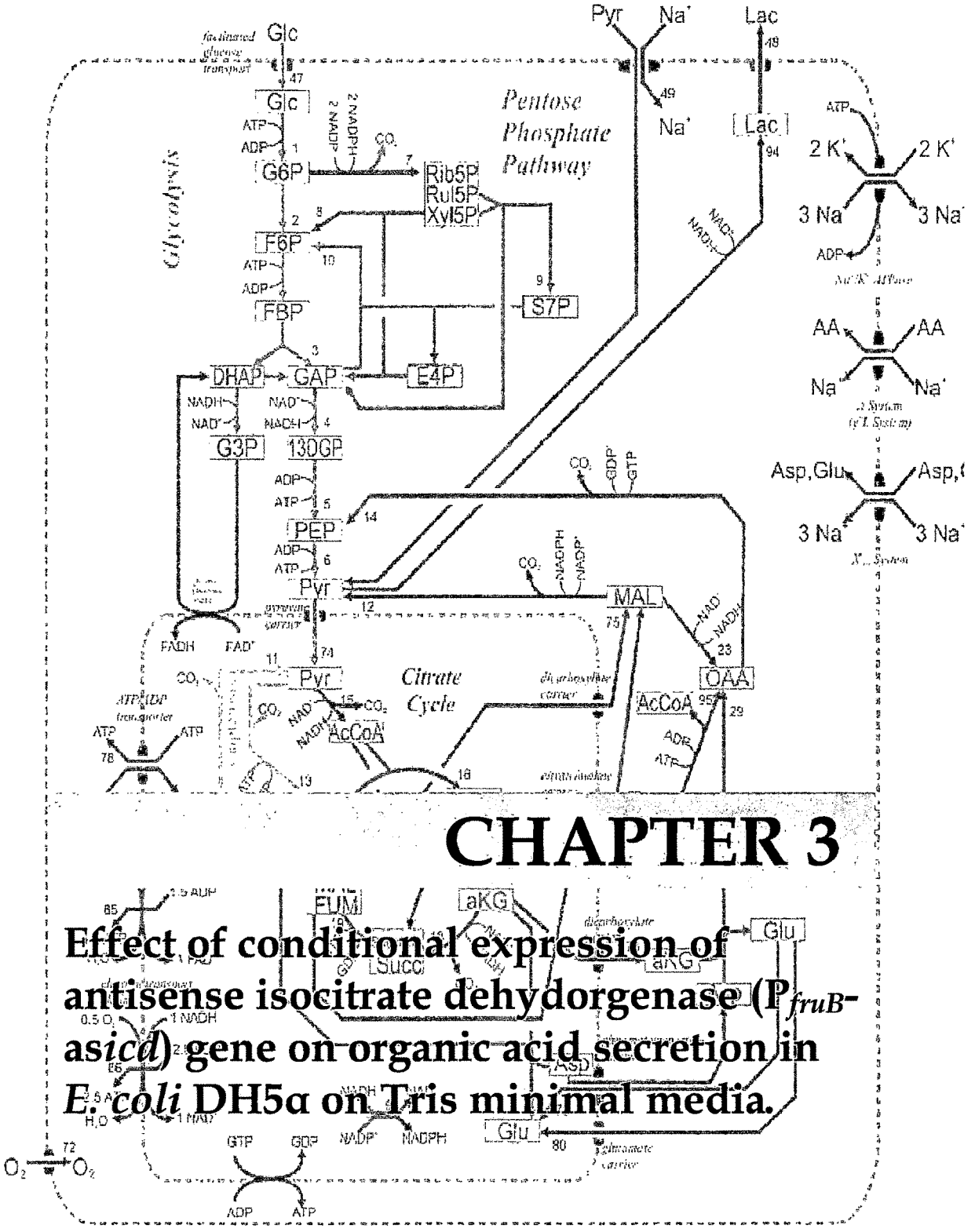


CHAPTER 3

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

3.1: INTRODUCTION

3.1.1: Conditional expression systems regulating Antisense RNA expression

In the post genomic era a vast pool of information has been made available with respect to genome sequences, bioinformatics programs and molecular biology tools. Gene inactivation/disruption strategies have being successfully employed to study the significance of a particular gene in the pathway, making the search for probable targets for metabolic engineering and drug discovery easier to accomplish. Disrupting genes playing an important role in survival and growth, and, recovering these mutants for further analysis pose a disadvantage to the strategy. Hence alternate strategies of using an antisense RNA both constitutive expression or under an inducible system have been preferred. Such a conditional gene expression can give a greater platform in developing quantitative data of gene products.

Antisense RNAs have been demonstrated to be an efficient tool to down-regulate gene expression thus validating potential targets for future modification in metabolic engineering (leading to improved product formation) or developing potential therapeutic drugs. Although this technique has been widely used for eukaryotes (Agarwal et al., 1997; Fire et al., 1998; Mizuta et al., 1999; De Backer et al., 2001) it has been less explored in prokaryotes (well studied for *E. coli* and *Bacillus*) (Guzman et al., 1995; Rappleye and Roth, 1997; Vagner et al., 1998). Antisense RNA mediated control of gene expression was reported to occur naturally, regulating plasmid copy number, phage and chromosomal replication (Wagner and Simmons, 1994; Brantl and Wagner, 2000).

Kernodle (1997) demonstrated potentials of antisense strategy in creating novel live attenuated strains of bacteria to be used as vaccine by using antisense for α-toxin (*hla*) gene against *Staphylococcus* pathogen. This strategy was further modified by expressing the antisense under a tetracycline (*tet*) inducible system (**Fig. 3.1**) (Steiger et al., 1999). Antisense gene cloned under xylose-tetracycline (*xyl/tet*) promoter-operator fusion system (Geissendrofer amd Hillen, 1990) showed a dose dependent down regulation of the *hla*

gene in *S. aureus*, thus reducing the lethality of the *S. aureus* infection (Yinduo et al., 2001; Dezhong and Yinduo, 2002). Generation of conditional mutants has also been well established for *Mycobacterium* using either acetamide inducible system or the *tet* inducible system (Parish and Stoker, 2001; Carroll et al., 2005).

