyruvate oxidase compliments pyruvate dehydrogenase activity in lowering pyruvate levels (Abdel-Hamid et al., 2001). This is supported by the high pyruvate oxidase activity on high glucose. In case of *E. coli* BL21, high pyruvate dehydrogenase activity in the early log phase lowers pyruvate levels (Phue et al., 2005). The expression levels of regulatory protein also varied on high glucose which marked high expression of *fru*R, *him*A and *him*D (positive regulators) and low levels of IclR negative repressor of *ace*BAK operon supporting the active glyoxalate shunt in *E. coli* BL21. The behaviour of the gluconeogenic enzymes viz *pps*, *acs* and *ace*BAK in *E. coli* JM109 is dependent on the glucose concentration. On lower glucose, *E. coli* JM109 behaves like *E. coli* BL21 while on high glucose these three genes are inactive (**Table 6.1**).

Characteristics on high glucose	*E. coli* K	*E. coli* B
Growth rate	slow	fast
Glucose utilization rate	high	Low
TCA flux	low	high
PoxB activity	high	low
Cra	Present	Dysfunction
Glyoxylate shunt, gluconeogensis	absent	constitutive
Acetate secretion	high	low
Low glucose	mimicks *E. coli* B (high glucose)	mimicks *E. coli* K (low glucose)

Table 6.1: Metabolic differences between *E. coli* K and *E. coli* B strain on high glucose (Noronha et al., 2000; Phue et al., 2005).

