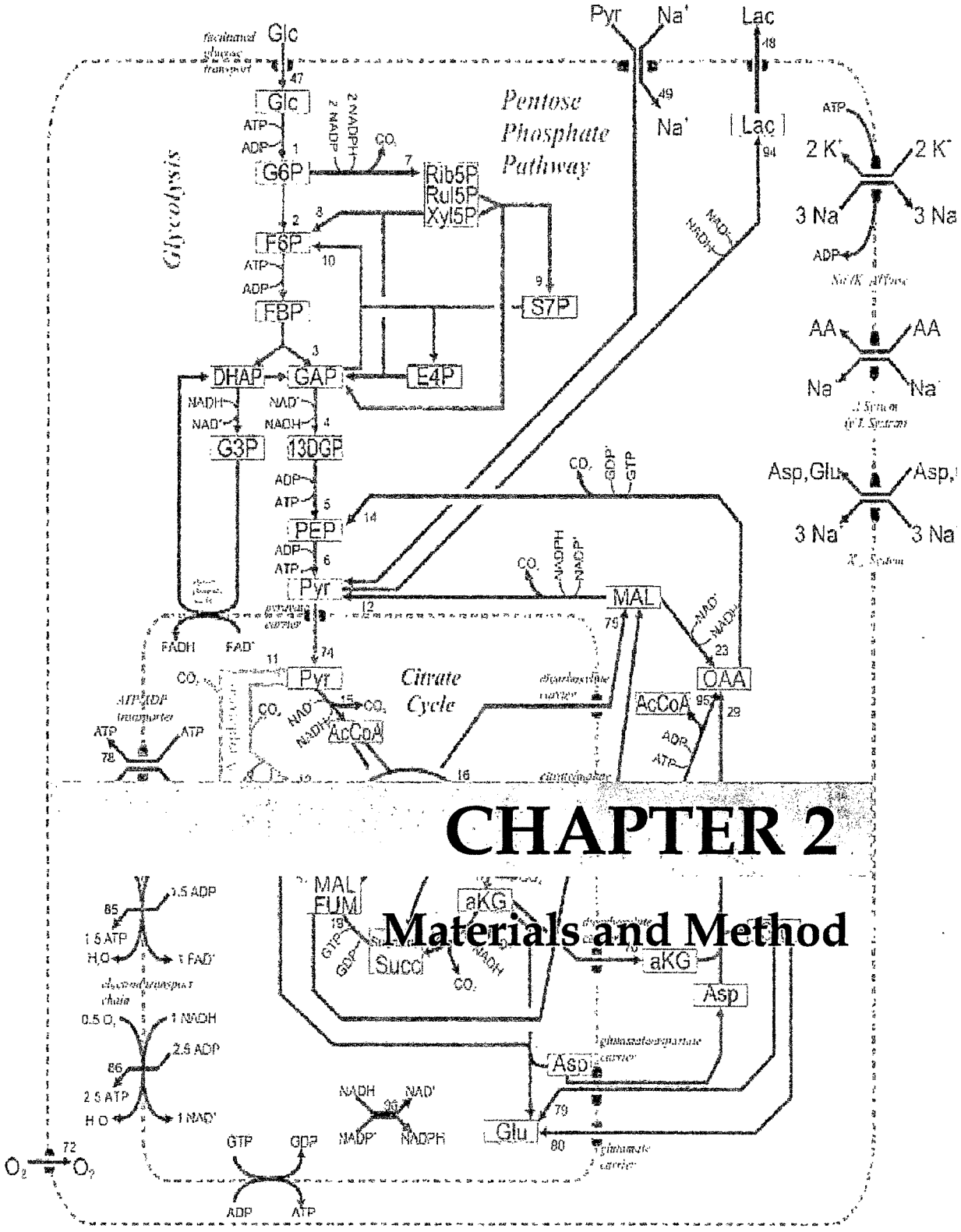


CHAPTER 2

Materials and Method

2.1: BACTERIAL STRAINS AND PLASMIDS

List of all wild type and genetically modified *E. coli* strains are given in **Table 2.1.** The plasmids used in the present study and their restriction maps are given in **Table 2.2** and **Fig. 2.1.** *E. coli* DH5α was used for all the standard molecular biology experiments wherever required. *E. coli* W620, citrate synthase gene (*glt*A) mutant was obtained from *E. coli* Genetic Stock Center (CGSC), Yale University, U.S.A. *E. coli* MA1935 *icd* mutant of *E. coli* BL21(λDE3) was a kind gift from Dr. Miho Aoshima, University of Tokyo, Japan.

E. coli strains	Genotype	References
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
E. coli BL21	F^-, *ompT*,$hsdS_B$ (r_{B-}, m^{B+}), *dcm*, *gal*	Sambrook and Russell, 2001
E. coli BL21(λDE3)	F^-, *ompT*,$hsdS_B$ (r_{B-}, m^{B+}), *dcm*, *gal*, (DE3).	Sambrook and Russell, 2001
E. coli MA1935	*icd* mutant of *E. coli* BL21(λDE3)	Aoshima, 2003
E. coli W620	CGSC 4278 - *glnV44 gltA6 galK30 LAM-pyrD36 relA1 rpsL129 thi*$^{-1}$; Str^r	*E. coli* Genetic stock centre
DH5α pTZ57R	T-vector, P_{lac}, Ap^r	Ch. 3,4,5
DH5α pVS2k3	P_{fruB}, of *fru*BKA operon , antisense isocitrate dehydrogense (as-*icd*) for *E. coli* , Ap^r	Ch. 3,4,5
DH5α pVSD1	P_{lac}, H^+-Citrate transporter from *Klebsiela pneumoniae*, Chl^r	Ch.3,4,5,7,8,9, 10,11,12
DH5α pTZ57R:pVSD1	T-vector, citrate transporter, Ap^r, Chl^r	Ch. 4,5
DH5α pVSk3:pVSD1	P_{fruB} as-*icd*, citrate transporter, Ap^r, Chl^r	Ch. 4,5
DH5α pTTQ18	P_{tac} promoter, Amp^r	Ch. 7