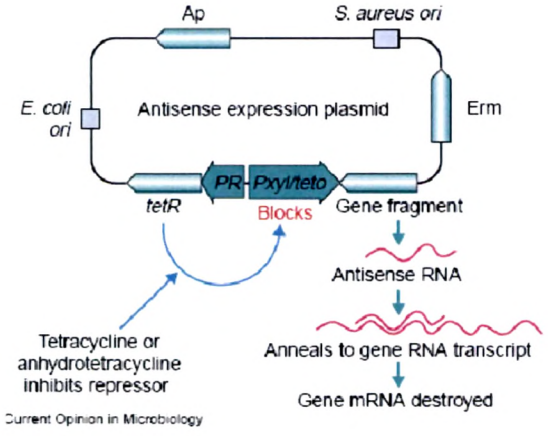


Fig. 3.1: Mechanism of Tetracyline (*tet*) inducible antisense expression system (Dezhong and Yinduo, 2002**).**

Author demonstrated a dose dependent increase in antisense RNA expression (50-100 folds) in response to tetracycline and decrease (14 fold) in α-toxin production in *S. aureus*.

Conditional mutagenesis involves various strategies like use of temperature sensitive mutations directing the regulation of certain proteins (Schmid et al., 1989). This strategy has a drawback as it is difficult to have protein responding to temperature sensitive phenotype ie. suboptimal activity is obtained under permissive temperature. Amber suppressor t-RNA expressed from an arabinose inducible promoter demonstrated the translational control of non-essential genes containing amber mutation with change in medium composition from arabinose to glucose (Herring and Blattner, 2004).

The present study explores developing a conditional expression system for expression of antisense RNA thus studying the significance of the gene in context to regulating the TCA cycle and citrate accumulation.

3.1.2: Isocitrate dehydrogenase a target for citrate accumulation

$NADP^+$-dependent isocitrate dehydrogenase (ICDH), catalyses the conversion of isocitrate to α-ketoglutarate, plays a very critical role in directing the carbon flow towards krebs cycle and glyoxalate bypass. Regulation of ICDH is important for growth on acetate (Dean et al., 1989). In the absence of this regulation, acetate is completely catabolized via tricarboxylic acid cycle leaving no carbon for growth. The glyoxylate bypass, directs isocitrate to form succinate and malate, and avoids CO_2 formation thus providing carbon for growth. Covalent modification (inactivation/activation by ICDH kinase/phosphatase) of ICDH decreases the activity there by increasing the isocitrate pools and 30% is directed to the bypass suggesting that this enzyme regulates carbon flow for growth and energy production (Walsh and Khosland, 1984; Le Porte et al., 1985).

Knockout of *icd* gene in *E. coli* reduced the growth rate (lower ATP/ADP and $NADPH/NADP^+$ ratio) and specific glucose consumption rate (Kabir and Shimizu, 2004). Specific CO_2 evolution rate was found to be high in the mutants, which was supported by the high activities of 6-phoshogluconate dehydrogenase, phosphoenol pyruvate carboxykinase and other NADP-dependent malic enzymes. Upregualtion of the glyoxalate bypass was also observed in these mutants on glucose. *E. coli icd* gene has a 42bp ArcA repressor binding region thus under anaerobic conditions this enzyme reported lower activity (Chao et al., 1997). Expression of *icd-lacZ* gene fusion varied in response to oxygen and carbon availability which provided an insight into the regulation of *icd* gene by global regulators like ArcA and Cra (Prost et al., 1999).

Physiological studies altering carbon metabolism increased the activities of TCA cycle enzymes and the release of citrate and malate (Hoffland et al., 1992; Johnson et al., 1994; 1996). Hence, engineering *E. coli* to accumulate and/or secrete citric acid in high amounts could be a great challenge. *E. coli icd* mutants grew slower than wild type cells but had remarkable increase in the intracellular accumulation of citrate (Lakhsmi and Helling, 1976). These mutants, however, failed to grow on glucose as a sole carbon source and

needed glutamate supplementation. Mutations of the *icd* gene in *Bacillus* (Matsuno et al., 1999) and *Rhizobium* (MacDermott and Kahn, 1992) and *E. coli* BL21 (Aoshima et al., 2003) also have showed citrate accumulation, poor growth and glutamate auxotrophy. Moreover, high levels of intracellular citrate and isocitrate have lead to spontaneous mutations in citrate synthase gene thus improving growth rate (Lakshmi and Helling, 1976).

Down regulation of NADP-ICDH gene expression using antisense RNA technique in transgenic potato plants increased citric acid levels in leaves (Kruse et al., 1999). Thus, down regulation of IICDH activity appears to be a good strategy for citrate accumulation. The present study explores the down regulation of the *icd* gene by an antisense RNA regulated by an inducible promoter.

3.1.3: *fruBKA* operon and CRA regulation

Cra protein of enteric bacteria was initially characterized as the fructose repressor (FruR). Mutants defective in the *cra* gene showed a phenotype that was unable to grow with gluconeogenic substrates as a sole carbon source but showed higher glucose uptake rate and higher acetate outflow (Chin et al., 1987; Sarkar et al., 2008). In *cra* mutant, TCA and glyoxylate bypass were down regulated while pentose phosphate (PP) pathway and Entner Duodroff (ED) pathway where up-regulated (**Table 3.1**) (Sarkar et al., 2008). Thus, Cra acts a global regulator for various genes involved in carbon and energy metabolism (Chin et al., 1987; Geerse et al., 1989; Saier and Ramseier, 1996).

