Effect of constitutive helerologous overexpression of E. coli NADH insensitive cs gene on the physiology and glucose metabolism of P. fluorescens PfO-1

CHAPTER 3

3 Chapter 3

3.1 INTRODUCTION

Citrate synthase (CS) is a ubiquitous enzyme that catalyzes the first committed step of tricarboxylic acid (TCA) cycle involving condensation of oxaloacetate (OAA) and acetyl-CoA to form citrate. Hence it is the key enzyme governing the carbon flux into the TCA cycle which plays a dual function in the production of cellular energy and biosynthetic precursors under aerobic conditions and only latter under anaerobic conditions, respectively (Park et al., 1994).

3.1.1 Citrate synthase and NADH sensitivity

The CS of gram-negative bacteria are allosteric enzymes designated as type II. Type II CS is a homo-hexamer of identical subunits with monomer size of ~48kDa, and is strongly and specifically inhibited by NADH (Weitzman 1981; Nguyen et al., 2001). *Escherichia coli* and *Acinetobacter anitratum* CS are strongly homologous in amino acid sequence and more distantly resemble the nonallosteric Type I citrate synthase of eucaryotes (Bhayana et al., 1984; Donald et al., 1987; Julie et. al., 2006). *Pseudomonas* CS are also allosteric and their kinetic properties suggest as intermediate between *E. coli* and *A. anitratum* enzymes (Massarini et al., 1975; Higa et al., 1978). Two forms of CS (EC 4.1.3.7) have been found in several species of ***Pseudomonas,*** a 'large' form ***(Mr~ 1:* 250000)** which **is** generally inhibited by NADH and a 'small' form ***(Mr~ 1:*** 100000) which is insensitive to these nucleotide effectors. Hence the NADH sensitivity of gram negative bacterial *CS* is attributed to subunit size (**Table 3.1, Fig. 3.1**). A mutant of ***Pseudomonas aeruginosa* PAC514** has been found to contain both a 'large' (CSI) and a 'small' (CSII) isozymes (Solomon & Weitzman, 1983; Mitchell et al., 1995).

Table 3.1: Distribution of large and small citrate synthases in *Pseudomonas* species (Coling et.al., 1986

Organism*	RNA homology group	Size of citrate synthase†	Percentage of 'small' citrate synthase‡	
			Gel filtration	FPLC
P. aeruginosa NCIB 8295	I	L + S	14	21
P. aeruginosa 8602		L + S	6	18
P. aeruginosa PAC 514		L + S	90	98
P. aeruginosa PAO 1		L + S	16	NT
P. fluorescens		L + S	25	25
P. putida		L + S	21	22
P. stutzeri		L + S	31	23
P. alcaligenes		S		
P. chlororaphis		S		
P. acidovorans	III	L		
P. saccharophila		S		
P. testosteroni		L		
P. diminuta	IV	L		
P. maltophilia	V	S		