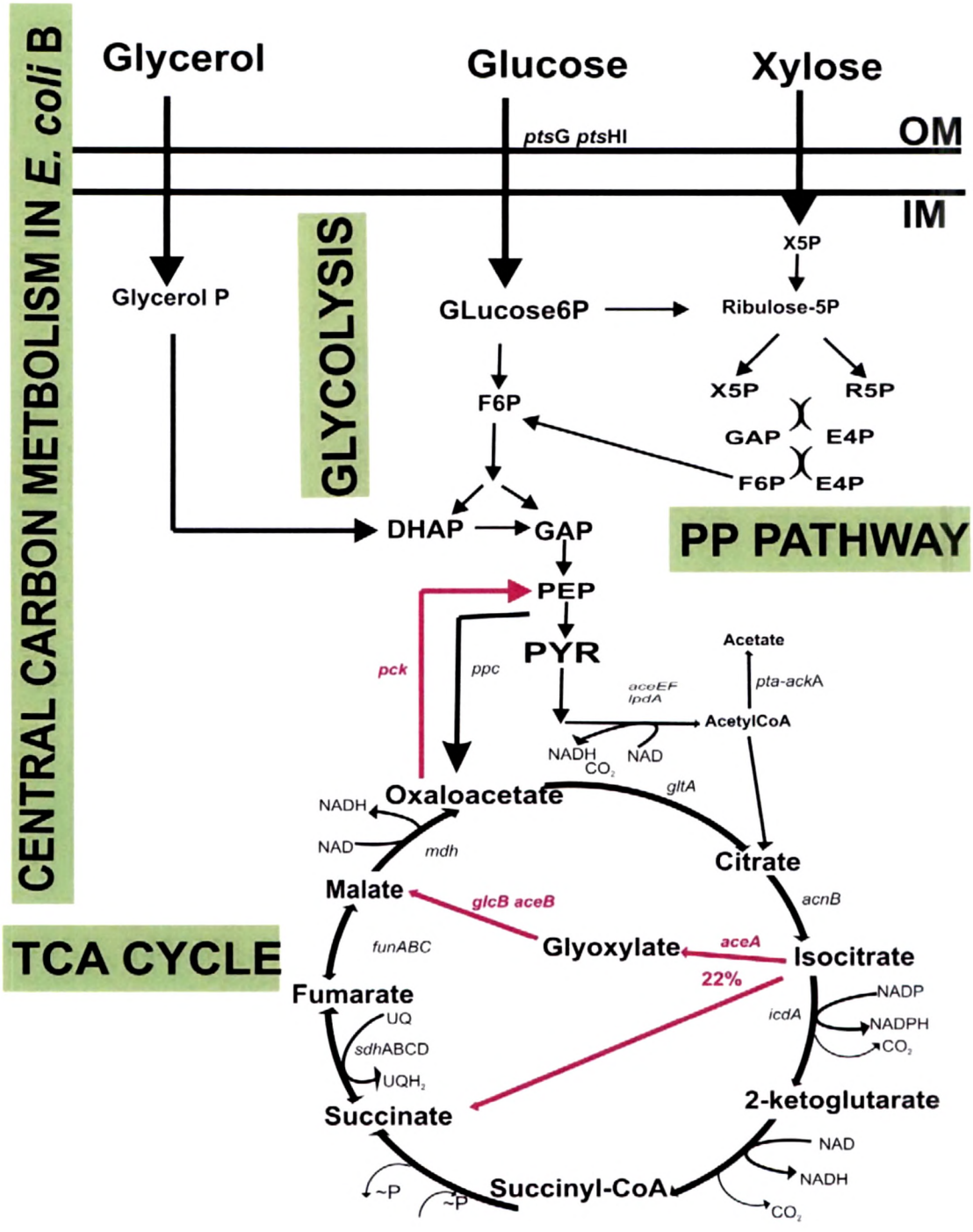

Fig 6.1: Carbon metabolism in *E. coli* BL21. Gene marked (red) are constitutively expressed on glucose, *pck* phosphenol pyruvate caboxykinase, *ace*A-isocitrate lyase, *ace*B-malate synthase, 22% is the precent activity of glyoxylate bypass on glucose.

6.1.3: Rationale for the present study

The present study investigates the efficacy of the P_{fruB} promoter in regulating the expression of antisense for isocitrate dehydrogenase in *E. coli* BL21 (λDE3).

6.2: WORK PLAN

6.2.1: Bacterial strains used

E. coli strains	Genotype	References
BL21(λDE3)	*F', ompT, hsdS$_B$ (r_{B-}, m^{B+}), dcm, gal, (DE3)*	Sambrook and Russell, 2001
BL21(λDE3) pTZ57R	T-vector, ap^r	Present study
BL21(λDE3)pVS2k3	P_{fruB}, as-*icd*, ap^r	Present study

Table 6.2: List of bacterial strains used for the present study. Details of plasmids and antibiotics concentration used for both rich and minimal medium are given in table 2.2 and 2.3.

6.2.2: Incorporation of pVS2k3 in *E. coli* BL21(λDE3).

pVS2k3 and pTZ57R plasmids were isolated form *E. coli* DH5α and transformed into *E. coli* BL21(λDE3) through the protocol mentioned in section 2.3.2 and were selected on ampicilin plates.

6.2.3: Monitoring growth parameters of *E. coli* BL21(λDE3):pVS2k3.

E. coli BL21(λDE3): pVS2k3 transformants were subjected to physiological experiments on M9 minimal media with micronutrients and either glucose (30mM, 100mM) or acetate (50mM) as the carbon source. Samples were collected at regular intervals for O.D $_{600nm}$, 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 at late log phase and stationary phase, G-6-PDH, CS and ICL at stationary phase.

6.3: RESULTS

6.3.1: Effect of pVS2k3 on physiological parameters in *E. coli* BL21 (λDE3) on glucose and acetate as carbon source.

E. coli BL21(λDE3) on high glucose showed a growth rate of 0.74 ± 0.10 h^{-1}. Expression of as-*icd* under P_{fruB} had not influenced the growth rate (0.64 ± 0.34 h^{-1}) when compared to plasmid control (0.60 ± 0.15 h^{-1}). Acidification of the medium below 5 was observed at the end of 14 h. Lowering glucose concentrations to 30mM or increasing it to 100mM did not alter the growth rate in either of the cases. No acidification was observed with lesser glucose. *E. coli* BL21(λDE3) showed a slower growth rate (0.38 ± 0.06 h^{-1}) on acetate as compared to that on glucose as a carbon source. Moreover the media turned alkaline at the end of 20 h (**Table 6.3, Fig. 6.2 (i)-(vi)**).