Cra activates and represses gene depending upon where it binds to the DNA with respect to the RNA polymerase binding site. Certain catabolites derived from glucose or fructose, *i.e.* fructose-1-phosphate and fructose-1, 6-bisphosphate, bind to Cra and remove it from DNA therefore diminishing its effect which results in repression of genes that are activated by Cra and activation of genes repressed by Cra (**Fig. 3.2**) (Saier and Ramseier, 1996). The promoter region of FruBKA (P_{fruB}) operon was employed to quantify the amount

of carbon utilization in the phyllosphere (Leveau and Lindou, 2000). Present study explores the use of this promoter for regulation of antisense RNA for *icd* gene.

Enzymes positively regulated by *cra*	Enzymes negatively regulated by *cra*
PEP synthase (*ppsA*)	Fructose catabolic enzymes (*fru BKA*)
PEP carboxykinase (*pckA*)	HPr Enzyme I (*ptsHI*)
Malate synthase; isocitrate lyase (*aceBA*)	Entner-Doudoroff enzymes (*edd-eda*)
Isocitrate dehydrogenase (*icd*)	Phosphofructokinase (*pfk*)
Fructose-1,6-bis phosphatase (*fdp*)	manitol catabolic enzymes (*mtlADR*)
Cytochrome d oxidase (*cyd*)	Erythrose-4-phosphate dehydrogenase (*gapB*)
	Pyruvate kinase (*pykF*)

Table 3.1 : Cra protein transcriptional regulation of genes encoding enzymes of carbon and energy metabolism in enteric bacteria (Saier and Ramsaier, 1996).

3.1.4: Transport mechanisms for citrate

E. coli icd mutants have shown poor growth rate in response to significantly high citrate accumulation (Lakhsmi and Helling, 1976) but it failed to secrete it into the extracellular medium suggesting the absence of citrate transport mechanism in *E. coli* (Lara and Stokes, 1952). This can also be supported as *E. coli* fails to grow on citrate as sole carbon source under aerobic condition (Koser, 1924). Apart from *E. coli*, other organisms of the *Enterobacteriaceae* family can utilize citrate and it also one of the tests of differentiating *E. coli* from other *Enterobacters* (Martin and Washington, 1980). However, some *E. coli* strains are capable of utilizing citrate under aerobic conditions; they have plasmid entitling them this property (Ishiguro and Sato, 1985; Sasatsu et al., 1985).

Citrate efflux in bacteria is a less known phenomenon although a few bacteria like *Citrobacter*, *Bacillus*, *Pseudomonas* (in response Al-toxicity) and *Bradyrhizobium japonicum* (siderophore for iron chelating) secrete citrate (Carson et al., 1992; Rohr et al., 1996; Gyaneshwar et al., 1998; Khan et al., 2006). Citrate uptake mechanisms have been well characterized for *Klebsiella pneumoniae* (van der Rest et al., 1992; Pos and Dimroth, 1996), *Bacillus* (Warner et al., 2000) and *Lactobacillus*. These involve proton–citrate symport (H^+- citrate), Na^+-dependent symport (anaerobic condition), Mg^{2+} dependent symport or a malate-citrate antiport. Even in higher organisms the citrate efflux in the mitochondria is antiport with malate (yeast) and Mg^{2+} dependent symport in *Aspergillus niger* (Burgstaller, 2006). Hence, the present studies dealt with incorporation of citrate transporter in *E. coli* and monitor its effect with antisence *icd* gene.

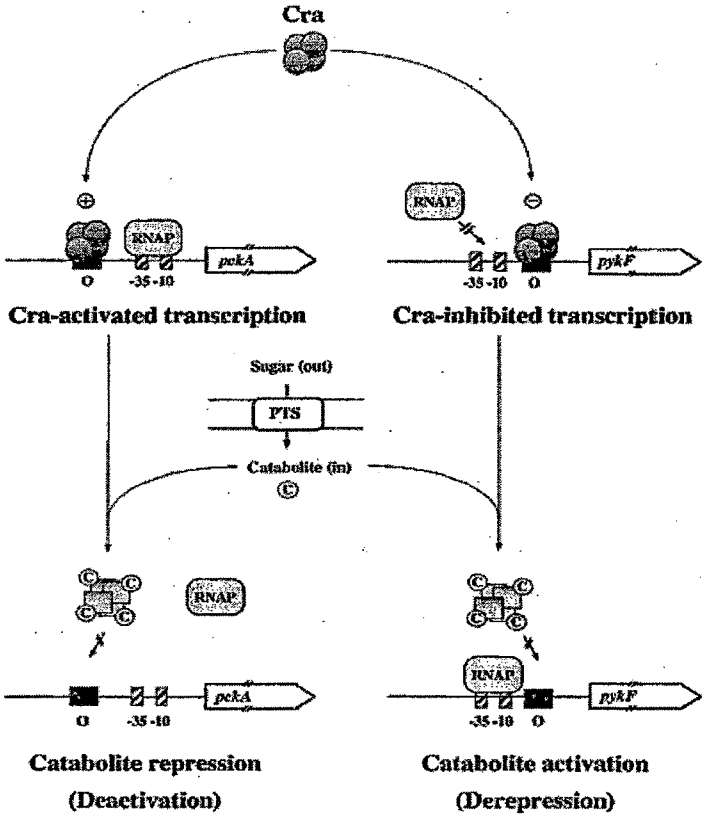


Fig. 3.2: A model of transcriptional regulation of gene transcription by Cra (Saier and Ramseier, 1996).

3.1.5: Rationale for present work

The present work is directed to enable *E. coli* to accumulate and secrete citrate in response to the availability of the carbon source. This work is divided into three parts : (i) Developing an antisense for *icd* gene of *E. coli* (ii) Regulating the antisense expression on carbon sources like glucose and fructose (**Fig. 3.3**). To achieve this *icd* gene was cloned under the promoter of *fruBKA* operon known P_{fruB}. (iii) Subcloning of proton-citrate symporter of *Klebsiella pneumoniae* under *lac* promoter and expressed in *E. coli*.