DH5α pVSppc	*ppc* gene from *Synechococcus elongatus* PCC 6301, P_{tac} promoter, tetracycline resistance gene, Amp^r, Tc^r	Ch.8
DH5α pJE1	pTTQ18, kanamycin resistance (kan^r) gene, P_{tac} promoter, Amp^r, Km^r	Ch. 9
DH5α pJE2	pTTQ18 carrying *gltA* gene from *E. coli*, P_{tac} promoter, kanamycin resistance (*kan*^{r}) gene, Amp^r, Km^r	Ch. 9
DH5α pJE3	pTTQ18, tetracycline resistance gene, P_{tac} promoter, Amp^r, Tc^r	Ch. 8
DH5α pJE4	*as-icd*, P_{tac} promoter, Amp^r	Ch. 7
DH5α pJE6	*as-icd*, P_{tac} promoter, Amp^r	Ch. 7
DH5α pJE7	pTTQ18 , genatamycin resistance (*aac*) gene, P_{tac} promoter, , Amp^r, Gm^r	Ch.11
DH5α pJE8	pTTQ18 carrying *as-icd*, P_{tac} promoter, genatamycin resistance (*aac*) gene, Amp^r, Gm^r	Ch. 11
DH5α pTZ57Rg	pTZ57R , gentamycin resistance (*aac*) gene, P_{lac} promoter, Amp^r, Gm^r	Ch. 12
DH5α pVS2k3g	pTZ57R carrying P_{fruB} *as-icd*, P_{lac} promoter, gentamycin resistance (*aac*) gene, Amp^r, Gm^r	Ch. 12
BL21(λDE3) pTZ57R	T-vector, P_{lac}, Ap^r	Ch. 6
BL21(λDE3) pVS2k3	P_{fruB}, of *fru*BKA operon , antisense isocitrate dehydrogense (as-*icd*) for *E. coli* , Ap^r	Ch. 6
BL21 pTTQ18	P_{tac} promoter, Amp^r	Ch. 7
BL21 pJE6	*as-icd*, P_{tac} promoter, Amp^r	Ch. 7
BL21 pVSD1	P_{lac}, H^+-Citrate transporter from *Klebsiela pneumoniae*, Chl^r	Ch. 7

BL21 pTTQ18:pVSD1	T-vector, citrate transporter, Ap^r, Chl^r	Ch.7
BL21 pJE6: pVSD1	as-*icd*, citrate transporter, Ap^r, Chl^r	Ch. 7
MA1935 pVSD1	P_{lac}, H^+-Citrate transporter from *Klebsiela pneumoniae*, Chl^r	Ch. 8
MA1935 pJE3:pVSD1	pTTQ18, citrate transporter, Amp^r, Chl^r, Tc^r	Ch. 8
MA1935 pVSppc:pVSD1	*ppc* gene, citrate transporter, Amp^r, Chl^r, Tc^r	Ch. 8
MA1935 pJE1:pVSD1	pTTQ18, citrate transporter, Amp^r, Chl^r, Km^r	Ch. 9
MA1935 pJE2:pVSD1	*gltA* gene, citrate transporter, Amp^r, Chl^r, Km^r	Ch. 9
MA1935 pJE1:pJE3:pVSD1	pTTQ18, citrate transporter, Amp^r, Chl^r, Km^r, Tc^r	Ch. 10
MA1935pJE2:pVSppc:pVSD1	*ppc* gene, *gltA* gene, citrate transporter, Amp^r, Chl^r, Km^r, Tc^r	Ch.10
BL21pJE1:pVSD1	pTTQ18, citrate transporter, Amp^r, Chl^r, Km^r	Ch. 11
BL21pJE2:pVSD1	*gltA* gene, citrate transporter, Amp^r, Chl^r, Km^r	Ch. 11
BL21pJE1:pJE7:pVSD1	pTTQ18, citrate transporter, Amp^r, Chl^r, Km^r, Gm^r	Ch. 11
BL21pJE2:pJE8:pVSD1	P_{tac} as-icd, *gltA* gene, citrate transporter, Amp^r, Chl^r, Km^r, Gm^r	Ch. 11
DH5α pJE1:pVSD1	pTTQ18, citrate transporter, Amp^r, Chl^r, Km^r	Ch. 12
DH5αpJE2:pVSD1	*gltA* gene, citrate transporter, Amp^r, Chl^r, Km^r	Ch.12
DH5α pJE1:pTZ57Rg: pVSD1	pTTQ18, pTZ57R, citrate transporter, Amp^r, Chl^r, Km^r, Gm^r	Ch. 12

DH5α pJE2: pVS2k3g:pVSD1	P_{fruB} as-icd, *gltA* gene, citrate transporter, Amp^r, Chl^r, Km^r, Gm^r	Ch. 12
W620 pJE1	pTTQ18 Amp^r, Str^r, Km^r	Ch. 9
W620 pJE2	*gltA* gene, Amp^r, Str^r, Km^r	Ch. 9
Enterobacter asburiae PSI3	Pigeon pea rhizosphere isolate	Gyaneshwar et al., 1999

Table 2.1: List of *E. coli* strains used in the present study. Ap=Ampicillin; Km=Kanamycin; Tc =Tetracycline; Str =Streptomycin; Gm =Gentamycin; ^{r} = resistant.

Plasmids	Features	References
pTZ57R	P_{lac} promoter, Amp^r	MBI Fermentas
pTTQ18	P_{tac} promoter, Amp^r	Stark,1967
pVS2k3	pTZ57R carrying P_{fruB} *as-icd*, P_{lac} promoter, Amp^r	Ch. 3,4,5
pTZ57Rg	pTZ57R carrying gentamycin resistance (*aac*) gene, P_{lac} promoter, Amp^r, Gm^r	Ch. 12
pVS2k3g	pTZ57R carrying P_{fruB} *as-icd*, P_{lac} promoter, gentamycin resistance (*aac*) gene, Amp^r, Gm^r	Ch. 12
pJE1	pTTQ18 carrying kanamycin resistance (*kan^r*) gene, P_{tac} promoter, Amp^r, Km^r	Ch. 9
pJE2	pTTQ18 carrying *gltA* gene from *E. coli*, P_{tac} promoter, kanamycin resistance (*kan^r*) gene, Amp^r, Km^r	Ch. 9
pJE3	pTTQ18 carrying tetracycline resistance gene, P_{tac} promoter, Amp^r, Tc^r	Ch. 8
pJE4	pTZ57R carrying *as-icd*, P_{lac} promoter, Amp^r	Ch. 7
pJE6	pTTQ18 carrying *as-icd*, P_{tac} promoter, Amp^r	Ch. 7
pJE7	pTTQ18 carrying gentamycin resistance (*aac*) gene, P_{tac} promoter, , Amp^r, Gm^r	Ch. 11
pJE8	pTTQ18 carrying *as-icd*, P_{tac} promoter, gentamycin resistance (*aac*) gene, Amp^r, Gm^r	Ch. 11

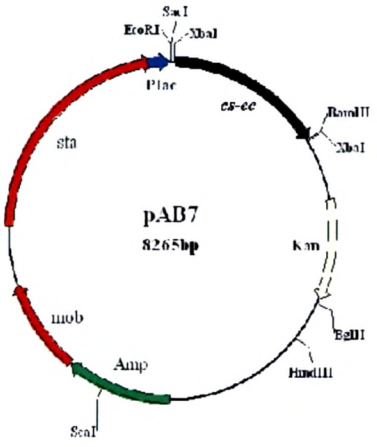
pVSppc	pTTQ18 carrying *ppc* gene from *Synechococcus elongatus* PCC 6301, P_{tac} promoter, tetracycline resistance gene, Amp^r, Tc^r	Sharma, 2008
pAB7	pUCPM18 carrying *gltA* gene from *E. coli*, P_{lac} promoter, kanamycin resistance (*kan*^{r}) gene, Amp^r, Km^r	Buch, 2008
pAB5	pUCPM18 carrying tetracycline resistance gene, P_{lac} promoter Amp^r, Tc^r	Buch, 2008
pGM160	gentamycin resistance (*aac*) gene	Muth, et al., 1989
pVSD1	pACYC184 carrying H^+citrate transporter from Klebsiella pneumoniae, P_{lac} promoter, chloroform resistance gene. Chl^r	Patel, 2001