E. coli strains	Growth rate on 100 mM glucose $\mu(h^{-1})$	Growth rate on 30 mM glucose $\mu(h^{-1})$	Growth rate on acetate $\mu(h^{-1})$
BL21(λDE3)	0.71 ± 0.10	0.74 ± 0.10	0.38 ± 0.06***[a]
BL21(λDE3) pTZ57R	0.58 ± 0.17	0.60 ± 0.15	0.31 ± 0.10
BL21(λDE3)pVS2k3	0.60 ± 0.24[ns]	0.64 ± 0.34	0.42 ± 0.10

Table 6.3: Effect of pVS2k3 expression on the growth rate of *E. coli* Bl21(λDE3) on glucose and acetate. All values expressed as Mean ± SD of 4-6 independent observations. [a] comparison between *E. coli* BL21(λDE3) grown on glucose and acetate. *** $p<0.001$, ns- non significant.

6.3.2: Effect of pVS2k3 on ICDH, CS and G-6-PDH activities.

E. coli BL21(λDE3) showed 1.15 ± 0.14 U of ICDH activity. *E. coli* BL21(λDE3) control and expressing as-*icd* showed 1.52 ± 0.12 U and 1.7 ± 0.17 U ICDH activity, respectively, in late log phase and a lower ICDH activity (0.55 ± 0.10 U and 0.8 ± 0.12 U) was observed at stationary phase (**Fig 6.3 (i)-(vi)**). Similar results were observed in case of *E. coli* BL21(λDE3) when grown on 30mM glucose. ICDH activity in *E. coli* BL21(λDE3) (1.25 ± 0.10) when grown on acetate remained same as that on glucose. The G-6-PDH activity of *E. coli* BL21(λDE3) pTZ57R and *E. coli* BL21(λDE3) pVS2k3 were 0.29 ± 0.02U and 0.28 ± 0.03U, respectively. There was no change in the citrate synthase activity in either the plasmid bearing strain or plasmid free strain. *E. coli* BL21(λDE3) showed 5.72 ± 0.95 U of ICL activity which significantly decreased in presence of the plasmids (2.66 ± 0.24 U and 2.60 ± 0.25 U).

6.3.3: Effect of pVS2k3 on organic secretion.

In all these strains, acetate was observed as the major acid secreted into the extracellular medium both on high and low glucose media (**Fig. 6.4 (i) and (ii)**). On lesser glucose, comparatively higher acetate was observed. Although intracellular citrate levels did not vary in response to the expression of as-*icd*, an overall reduction in the citrate levels were observed for the plasmid bearing strain compared to *E. coli* BL21(λDE3). No extracellular citrate was observed in either of the cases (**Table 6.4**).

E. coli strains	Intracellular citrate (mM)	Acetate (mM)
BL21(λDE3)	2.1 ± 0.04	27.75 ± 0.75
BL21(λDE3) pTZ57R	0.78 ± 0.06***[a]	19.34 ± 3.4***[a]
BL21(λDE3)pVS2k3	**0.68 ± 0.12[b]**	**14.23 ± 3.4 [ns b]**

Table 6.4: Organic acid formation in *E. coli* BL21 (λDE3) transformants expressing pVS2k3. All the values are represented as Mean ± SD values of n= 4-8 observations. [a] comparison made between plasmid free strain and plasmid bearing strain; [b] comparison made between as-*icd* expressing strain and its plasmid control. ***p< 0.001, ns-non-significant.