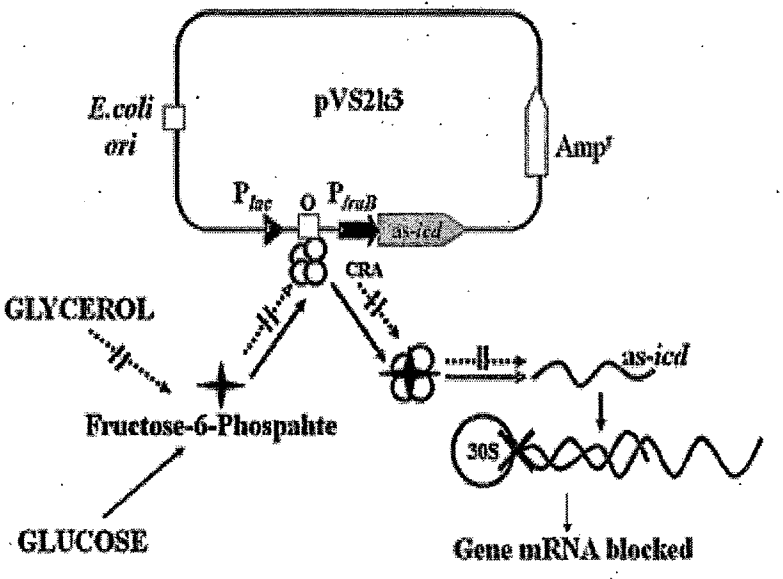


Fig. 3.3: Schematic representation of P_{fruB} regulating as-*icd* expression. **Dark arrows** mark as-*icd* expression on glucose, **broken lined arrow** mark as-*icd* expression on on glycerol. ✦ Fructose-1,6, bisphostate ⊗ CRA

3.2: WORK PLAN

3.2.1: Bacterial strains used for the 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, P_{lac}, Ap^r	Present study
DH5α pVS2k3	P_{fruB}, of *fru*BKA 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 pVS2k3:pVSD1	P_{fruB} as-*icd*, citrate transportet, Ap^r, Chl^r	Present study

Table 3.2: List of bacterial strains used in the present study. Details of the plasmids and the concentration of antibiotics used are given in Table 2.2 and Table 2.3.

3.2.1: PCR primer design for construction of P_{fruB} as-*icd* chimera

Primers (oligos from Sigma) were designed, corresponding to the upstream and downstream regions of the targeted regions of the *icd* gene and *fruB* promoter (Table 2). Sequence information of *E. coli* K-12 strain was used for generating primers. EC*fruB* L1 primer (5' c**ggaattc**tgctcataactttacggctt 3') carried an EcoR1 site at the 5' end (bold highlighted in the sequence) followed by 20 nucleotides corresponding to –158 to –139 region of *FruB* promoter. The EC *fruB* R1 (5' gttccggcacaaggcggataactgga 3') primer contained 14 nucleotides corresponding to +19 to +33 region of *icd* gene (underlined in sequence) followed by 11 nucleotides corresponding to +15 to +5 of *fruB* gene. EC*icd* L1 (5' **atgttccagttatcc**gccttgtgccggaa 3') primer contains 15bp corresponding to atg +5

to +15 (15 nucleotides) of *fruB* gene at the 5' region (bold highlighted in sequence) followed by 14 nucleotides corresponding to +33 to +19 nucleotides corresponding to *icd* gene.

The EC *icd*R1 (5' c**ggg**a**tcc**tatcgcaggacgcaaac 3') primer carried the BamH1 site (bold highlighted in the sequence) followed by 17 nucleotides corresponding to –72 to –56. Primers EC*fruB* R1 and EC*icd* L1 have 14 nucleotides corresponding to *icd* and 11 nucleotides corresponding to *fruB* that are complementary to each other.

3.2.2: Construction of pVS2k3 carrying P_{fruB} as-*icd*

Construction of P_{fruB} as-*icd* was carried out as depicted schematically in **Fig. 3.4.** First, PCR amplification of *E. coli* DH5α genomic DNA was carried out separately using pairs of EC*fruB*L1 and EC*fruB*R1 as well as EC*icd*L1 and EC*icd*R1 primers. The fragments corresponding to 192bp region encompassing the *fruB* promoter and 122bp region of the *icd* gene were obtained. The 192bp amplicon of *fruB* contained 170bp region of *fruB* promoter, 14bp of *icd* region at one end and additional 8bp containing an *Eco*R1 site on the other end. Similarly, *icd* amplicon had 103 bp regions of *icd* with 11bp corresponding to *fruB* and 8bp extra with a BamH1 recognition site on either ends. Recombinant PCR was carried out using the EC*fruB* L1 and EC*icd*R1 primers and the previously obtained amplicons. This resulted in a 289bp fragment containing *fruB* promoter and as-*icd* flanked by EcoR1 and BamH1 sites. The amplified product was cloned into pTZ57R using InsT/Aclone™ PCR Product Cloning Kit, MBI Fermentas and transformed into *E. coli* DH5α. The presence of the appropriate plasmid was checked by PCR using EC*fruB*L1 and EC*icd* R1 primers. Digestion of the plasmid with EcoR1 released an insert of 290bp confirming correctness of the construct, which was denoted as pVS2K3.

3.2.3: Construction of pVSD1 vector carrying citrate transporter (Patel, 2001).

Plasmid pGR_3 containing citrate carrier gene (*cit* $^+$) of *Klebsiella pneumoniae* was digested with SalI followed by HindII and 4,068bp fragment was ligated with the 3,781bp

fragment of pACYC184 obtained by digestion with SalI and EcoRV and transformed to *E. coli* DH5α.