Table 2.2: List of plasmids used in the present study. All the plasmids were stored as stocks at -20°C in the form of ethanol precipitates.

pTZ57R is a pUC based vector designed to clone PCR products that are amplified using Taq polymerase (generates a A overhang). This vector is provided by MBI Fermentas, InsT/Aclone™ PCR Product Cloning Kit (**Fig. 2.1 (vii)**). It provides blue and white selection and ampicilin resistance for screening. This vector was used for cloning P_{fruB} as-*icd* (**pVS2k3**) and as-*icd* (**pJE4**) (**Fig. 2.1 (viii) and (xv)**). **pTZ57Rg** and **pVS2k3g** (carrying P_{fruB} as-*icd*) (**Fig. 2.1 (xi) and (xii)**) are derivatives of pTZ57R carrying genatmycin resistance (*aac*) gene from **pGM160 (Fig. 2.1 (iii))**.

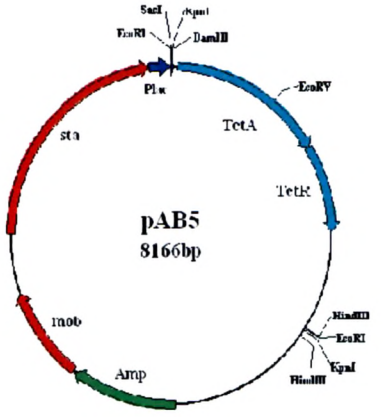
pTTQ18 was constructed carrying the cloning sites from pUC18 and the strongest promoter available for *E. coli* promoter *tac* promoter (P_{tac}) and terminator (the *rrn*B ribosomal RNA terminator) and then *lacI*^{Q} gene was added. The *LacZα* under the *tac* promoter enables blue white selection (**Fig. 2.1 (iv)**) (Stark, 1987). pJE1, pJE3 and pJE7 are derivatives of pTTQ18 carrying kanamycin resistance (*kan*^{r}) gene, tetracycline resistance (Tc^r) gene and genatamycin resistance (*aac*) gene respectively (**Fig. 2.1 (xii), (xi) and (xiv)**). pJE2, pJE6 and pVSppc are the vectors carrying *gltA* gene, as-*icd* and *ppc* gene under P_{tac} promoter respectively (**Fig. 2.1 (v), (xiii), (xvi) and (xvii)**).

(i)



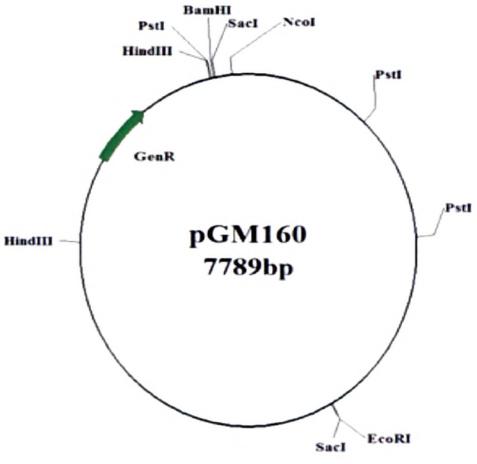
pAB7- carrying gltA gene from *E. coli* under P_{lac} promoter (**Buch,**

(ii)



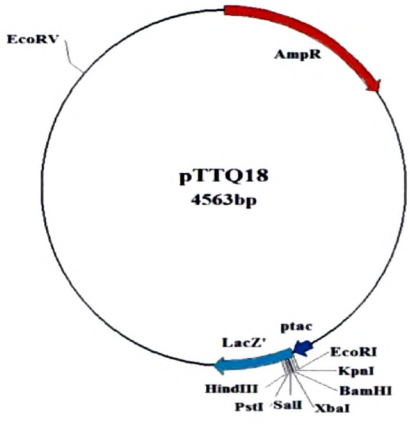
pAB5- carrying tetracycline resistance (Tc^r) gene. (**Buch, 2008).**

(iii)



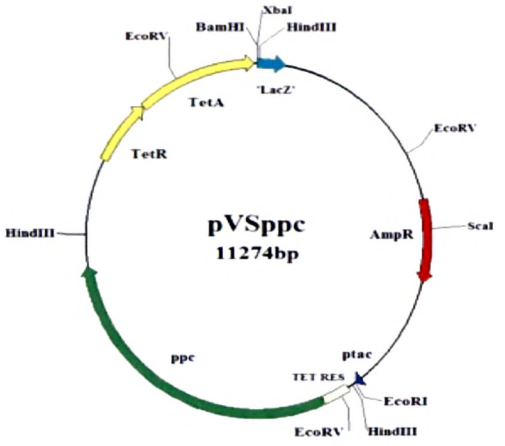
pGM160- Generous gift from **Dr. Günther Muth,** University of Bielfeld, Germany

(iv)



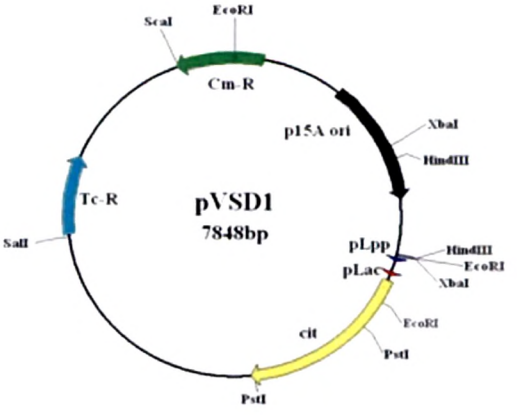
pTTQ18- Generous gift from **Dr. Micheal J. R. Stark**, University of Leichester

(v)



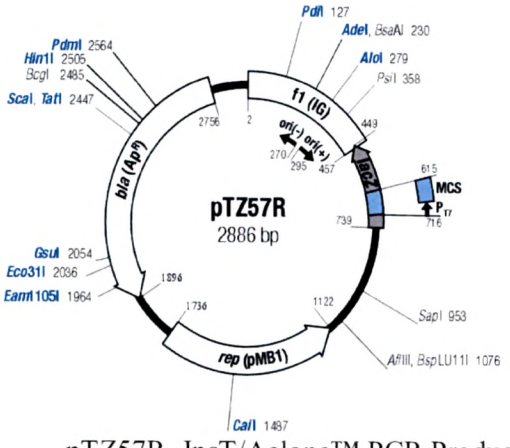
pVSppc- *ppc* gene from S. elongates PCC 6301, **(Sharma, 2008)**

(vi)



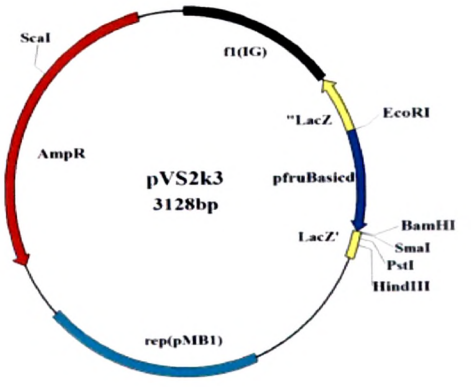
pVSD1- H^+ citrate transporter from Klebsiella pneumonia **(Patel, 2002)**