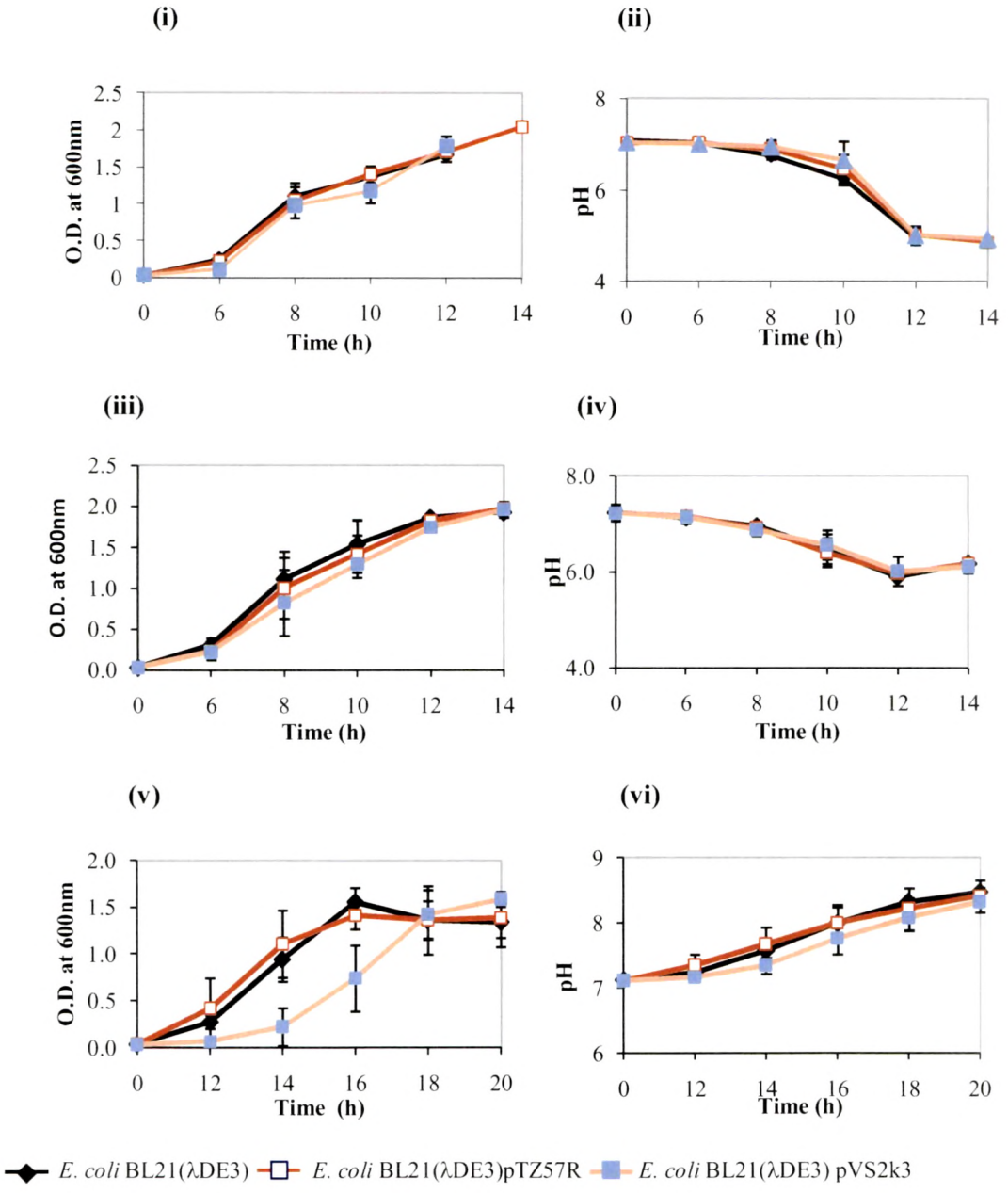

Fig. 6.2: Growth and pH profiles of *E. coli* Bl21(λDE3) transformants on M9 minimal media with varying glucose concentrations and 50mM acetate. (i) & (ii) $O.D_{600nm}$ and pH on 100mM glucose (iii) & (iv) $O.D_{600nm}$ and pH on 30mM glucose (v) & (vi) $O.D_{600nm}$ and pH on 50mM acetate. The values plotted represent the Mean ± SD of 4-8 independent observations.