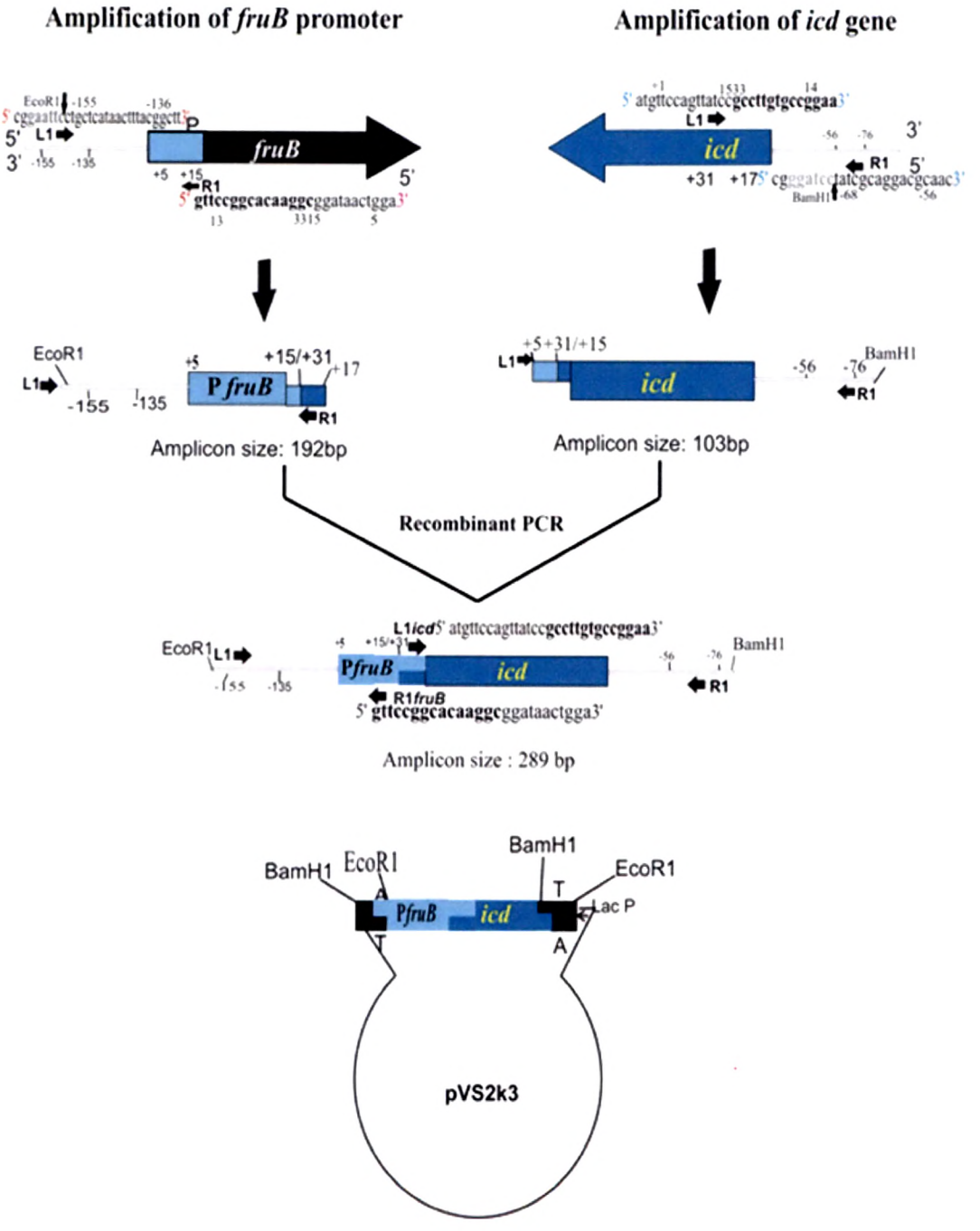


Fig. 3.4: Schematic representation - Construction of P_{fruB} as-*icd*.

The transformants were selected on LB agar containing chloramphenicol (20μg/ml) and further confirmed for tetracycline sensitivity since SalI site lies in tetracycline resistance gene of pACYC184. The transformants were further confirmed for growth on Koser's citrate agar plate containing sodium citrate as the sole carbon source. PstI (two sites within the (*cit* $^{+}$gene) digestion of the plasmid gave two fragments of approximately 6Kb and 900bp confirming the presence of the *cit* $^{+}$ in the plasmid pVSD1 (7,848bp).

3.2.4: Developing *E. coli* DH5α carrying P_{fruB} as-*icd* and citrate transporter

E. coli DH5α was transformed (section 2.3.2) with pVS2k3 carrying the antisense for *icd* gene under *fru*B promoter. It was co-transformed with pVSD1 vector carrying citrate transporter along with pVS2k3 to confer citrate efflux property to *E. coli* DH5α. Transformants carrying citrate transporter were confirmed for their citrate utilization on Kosers medium (section 2.2.1).

3.2.5: Monitoring the effect of pVS2k3 on the metabolism of *E. coli* DH5α

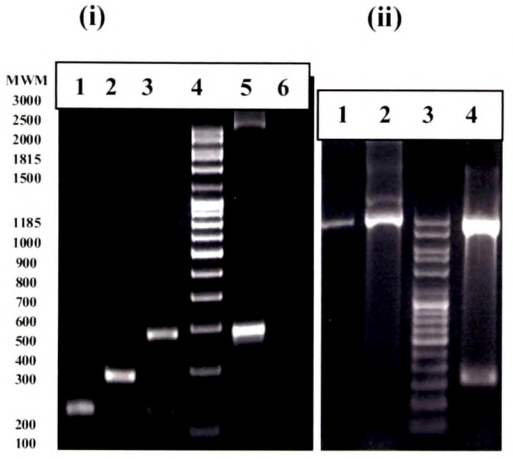
E. coli DH5α transformants were grown on 100mM Tris minimal medium (TrP) with acetate (50mM), glucose, fructose, glycerol and xylose as carbon source at 100mM each. IPTG (0.1mM) was used for induction of expression of citrate transporter gene in plasmid pVSD1 (section 2.2.3). Samples were collected at regular interval for O.D. $_{600nm}$, and organic acid secreted in the medium. ICDH assay performed at stationary phase as per protocol mentioned in (section 2.6.2.4).