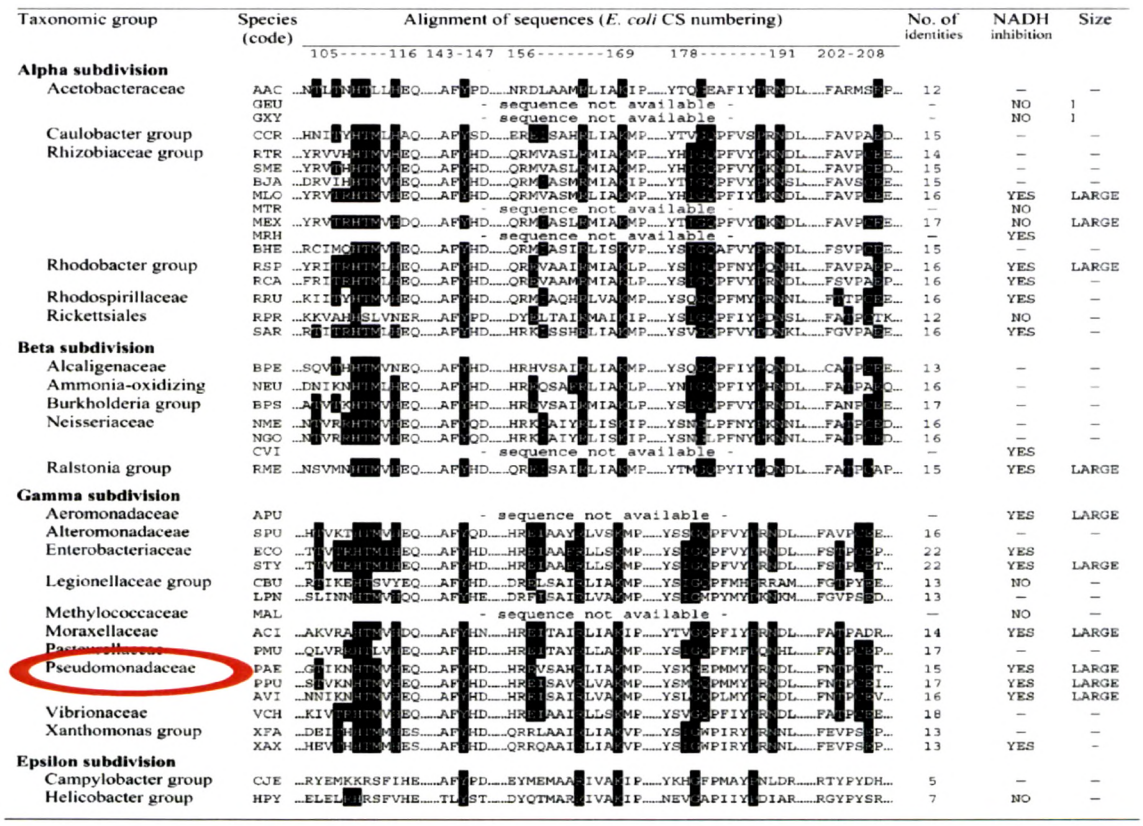

Figure 3.1: Correlation of Gram-Negative Bacterial Citrate Synthase Subunit Size and NADH Sensitivity with the Presence of NADH-Interacting Residues As Identified in the *E. coli CS*-NADH Complex (Robert et al., 2003)

3.1.2 Homology between *E. coli* and *P. fluorescens* citrate synthase

Clustal W multiple alignments show 71% homology between *E. coli* and *P. fluorescens cs* and posses similar regulatory properties.Individual *P. fluorescens* strains also exhibit variations in the coding region of cs (**Fig. 3.2-3.3**)

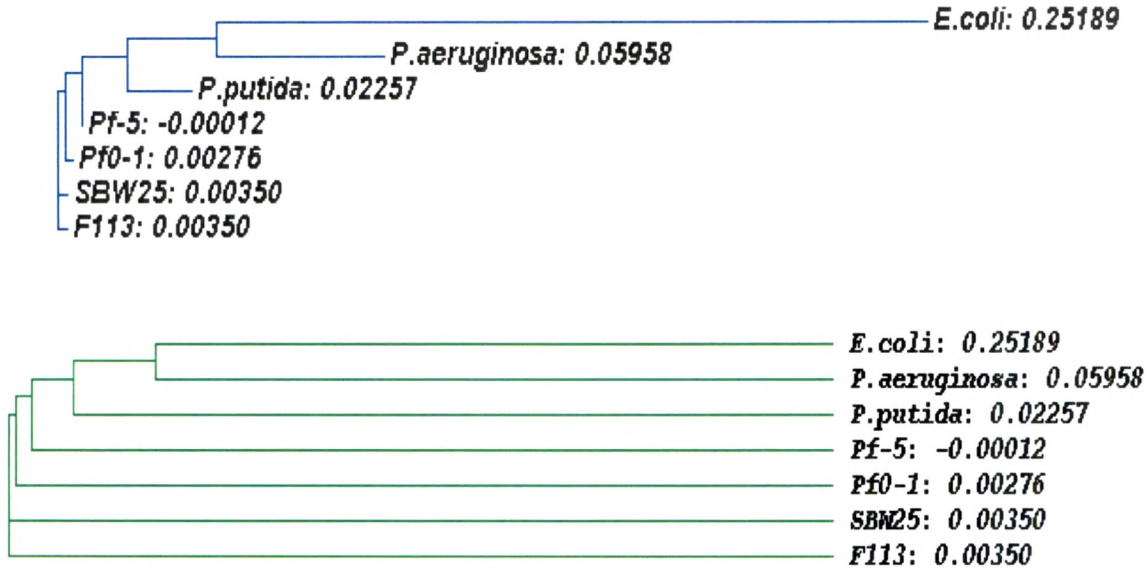

Figure 3.2: Phylogram and cladogram tree

3.1.3 Citrate overproduction through citrate synthase overexpression:

The study of the effect of *cs* gene manipulation on citric acid secretion and overall cellular metabolism can have tremendous applications in agriculture. *E. coli* lacking functional *cs* gene failed to utilize glucose unless supplemented with glutamate (or other TCA cycle intermediates) and had reduced growth as compared to the wild type (Vandedrinck et al., 2001; De Maeseneire et al., 2006). On the other hand, *cs* gene overexpression or underexpression in *E. coli* had no effect on growth on glucose while on acetate as sole carbon source; CS levels strongly affected the growth rate (Vandedrinck et al., 2001). Transgenic tobacco plants overexpressing *Pseudomonas aeruginosa cs* gene under the control of CaMV promoter increased excretion of citrate (Bucio et al., 2000). These citrate secreting plants enhanced the P solubilization efficiency and yielded more leaf and fruit biomass when grown under P-limiting conditions.