(vii)

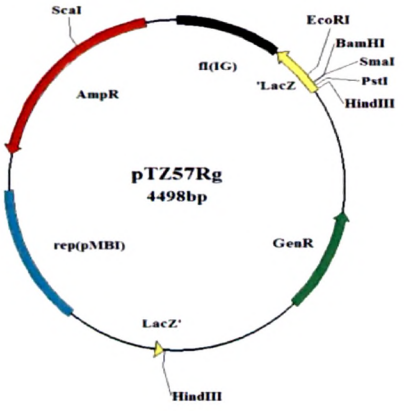


pTZ57R- InsT/Aclone™ PCR Product Cloning Kit **(MBI Fermentas)**

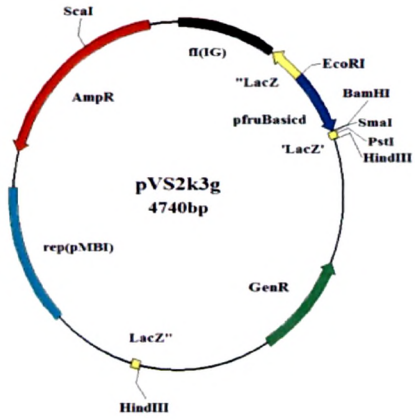
(viii)



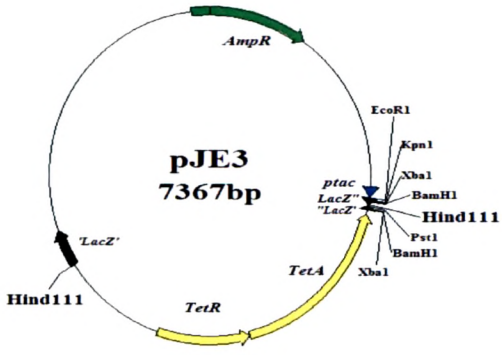
(xi)



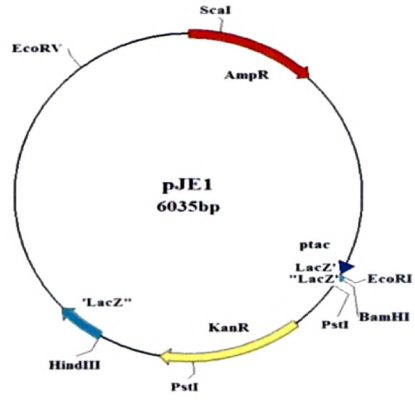
(x)



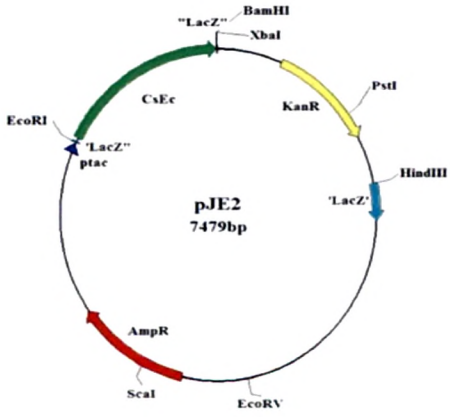
(xi)



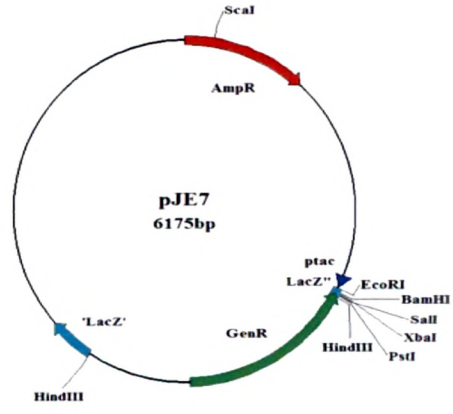
(xii)



(xiii)



(xiv)



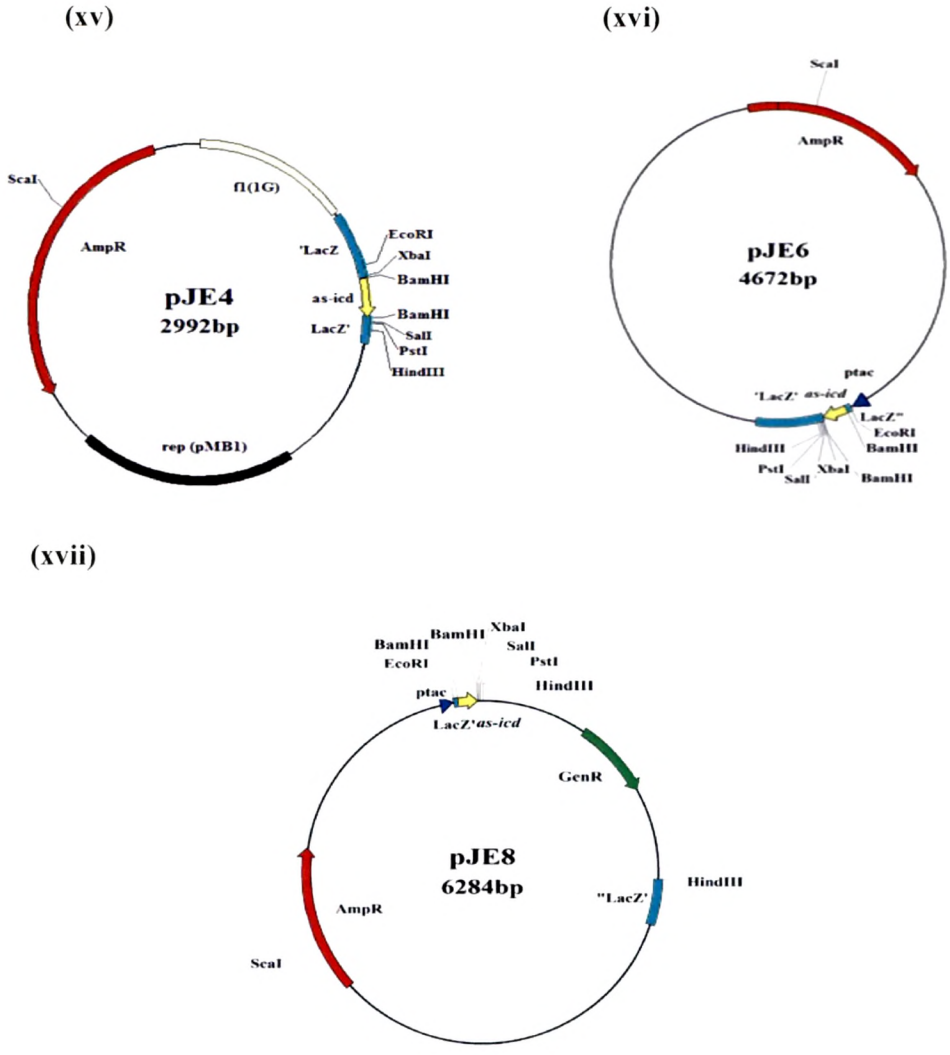


Fig. 2.1: Restriction maps of the plasmids used in this study. (i) plasmid used to obtain *gltA* gene and Km^r , (ii) and (iii) are plasmids used to obtain Tc^r and Gm^r genes (iv) plasmid used to clone *gltA* , *ppc* gene and as-*icd* under *tac* promoter (v)-(xvii) are final construct used for physiological studies in *E. coli*.