3.3: RESULTS

3.3.1: Developing *E. coli* DH5α expressing both pVS2k3 and pVSD1

pVS2k3 plasmid was incorporated into *E. coli* DH5α and the transformants were confirmed using colony PCR method and restriction digestion **(Fig.3.5 (i) and (ii))**. pTZ57R the vector used for cloning was used as a control for pVS2k3 in all further

experiments. *E. coli* DH5α carrying pVS2k3 was co-transformed with pVSD1 and selected on Ap^r and Chl^r. These transformants were then checked for their citrate utilization ability (**Fig. 3.6**) on Koser's citrate agar plate.



(i) PCR amplification of pVS2k3: Lane 1: as-icd (122bp), Lane 2: P_{fruB} promoter (192bp), Lane 3: P_{fruB} as-*icd* (290bp) Lane 4: Low range DNA ruler, Lane 5: pVS2k3 (colony PCR, 290bp), Lane 6: pTZ57R (colony PCR, control).

(ii)Restriction digestion pattern of pVS2k3: Lane 1: pTZ57R EcoRI digest (2,886bp), Lane 2: pVS2k3 HindIII digest (3,128bp), Lane 3: Low range DNA ruler, Lane 4: pVS2k3 EcoRI digest (2,886bp, 290bp)

Fig. 3.4: Construction of pVS2k3.

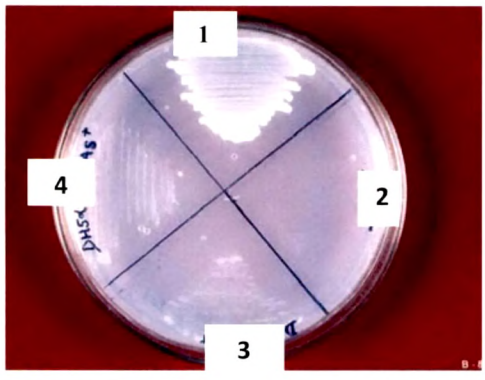


Fig. 3.6: *E. coli* transformant with P_{fruB} as-*icd* and citrate transporter on Koser's citrate agar plate.

1. *E. asburiae* (control)
2. *E. coli* DH5α
3. *E. coli* DH5α pVSD1
4. *E. coli* DH5α pVS2k3:pVSD1

3.3.2: Effect of P_{fruB} as-*icd* expression *in E. coli* DH5α in presence of citrate transporter on 100mM Tris minimal media with various carbon sources.

E. coli DH5α pVS2k3:pVSD1 showed a relative slow growth on TrP medium with fructose, xylose and glycerol as carbon sources as compared to glucose. The acidification of the medium (pH< 5) was observed at 72-96h on glucose and 96-120 h on fructose and xylose. No acidification was observed with glycerol and acetate (**Fig. 3.7**).

E. coli strain	100mM TrP medium with 100mM glucose			
	$O.D_{600nm}$		ICDH (µmoles/mg. protein/min)	
	48 (h)	72 (h)	48 (h)	72 (h)
DH5α	0.58 ± 0.04	0.62 ± 0.05	0.23 ± .060	0.24 ± 0 .06
DH5αpVS2k3:pVSD1	0.60 ± 0.13	0.59 ± 0.13	0.01 ± 0.05***	0.02± 0.01***
	100mM TrP medium with 100mM Fructose			
	72 (h)	96 (h)	72 (h)	96 (h)
DH5α	0.37 ± 0.29	0.37 ± 0.23	0.39 ± 0.8	0.33 ± 0.03
DH5αpVS2k3:pVSD1	0.55 ± 0.25	0.59 ± 0.19	0.09 ± 0.03***	0.11± 0.01***
	100mM TrP medium with 100mM xylose			
DH5α	0.71 ± 0.09	0.66 ± 0.11	0.22 ± 0.03	0.18 ± .015
DH5αpVS2k3:pVSD1	0.47 ± 0.25	0.66 ± 0.11	0.18 ± 0.06	0.13 ± 0.04
	100mM TrP with 50mM acetate			
DH5α	0.46 ± 0.17	0.49 ± 0.09	0.01 ± 0.01[a]***	0.028 ± .022
DH5αpVS2k3:pVSD1	0.15 ± 0.18	0.28 ± 0.14	0.013 ± 0.01	0.02 ± 0.027
	100mM TrP medium with 100mM glycerol			
	96 (h)	120 (h)	96 (h)	120 (h)
DH5α	0.59 ± 0.26	0.63 ± 0.26	0.20 ± 0.03	0.22 ± 0.02
DH5αpVS2k3:pVSD1	0.69 ± 0.11	0.64 ±0.08	0.19 ± 0.02	0.18 ± 0.01

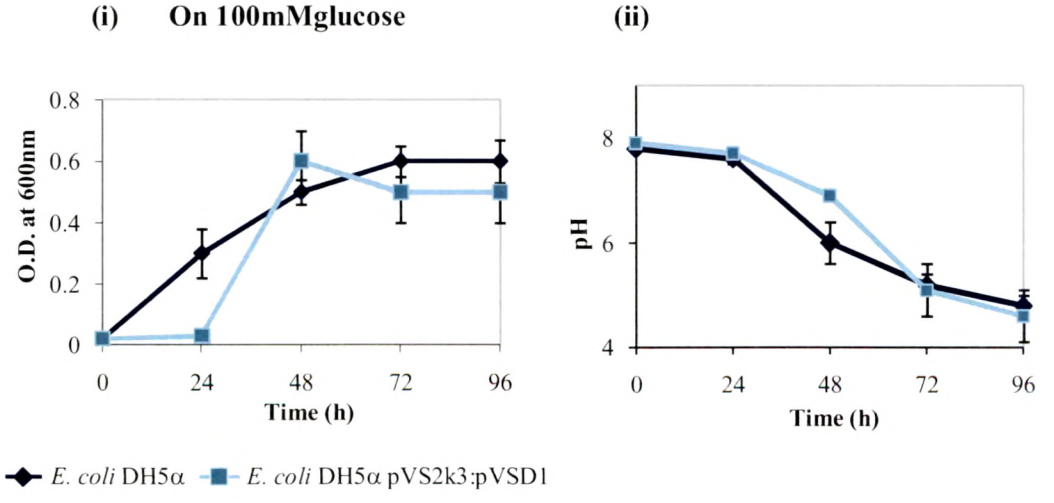
Table 3.3: Effect of P_{fruB}- a*sicd* expression in *E. coli* DH5α in presence of citrate transporter on growth and ICDH activity on various carbon sources. [a] comparision with ICDH activity on glucose. *** $p<0.001$.