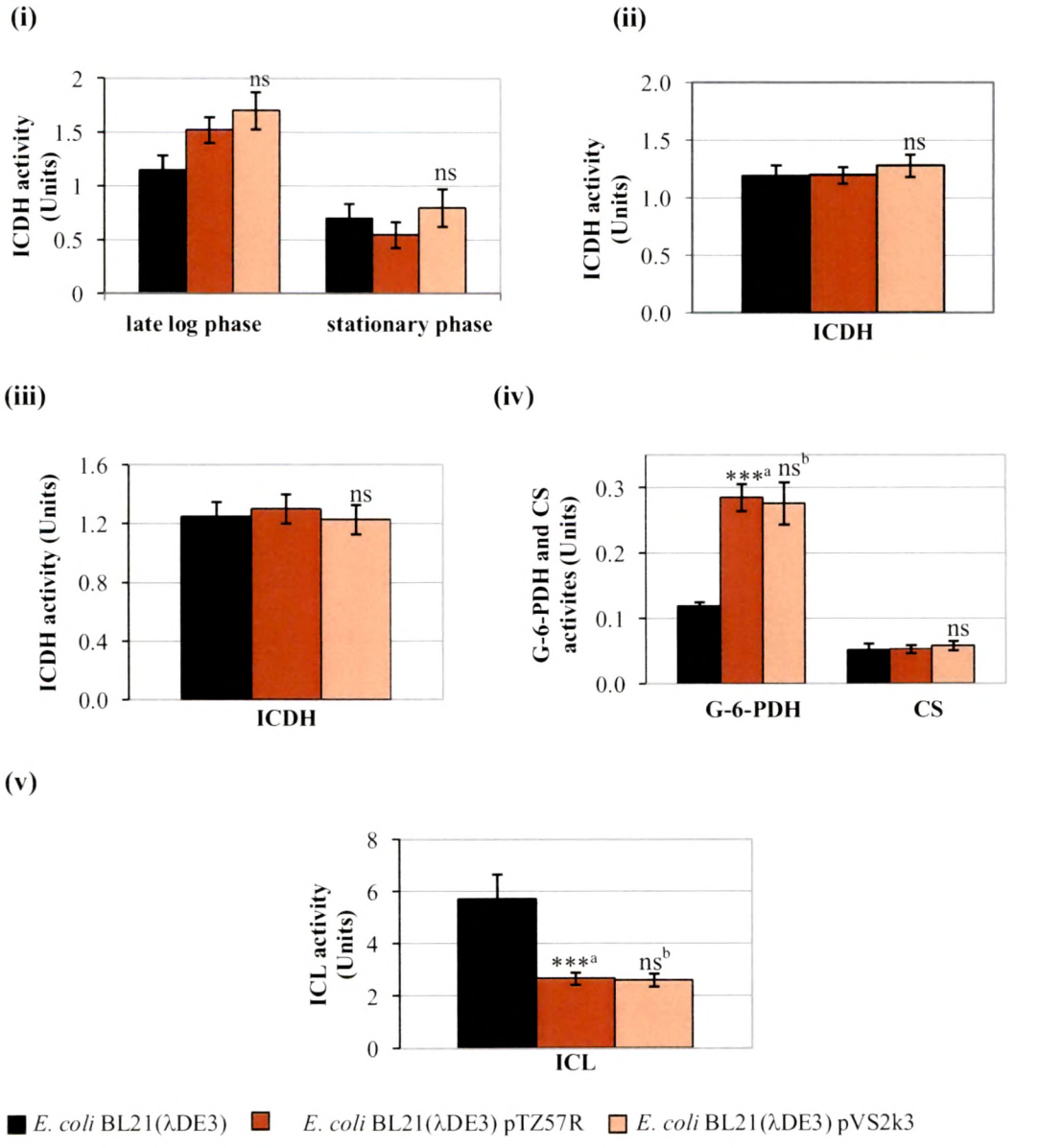

Fig 6.3: Effect of pVS2k3 expression on various key enzymes of the central metabolic pathway. (i) (ii) & (iii) ICDH activity on high glucose, lesser glucose and acetate (Units- μmoles/mg. protein/min) (iv) CS and G-6-PDH activity on high glucose (v) & (vi) ICL activity on high glucose and acetate. Units of Specific activity are μmoles/mg. protein/min except for ICL it is ηmoles/mg. protein/min. All the values are represented as Mean ± SD values of n= 4-8 observations. [a] comparison between plasmid free and plasmid bearing strain, [b] comparision between plasmid control and test. *** $p<0.001$, ns- non significant.