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2.1.1: Construction of pVSppc and its functional validation.

Plasmid pA172A vector carrying *ppc* gene of *S. elongates* PCC 6301 was used as a source for subcloning the gene under a strong promoter P_{tac}. Both the vectors pA172A and pTTQ18 were digested by EcoRI/BamHI. pA172A released 3,914 bp fragment

corresponding to *ppc* gene while pTTQ18 was linearised; both the vector and insert were gel purified (protocol mentioned in section 2.3.5) and ligated, the plasmid was designated as pVS1 (8,477bp). Tetracycline (*Tc*^{r}) resistant gene (2,817bp) from pME6010 (GenBank accession number: AF118810) was introduced into pVSI plasmid at the BamHI site. The resultant plasmid was designated as **pVSppc** (11,124bp). pVSppc gene could functionally complement *E. coli ppc* mutant (Sharma, 2008). pGM160, an *E. coli*-Streptomycetes shuttle vector was a source for genatmycin resistance (*aac*) gene (Muth et al., 1989). **pAB7**, a derivative of pUCpm18 plasmid (a pUC based broad host range plasmid) was the source for *gltA* gene from *E. coli* and kanamycin resistance (*kan*^{r}) gene. **pAB5** is a derivative of pUCpm18 plasmid carrying tetracycline (*Tc*^{r}) resistant gene hence was a source for *Tc*^{r} gene (Buch, 2008).

2.2: MEDIA AND CULTURE CONDITIONS

The *E. coli* strains were cultured and maintained on Luria Agar (LA) (Hi-Media aboratories, India). *E. coli* cultures were grown at 37°C, for growth in liquid medium; cultures were rotated at 200rpm. The plasmid transformants of *E. coli* were maintained using respective antibiotics at the final concentrations as mentioned in **Table 2.3** as and when applicable. *E. coli* wild type strains and plasmid transformants were grown in 3ml Luria broth (LB) with appropriate antibiotics to prepare glycerol stocks which were stored at -20°C.

The compositions of different minimal media used in this study are as described below. Antibiotic concentrations in all the following minimal media were reduced to 1/4$_{th}$ of that used in rich media (**Table 2.3**).

2.2.1: Koser's Citrate medium

Composition of the Koser's Citrate medium contained, magnesium sulphate, 0.2g/L; monopotassium phosphate, 1.0g/L; sodium ammonium phosphate, 1.5g/L; sodium citrate, 3.0g/L and agar, 15g/L. The readymade media was obtained from Hi- Media Laboratories, India, and was used according to manufacturer's instructions.

Antibiotics	Rich Medium	Minimal medium
Tetracycline	30μg/ml	7.5μg/ml
Kanamycin	50μg/ml	12.5μg/ml
Streptomycin	10μg/ml	2.5μg/ml
Ampicillin*	50μg/ml	12.5μg/ml
Chloramphenicol*	20μg/ml	10μg/ml
Gentamycin*	20μg/ml	10μg/ml

Table 2.3: Recommended doses of antibiotics used in this study (Sambrook and Russell, 2001). The antibiotic dozes for *E. coli*. All the antibiotics were prepared in sterile distilled water or recommended solvent at the stock concentrations of 1000x or 2000X (for antibiotics marked with *) and were used accordingly to have the desired final concentrations.

2.2.2: M9 minimal medium

Composition of M9 minimal broth was according to Sambrook and Russell (2001) including Na_2HPO_4 $7H_2O$, 34g/L; KH_2PO_4, 15g/L; NH_4Cl, 5g/L; NaCl, 2.5g/L; 2mM $MgSO_4$; 0.1mM $CaCl_2$ and micronutrient cocktail. The micronutrient cocktail was constituted of $FeSO_4.7H_2O$, 3.5 mg/L; $ZnSO_4.7H_2O$, 0.16 mg/L; $CuSO_4.5H_2O$, 0.08 mg/L; H_3BO_3, 0.5 mg/L; $CaCl_2.2H_2O$, 0.03 mg/L and $MnSO_4.4H_2O$, 0.4 mg/L. Thiamine (100 μg/ml, stock-1000X) and glutamate (340 μg/ml, stock-1000X) were added as and when required. Carbon sources used were glucose, glycerol and acetate as and when required. For solid media, 15g/L agar was added in addition to above constituents. 5X M9 salts, micronutrients (prepared at 1000X stock concentration) and carbon source (2M stock) were autoclaved separately. Fixed volumes of these were added aseptically into pre-autoclaved flasks containing distilled water to constitute the complete media with the desired final concentrations. Volume of water to be autoclaved per flask was calculated by subtracting the required volumes of each ingredient from the total volume of the media to be used.

2.2.3: Tris buffered medium

The media composition included Tris-Cl (pH=8.0), 100mM; NH_4Cl, 10mM; KCl, 10mM; $MgSO_4$, 2mM; $CaCl_2$, 0.1mM; micronutrient cocktail; Glucose, 100mM and phosphate (P) sources (Sharma et al., 2005). KH_2PO_4 were used soluble P sources respectively. Each ingredient was separately autoclaved at a particular stock concentration and a fixed volume of each was added to pre-autoclaved flasks containing sterile distilled

water (prepared as in Section 2.2.2) to constitute complete media. Carbon sources used were glucose, fructose, xylose, acetate and glycerol as and when required.

2.3: MOLECULAR BIOLOGY TOOLS AND TECHNIQUES.

2.3.1: Isolation of plasmid and genomic DNA

The plasmid DNA was isolated by the boiling lysis method using CTAB (3ml) and by alkaline lysis method and purification by phenol chloroform method was carried out for plasmid isolated from more than more than 10ml cultures. Genomic DNA was isolated using NaCl-CTAB method. (Sambrook and Russell, 2001).

2.3.2: Transformation of plasmid DNA in *E. coli*

The transformation of plasmids in *E. coli* using $MgCl_2$-$CaCl_2$ method and bluewhite selection of the transformants using IPTG and X-Gal (as and when applicable) was carried out according to Sambrook and Russell (2001).

2.3.3: Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% and 2% agarose (as when required) (containing 1μg/ml ethridium bromide) gel in Tris-acetate-EDTA (TAE) buffer at 5v/cm for 0.5-2h. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator.

2.3.4: Restriction enzyme digestion analysis

0.5-1.0μg DNA sample was used for each restriction enzyme digestion. 1-3U of the restriction endonuclease (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10μl. The reaction mixture was incubated overnight at 37°C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% and 2% agarose gels (as and when required) and were subsequently photographed. In case of double digestion, a compatible buffer for the two REs was

essentially checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers.