3.3.3 Effect on ICDH activity on various carbon sources.

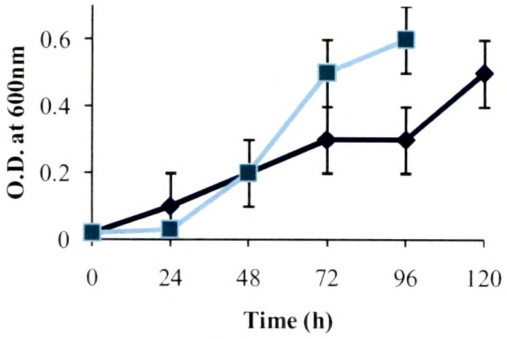
ICDH activity on various carbon sources for *E. coli* DH5α pVS2k3: pVSD1 and *E. coli* DH5α control is summarized in the (**Table 3.2, Fig. 3.8**). On glucose, *E. coli* DH5α (0.23 ± .060 U) showed 5-6 fold (0.011 ± 0.05 U) decrease in ICDH activity in presence of pVS2k3 (P_{fruB} as-*icd*) as compared to 3-4 fold (0.099 ± 0.03 U) on fructose. On glycerol ICDH activity was (0.18 ± 0.02 U U) and xylose (0.18 ± 0.06 U) which was similar to the control. This confirmed both the functionality of the promoter and the ability of antisense to bring about down regulation of *icd* gene. On acetate, *E. coli* DH5α showed a very low ICDH (0.013 ± 0.01 U) activity suggesting that on acetate the enzyme gets phosphorylated and thus gets inactivated.

3.3.4 Effect of *E. coli* DH5α pVS2k3:pVSD1 on the organic acid secretion.

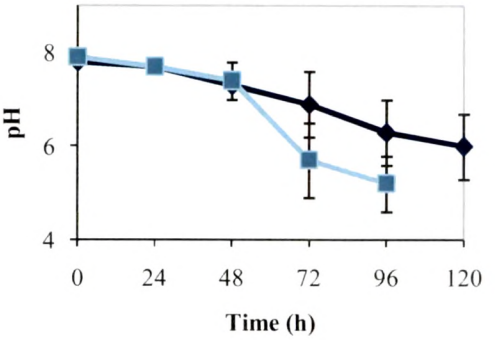
At the stationary phase, extracellular medium of *E. coli* DH5α pVS2k3:pVSD1 grown on glucose and fructose contained acetate as a major acid secreted (23.99 ± 0.10 mM) and small amounts of pyruvate were also observed in all the samples. On xylose, however, the acetate levels decreased to less than half (10.68 ± 0.46 mM) as compared to the control (24.07 ± 0.65 mM). *E. coli* DH5α showed very less acetate (8.01 ± 0.43 mM) secretion on glycerol and no organic acid was secreted on acetate.

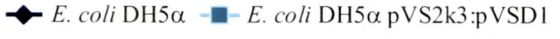


(iii) On 100mM fructose

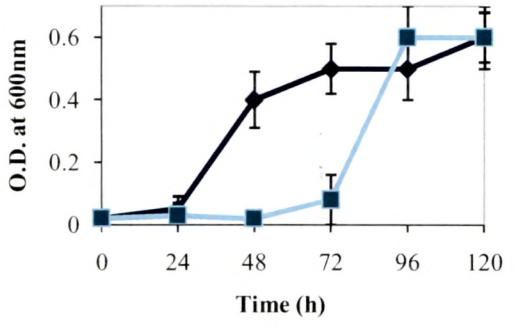


(iii)

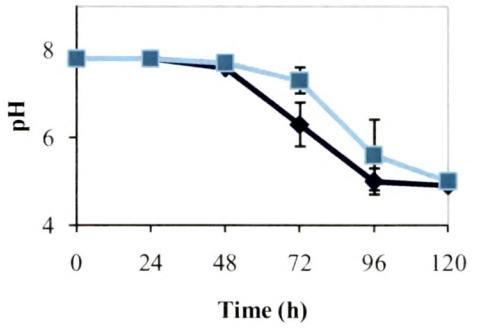


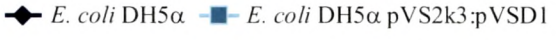


(v) On 100mM xylose

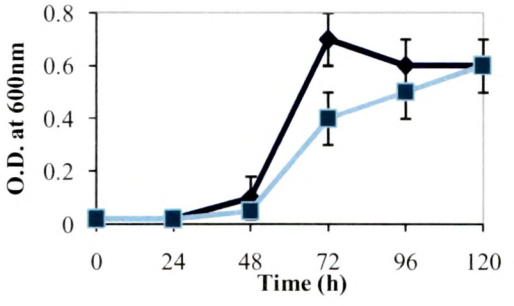


(vi)

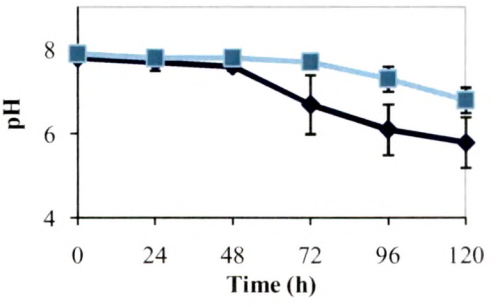


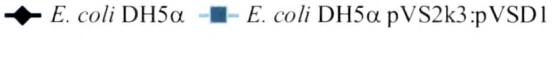


(vii) On 100mM glycerol



(viii)