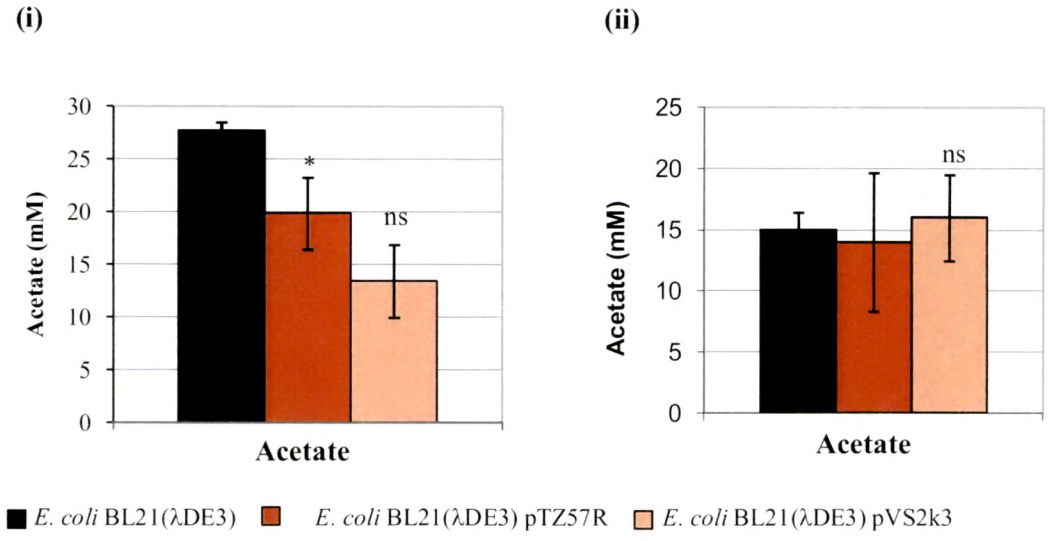

Fig. 6.4: Organic acid formation in *E. coli* BL21 (λDE3) transformants expressing pVS2k3. (i) and (ii) acetate secretion on high (100 mM) and lesser glucose (50mM). All the values are represented as Mean ± SD values of n= 4-8 observations. * $p< 0.05$, ns-non significant.

6.4: DISCUSSION

E. coli BL21(λDE3) bears the advantage of being a low acetate secreting strain which is mainly attributed to the constitutive glyoxylate bypass and gluconeogenic pathway operating in the host as a result of an altered Cra. Unaltered ICDH activity in the *E. coli* BL21(λDE3):pVS2k3 suggested that P_{fruB} as-*icd* is not expressed in the host. It is supported by the fact that Cra expression is altered in *E. coli* BL21 strain (Phue et al., 2005) and further supported by the high ICDH activity on acetate as carbon source. Previous studies have reported for *E. coli* K strain that on acetate ICDH undergoes a post translational modification and thus resulting in lower activity on acetate (Thorsness and Koshland.1987). But the present study suggests that such protein modification may not be present in *E. coli* BL21(λDE3).

E. coli BL21(λDE3) showed a higher growth rate compared to the *E. coli* K-strain derivative. Expression of P_{fruB} as-*icd* had no impact on the growth rate. Incorporation of plasmid itself reduced growth rate. This result is in agreement with the earlier reports that high copy plasmid expression in *E. coli* BL21 reduced growth rate. It also suggests that comparing a plasmid free *E. coli* to a plasmid bearing strain may be inaccurate (Wang et al., 2006).

Presence of plasmid increased G-6-PDH activity by 2 fold when compared to the plasmid free *E. coli* BL21(λDE3). Expression of P_{fruB} as-*icd* did not alter G-6-PDH, CS and ICDH activities which are in agreement that high copy number plasmid did not alter the TCA cycle enzymes (Neubauer et al, 2002; Wang et al., 2006; De Gelder, 2007). However, 2 fold reductions in ICL activity was observed in the plasmid bearing *E. coli* BL21 (λDE3) strains which increased in response to acetate as carbon source suggesting that this enzyme is inducible on acetate and partially repressed on glucose.

No citrate accumulation was observed in *E. coli* BL21(λDE3) expressing P_{fruB} as-*icd*. But the presence of the plasmid resulted in overall reduction of intracellular citrate and acetate levels on high glucose. *E. coli* BL21(λDE3) demonstrated low acetate secretion on high glucose as compared to *E. coli* DH5α which is agreement with earlier work observing low acetate secretion in *E. coli* BL21(λDE3) (Phue et al., 2005). However, on 30mM glucose the levels of acetate did not vary much as compared to the high glucose medium which is in agreement with acetate secretion of *E. coli* BL21 under low glucose is similar to that of *E. coli* K strain on high glucose (Phue et al., 2005).

The present study points that P_{fruB} is not a suitable promoter for expression studies in BL21(λDE3). Presence of high copy plasmids influences the metabolism of *E. coli* even in minimal medium conditions. Hence, in order to determine the effect of as*icd* in *E. coli* BL21 it is necessary to employ strong inducible promoter.