2.3.5: Gel elution and purification

The DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band. The agarose piece was weighed in a sterile microcentrifuge tube and was solubilized in 2.5 volumes of 6M sodium iodide (NaI, freshly prepared) [e.g. for 200mg of agarose piece, 500µl of NaI was added]. Once the gel was completely dissolved, 15µl of silicon dioxide suspension (50% w/v, stored at 4°C) was added and was incubated at room temperature for 15-20 minutes. The DNA bound to silica was recovered by centrifuging at 9, 200x *g* for 2 minutes; the pellet was washed twice with 70% ethanol, dried and finally re-suspended in 20-30µl sterile double distilled water. The microcentrifuge tube was incubated at 55°C for 10 minutes to allow complete dissociation of DNA from silica beads into the solution and then was subjected to centrifugation at 9, 200x *g* for 2 minutes. The resultant supernatant was collected with sterile micropipette tip and transferred to fresh sterile tube. The purification efficiency was checked by running 2µl DNA solution on agarose gel and the sharp DNA band of desired size was visualized. The purified DNA was used for ligation experiments only if >50ng/µl DNA was recovered after purification.

2.3.6: Ligation

The ligation reaction was usually done in 10µl volume containing the following constituents: Purified vector and insert DNA (volume varied depending on the respective concentrations); 10X T4 DNA Ligase buffer, 1µl; T4 DNA ligase (MBI Fermentas), 0.5-1.0U and sterile double distilled water to make up the volume. The cohesive end ligation reaction was carried out at 16°C for 12-16h.

$$\text{pmoles of DNA} = \frac{\text{Amount of DNA (µg)} \times 1,515}{\text{Size of the DNA fragment (no. of base pairs).}}$$

The vector to insert molar ratio (molar concentrations calculated by the under mentioned formula) of 1:4 was maintained, with a total of 50-100ng of DNA in each ligation system.

2.3.7: Polymerase Chain Reaction (PCR)

The PCR reaction set up was based on the guidelines given in Roche Laboratory Manual. The assay system and the temperature profile used are described in **Table 2..**

Assay System		Temperature profile	
Sterile DDW	38 µl	Initial denaturation	94°C- 5 min
dNTP (10mM with 2.5mM each)	3 µl	Denaturation	94°C- 30 sec
Reverse Primer 20pmoles	1 µl	Annealing	62°C - 30sec
Forward Primer 20pmoles	1 µl		
Template DNA (100ng/µl)	1 µl	Elongation	72°C - 45sec
Taq PCR buffer (10X)	5 µl	Final Elongation	72°C- 10 min
Taq DNA Polymerase (1 unit/µl)#	1 µl	For plasmid	(30 cycles)
Total System	**50 µl**	For genomic DNA	(40 cycles)

Table 2.4: PCR conditions used in the present study. PCR amplifications were performed in Techne TC-312 thermal cycler. # Processivity of Taq polymerase is ~1000 bases per min. Taq DNA polymerase and its buffer, dNTPs and primers were obtained respectively from Bangalore Genei Pvt. Ltd., India and Sigma Chemicals Pvt. Ltd., respectively, and were used according to manufacturer's instructions.

The theoretical validation of the primers (absence of intermolecular and intramolecular complementarities) to avoid primer-primer annealing and hairpin structures and the appropriate %G-C were checked with the help of online primer designing software Primer 3. The PCR products were analyzed on 2.0% agarose gel along with appropriate molecular weight markers (Section 2.) The DNA sequencing service was obtained from MWG Biotech. Pvt. Ltd. (India) for ~400bp PCR product (details in Chapter 3.).

2.4: MUTANT COMPLEMENTATION PHENOTYPE

E. coli W620 *gltA* mutant was used to confirm the functionality of the cloned *gltA* genes. The recombinant plasmid containing *gltA* gene under *tac* promoter and the respective plasmid control were transformed into the mutant. The *gltA* transformants were selected on streptomycin and kanamycin. These transformants were grown on M9 minimal medium with 0.2% (11.11mM) glucose as carbon source in presence and absence of 340µg/ml sodium glutamate, under shaking conditions at 37°C for *E. coli* W620. Kanamycin, and streptomycin were used at the final concentrations of 12.5µg/ml, and 2.5µg/ml (1/4th of the concentration used for growth in LB broth. *gltA* mutants exhibited glutamate auxotrophy (Underwood et al., 2002) as they could not grow on glucose as the sole carbon source in the absence of glutamate supplementation. Similar protocol was followed to validate glutamate auxotropy for *E. coli* MA1935, *icd* mutant (Lakshmi and Helling, 1976).

2.5: PHYSIOLOGICAL EXPERIMENTS

The physiological experiments were carried out using various *E. coli* WT (lab strain) and *E. coli* transformants which included growth, pH profile and enzyme assays.

2.5.1: Inoculum preparation

The inoculum for M9 and Tris minimal media containing free Pi was prepared by growing the *E. coli* cultures overnight at 37°C in 3ml LB broth. Cells were harvested aseptically, washed thrice by normal saline and finally re-suspended in 1ml normal saline under sterile conditions. Freshly prepared inoculum was used for all the experiments.

2.5.2: Growth characteristics and pH profile

Growth parameters and pH profile of all the *E. coli* transformants were determined using two different media conditions including **(i)** Tris buffered medium with KH_2PO_4 as P source: The media composition was same as mentioned in section 2.2.4 free Pi was supplemented in the form of KH_2PO_4 (10.0mM) and 100mM of glucose, xylose, fructose, glycerol and acetate (50mM) were used as carbon source (only for **Chapter 3**); and **(iii)** M9 minimal medium (Section 2.2.3). 50mM or 100mM glucose was used as the carbon source (depending on the need of the experiment). In some experiments glycerol (100mM) and

acetate (50mM) were used as carbon source. In 150ml conical flasks, 30ml of relevant minimal broth containing free Pi was inoculated with cell suspensions to give an initial $O.D_{600nm}$ of 0.01-0.03 (0 hour O.D.). Similarly, for the TRP broth, the initial $O.D._{600nm}$ was about 0.03-0.04. The batch culture studies were performed under aerobic conditions in Orbitek rotary shaker maintained at 37°C with agitation speed kept constant at 200rpm. 1ml samples were aseptically collected at regular intervals (varying with every set of batch culture depending on media conditions) for various analytical techniques.

2.5.3: Analytical techniques

Cell densities were determined at 600nm spectrophotometrically. Change in absorbance was considered as the measure of growth and drop in pH of the media was taken as the measure of acid production. In all the cases, the observations were continued till the media pH reduced to less than 5 (where 100mM glucose was used) in case of 50mM glucose, 100mM glycerol or 50mM acetate the observations were stopped when the O.D reached more than 2 and remained constant. 1ml aliquots withdrawn aseptically at regular time intervals were immediately frozen at - 20°C until further used for biochemical estimations. The stored samples were centrifuged at 9, 200x *g* for 1 min at 4°C and the culture supernatants derived were used to estimate residual glucose and organic acid analysis using HPLC. For HPLC analysis, the culture supernatant was passed through 0.2µm nylon membranes (MDI advanced microdevices, India) and the secreted metabolites were quantified using RP-18 column. The column was operated at room temperature using mobile phase of 0.01M Na_2HPO_4 + 5% acetonitrile at a flow rate of 0.2 ml min^{-1} and the column effluents were monitored using a UV detector at 210 nm. Mobile phase was freshly prepared and degassed prior to use. Standards of organic acids were prepared in double distilled water, filtered using 0.2µm membranes and were subjected to chromatography for determining the individual retention time. Measurements of area under peak with an external standard were used for quantification. The glucose concentration in the medium was estimated using enzymatic kit (Reckon Diagnostics, India).