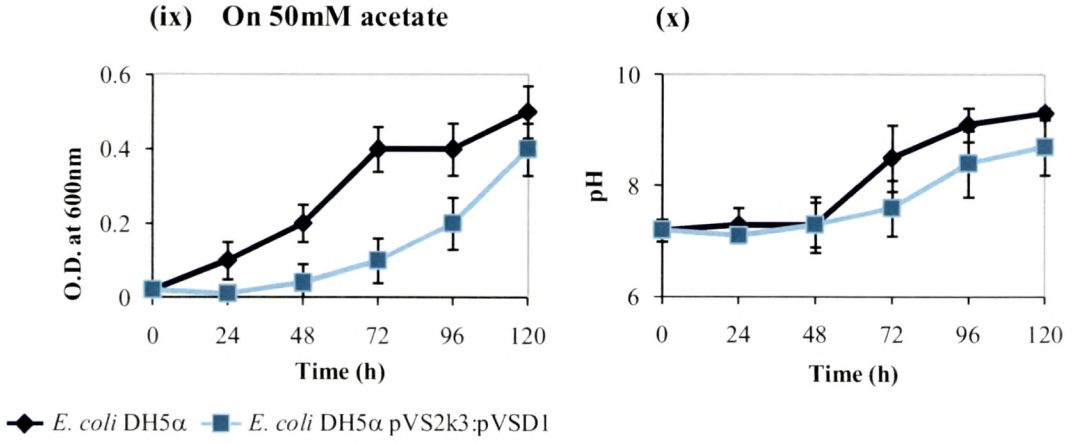


Fig. 3.7: Growth characteristics of *E. coli* DH5α pVS2k3: pVSD1 on different carbon sources. (i) & (ii) $O.D_{600nm}$ and pH on glucose (iii)&(iv) $O.D_{600nm}$ and pH on fructose (v)& (vi) $O.D_{600nm}$ and pH on xylose (vii) &(viii) $O.D_{600nm}$ and pH on glycerol (ix) & (x) $O.D_{600nm}$ and pH on acetate. All values plotted are represented as Mean ± SD for n= 4 to 7 observations.

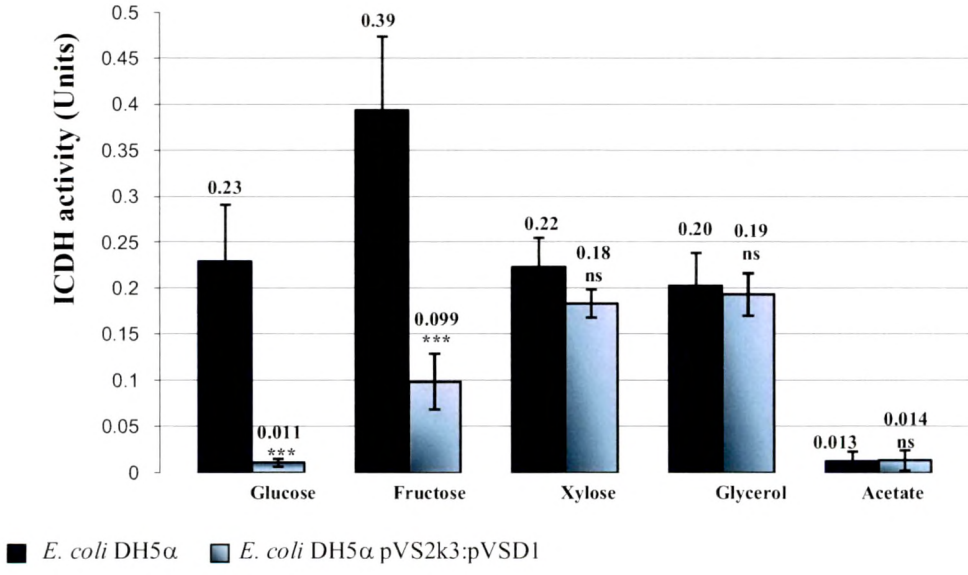


Fig. 3.8: Effect P_{fruB} as-*icd* on ICDH activity on various carbon sources. All values represented here are Mean ± SD for n= 5-8 observations. Units- µmoles/mg. protein/min. ***p<0.001, ns- non significant.

3.3 DISCUSSION

The present study on 100mM TrP medium with various carbon sources showed that the P_{fruB} could efficiently regulate the antisense expression in response to the availability of carbon sources like glucose and fructose. This result is in agreement with the regulation green fluorescent protein (*gfp*) expression by P_{fruB} in *Erwinia herbicola* (Leveau and Lindou, 2000). Absence of as-*icd* effect on glycerol and xylose also suggests that the F-1,6 bisphosphate levels are comparatively low as compared to that on glucose and fructose which is in agreement earlier report (Holms, 2001). Hence, the present study demonstrates the novelty of conditional antisense RNA strategy as it avoids the use of external inducers for the down- regulation of target genes and its regulation depends upon the nature of the available carbon source.

Lower ICDH activity on acetate supported the reports that ICDH enzyme undergoes post translation modification (ICDH kinase/phosphatase) leading to its inactivation. This kind of a regulation is very critical in directing the carbon flow towards growth and energy production when grown on acetate as a carbon source (Walsh and Koshland 1985a; Thorness and Koshland, Jr. 1987).

E. coli K strains have demonstrated high acetate overflow when grown excess glucose but surprisingly pyruvate was also present. Lack of KCl, $MgSO_4$, $CaCl_2$ and micronutrients may have caused some stress leading to pyruvate secretion. This view is also supported by the poor growth on this medium. Hence subsequent studies were carried out on M9 Minimal medium with glucose (100mM) and glycerol (100mM) as carbon source.