The physiological parameters like specific growth rate, specific total glucose utilization rate and biomass yield (as described by Chao and Liao, 1993) and the organic acid yield were calculated as described below.

(i) Specific growth rate (h^{-1}):

$$k = \frac{(Log_{10}Nt_1 - Log_{10}Nt_2) \times 2.303}{(t_1 - t_2)\ (h)} \quad \text{where,}$$

N1 and N2 are the number of cells at time t1 and t2 respectively and (t1-t2) is the corresponding time interval in hours. 2.303 is the factor derived from the formula- number of generations (n) = $(Log_{10}N - Log_{10}N_0)/Log_{10}2$. The number of cells was calculated from $O.D._{600nm}$ using the correlation 1 $O.D._{600nm} = 8.3 \times 10^8$ CFU/ml during the exponential growth phase (Zheng et al., 2005).

(ii) Specific total glucose utilization rate (Q_{Glc})

$$Q_{Glc} = \frac{\Delta Glucose\ (t_1 - t_2)\ (g/L)}{\Delta dcw\ (t_1 - t_2)\ (g/L) \times Time\ interval\ (t_1 - t_2)\ (h)} \quad \text{where,}$$

ΔGlucose (t_1-t_2) is the amount of glucose consumed over the time interval t_1-t_2; Δdcw (t_1-t_2) is the difference in the dry cell weight (dcw) of the cells over the time interval t_1-t_2. Q_{Glc} is expressed as g glucose utilized/g dcw/h). Dry cell weight was calculated using the correlation 1 O.D.600nm = 0.5mg/ml (Zheng et al., 2005).

(iii) Biomass yield

$$Y_{dcw/Glc} = \frac{\Delta dcw\ (t_1 - t_2)\ (g/L)}{\Delta Glucose\ (t_1 - t_2)\ (g/L) \times Time\ interval\ (t_1 - t_2)\ (h)} \quad \text{where,}$$

All the parameters were as described for Specific glucose utilization rate. $Y_{dcw/Glc}$ is expressed as g of dry cell weight produced/ g glucose utilized/h.

(iv) Organic acid yield

$$\text{Organic acid yield} = \frac{\text{Amount of organic acid produced (g/L)}}{\text{Total glucose utilized (g/L)} \times \text{dcw (g/L)}} \text{ where,}$$

The amount of total glucose utilized was obtained by deducting the value of residual glucose concentration from the initial glucose concentration supplied in the medium. The total glucose utilized was taken into account for calculating specific glucose utilization rate. The statistical analysis of all the parameters was done using Graph Pad Prism (version 3.0) software.

2.6: ENZYME ASSAYS

2.6.1: Preparation of cells and cell free extracts

Glucose or glycerol grown cells were harvested in appropriate growth phase from 30ml of cell culture by centrifugation 9,200x *g* for 2 minutes at 4°C. Citrate synthase (CS), isocitrate lyase (ICL) and isocitrate dehydrogenase (ICDH) were assayed in the stationary phase while PPC was assayed in late log phase cultures. The preparation of cell free extracts for PPC, CS, PYC, G-6-PDH, ICDH and ICL assays was carried out according to Kodaki et al (1985). The cell pellet was washed once with 250mM phosphate buffer (pH=7.0) followed by resuspension in same buffer containing 20% glycerol and 1mM DTT. For ICDH and G-6-PDH 10mM phosphate buffer (pH=7.0) was used as $MnCl_2$ and $MgCl_2$ used in the respective assay system. The cells were subjected to lysis by sonicating for maximum 1-1.5 minute in an ice bath, followed by centrifugation at 9,200x *g* at 4°C for 30 minutes to remove the cell debris. The supernatant was used as cell free extract (cell lysate) for the enzyme assays.

Note: *High concentration of phosphate in the buffer system often lead to the precipitation of* (Mg^{2+}) *and* (Mn^{2+}) *ions hence low concentration phosphate buffer were used for reactions involving these ions as cofactors.*

2.6.2: Enzyme Assay Protocols

2.6.2.1: PPC assay

PPC (EC 4.1.1.31) activity was estimated spectrophotometrically by monitoring the oxidation of NADH in a coupled assay with malate dehydrogenase (MDH) as described by Kodaki et al (1985) with modifications as follows. The coupled assay was divided in two steps. The assay mixture for first step of the reaction contained following ingredients in total volume of 0.9ml: Tris-$H2SO_4$ (pH 8.0), 100mM; potassium PEP, 2mM; $MgSO_4$, 10mM; $KHCO_3$, 10mM and the enzyme solution (cell lysate). After 20 minutes incubation at 30°C, the reaction was terminated using 25% TCA followed by 15 minutes incubation on ice bath. The supernatant recovered after centrifuging the resultant reaction mix at 9,200x *g* for 30 minutes at 4°C, was brought to pH 8.0 by using 7µl of 10N NaOH which was then used as the source of OAA in second step of the reaction for which the assay system contained 950µl of reaction mix from first step of reaction, 5units of MDH and 0.12mM NADH. The assay system volume was adjusted to 1ml using distilled water. The rate of oxidation of NADH recorded at 340nm was used to indicate concentration of OAA formed. Molar absorbance of NADH was taken as $6.22mM^{-1}cm^{-1}$ at pH 8.0.

Note : *The absence of glycerol in the sonication buffer led to complete loss of PPC activity* (Kodaki et al., 1985).

2.6.2.2: G-6-PDH assay

G-6-PDH (EC 1.1.1.49) activity was determined spectrophotometrically at room temperature by following the reduction of NADP at 340nm as a function of time (Eisenberg and Dobrogosz, 1967). Molar absorbance of NADP was taken as $6.22mM^{-1}cm^{-1}$ at pH 8.0. The reaction mixture of 1ml included: Tris-Cl (pH 8.2), 200mM; glucose-6-phosphate, 3.3mM; $MgCl_2$, 10mM; NADP, 0.1mM and cell extract.

2.6.2.3: ICL assay

ICL (4.1.3.1) activity was measured by a modified method of Dixon and Kornberg (1959). ICL catalyzes the hydrolysis of isocitrate into glyoxylate and succinate. The glyoxylate formed in the presence of phenylhydrazine was measured as glyoxylic acid phenylhydrazone at 324nm. The reaction mixture of 1ml consisted of following ingredients: potassium phosphate buffer (pH=7.0), 100mM; $MgCl_2$, 6mM; cysteine HCl, 12mM; phenylhydrazine, 4mM; isoctrate; 8mM and cell extract. Molar absorbance coefficient of phenylhydrazine was taken as 17.4 $mM^{-1}cm^{-1}$. The rate of increase in absorbance in the linear range was used to calculate ICL activity.

2.6.2.4: ICDH assay

ICDH (1.1.1.42) activity was measured by following NADPH formation at 340nm (Garnak and Reeves, 1979). The reaction mixture contained the following in 1ml: Tris-HCl (pH 7.5), 150mM; $MnCl_2$, 0.25mM; NADP, 0.65mM, isocitrate, 2.5mM and cell extract. Molar absorbance coefficient of NADP was taken as 6.22 $mM^{-1}cm^{-1}$.

2.6.2.5: CS assay

(i)

CS (4.1.3.7) activity was estimated by following the absorbance of 5, 5- dithiobis(2-nitrobenzoic acid) (DTNB) at 412nm which would change due to its reaction with sulfhydryl group of CoA (Serre, 1969). The assay mixture per cuvette contained following ingredients in 1.0ml: Tris-HCl (pH=8.0), 93mM; acetyl CoA, 0.16mM; OAA, 0.2mM; DTNB, 0.1mM and cell lysate. The reaction was started by addition of OAA. Molar absorbance coefficient was taken as 13.6 $mM^{-1}cm^{-1}$ at 412nm. The rate of increase in absorbance was used to calculate CS activity.

(ii)

An alternate method using DNPH was followed to estimate CS activity in *E. coli* MA1935 *icd* mutant (Else et al; 1998). The reaction mixture consisted of 20 mM-Tris/HCl (pH 8.0)/1 mM-EDTA, 0.2 mM-oxaloacetate, 0.15 mM-acetyl-CoA and cell extract. 50µl of aliquot were withdrawn every 1 min upto 5 min and added to 0.45 ml of 0.5 mM-DNPH

made in 1 M HCl and kept for incubation (10 min). The reaction was neutralized using 0.5 ml of 3 M-NaOH giving a change in colour (ketone group of oxaloacetate gave a yellow 2,4-dinitrophenylhydrazone) and incubated further for 5min and the absorbance was recorded at 450 nm. Molar absorbance coefficient was taken as 12.5 $mM^{-1}cm^{-1}$ and the utilization of the substrate OAA was measured to calculate CS activity.

***Note**: Estimation of CS activity could not be carried out for E. coli MA1935 icd mutant and its respective transformants using the above protocol probably due to high accumulation of oxoglutarate or NADH which are reported to inhibit CS activity.*

All the enzyme activities were determined at 30°C and were expressed per mg total protein. Total protein concentration of the crude extract as well as whole cell suspensions was measured by modified Lowry's method (Peterson, 1979) using bovine serum albumin as standard. Corrections were made for Tris buffer. Enzyme activities were calculated using following formula

$$\text{Specific enzyme activity (U)} = \frac{\Delta A_y \text{ nm/min}}{\epsilon \times \text{enzyme (sample) aliquot (ml)} \times \text{Total protein (mg/ml)}} \quad \text{where,}$$

ΔA_y nm is the difference in the absorbance at any given wavelengths (y nm) and ϵ is the millimolar extinction coefficient at *y* nm. One unit of enzyme activity was defined as the amount of protein required to convert 1 nmole of substrate per minute unless stated in the figure legend.

2.7: ENZYMATIC ESTIMATION OF INTRACELLULAR AND EXTRACELLULAR CITRIC ACID

E. coli transformant cultures grown on minimal medium were harvested in the late stationary phase and pelleted by centrifuging at 9, 200x *g* for 2 minutes at 4°C. The pellet was washed with 1ml of 250mM phosphate buffer (pH=7.0) and resuspended in the same buffer containing 20% glycerol. This homogenous cell suspension was sonicated for maximum 1-1.5 minute on an ice bath for complete cell lysis. To remove the cell debris centrifugation at 9, 200x *g* and 4°C for 30 minutes was carried out. The recovered

supernatant was filtered through 0.2µm nitrocellulose membrane and frozen immediately till further analysis (Petrarulo et al., 1995; Aoshima et al., 2003). This filtrate was used to estimate the amount of intracellular citric acid using the method described by Petrarulo et al (1995) with minor modifications. According to this method citrate lyase converts citrate to OAA and acetate and the resultant OAA can rapidly react with phenylhydrazine at slightly acidic pH to form the corresponding phenylhydrazone which can absorb ultraviolet (UV) light. Increase in the absorbance at 330nm would be an expression of the citrate concentration. The assay system per cuvette contained- 50mM phosphate buffer mix, 1.0ml; 246mM phenylhydrazine, 0.02ml; citrate lyase, 0.27U (0.02ml of 13.3 units/ml stock); citric acid standard or test sample. Buffer mix contained 50mM phosphate buffer (pH=6.5), 0.1mM $ZnSO_4.7H_2O$ and 0.2g/L sodium azide. Citric acid standards of 5µM, 7.5µM, 10µM, 15µM and 20µM concentrations were used to generate standard curve. Difference in O.D.330nm after 3 minutes of addition of citrate lyase was used for the calculations. To calculate the intracellular citrate concentration (in mM), cellular volume was assumed to be 1.63µl mg dcw^{-1} (Emmerling et al., 1999).

$$\text{Intracellular concentration (mM)} = \frac{\text{X nmoles/ml}}{\text{Dry cell mass (mg)} \times \text{Cf} \times 1.63}$$ where,

X nmoles/ml is calculated as follows- $\frac{\Delta \text{O.D.330nm /3min (test)} \times \text{Std (nmoles)}}{\text{Test aliquot (ml)} \times \Delta \text{O.D.330nm /3min (Std.)}}$

Cf = concentration factor of the 25 ml cell culture

Dry cell mass (dcw) = $O.D_{600nm}$ x 0.5, where 0.5 is the factor correlating $O.D_{600nm}$ was with dry cell weight (Zheng et al., 2005).

Extracellular concentration (mM) = X nmoles/ml

Note:

(i) All the centrifugations were carried out using Heraeus Biofuge *Stratos*. The spectrophotometric measurements of $O.D._{600nm}$ were carried out using Helios γ spectrophotometer, Thermospectronics (path length of 1cm) while the enzymatic determinations were done using JASCO V-530 spectrophotometer and Shimadzu UV-VIS spectrophotometer. The cell-lysis by sonication was achieved using Branson Sonifier Model 450. HPLC equipment from Merck, India was used for organic acid analysis.

(ii) The fine chemicals like NADH, NADP, acetyl-CoA, PEP potassium salt, DCIP, pure enzymes like MDH, LDH and citrate lyase were obtained from Sigma Chemicals Pvt. Ltd. While other routine chemicals and solvents were procured from Hi-media Laboratories, Qualigens Fine Chemicals, Sisco Research Laboratories and Merck, India.

(iii) All molecular biology grade reagents and kits were procured from Hi-Media Laboratories, India; Sisco Research Lab (SRL), India; Bangalore Genei Pvt. Ltd., India, or Sigma Chemical Pvt. Ltd. until and unless specified.