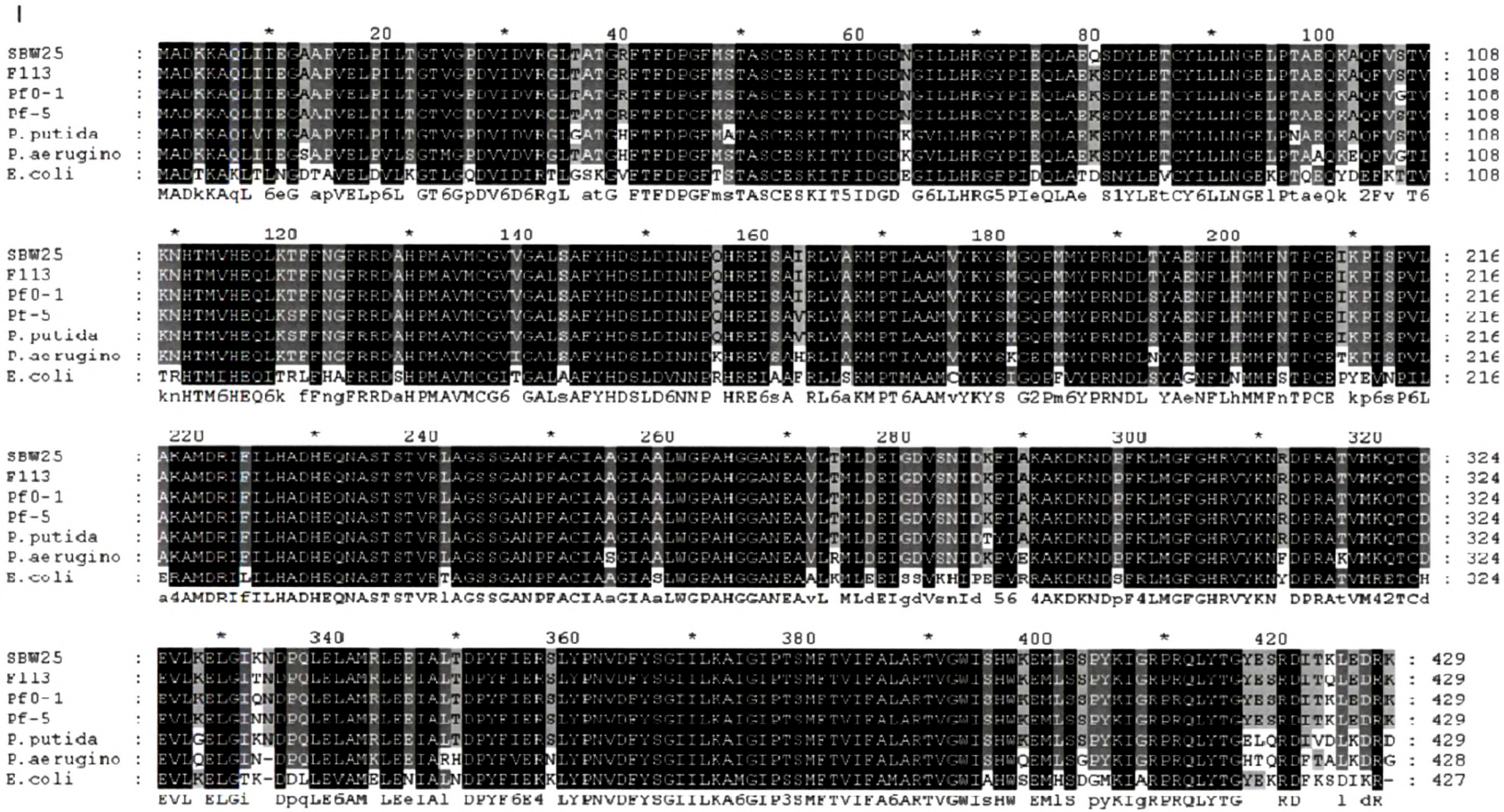

Figure 3.3: CS homology by ClustalW analysis between *E. coli* and fluorescent pseudomonads. Enclosed black areas indicate identical amino acid residues.

Overexpression of mitochondrial citrate synthase in *Arabidopsis thaliana* improved growth on a phosphorus-limited soil and improved the growth of carrot cells on Al-phosphate medium; the effect suspected to be due to enhanced secretion of citric acid (Koyama et al., 1999; 2000). Similar studies in *Nicotiana benthamiana* elicited Al induced citrate excretion (Deng et al., 2009) and conferred Al-tolerance in *S. cerevisiae* and canola (*Brassica napus cv Westar*) (Anoop et al, 2003). However, up to 11-fold overproduction of CS did not increase the rate of citric acid production by *Aspergillus niger*, suggesting that citrate synthase contributes little to flux control in the pathway involved in citric acid biosynthesis by the strain (Ruijter et al., 2000). On the other hand, *E. coli* K and B isocitrate dehydrogenase (*icd*) mutants accumulated high levels of citrate when grown on glucose with a concomitant increase in *CS* activity upto more than 2 fold (Lakshmi and Helling, 1976; Aoshima et al., 2003). Similarly, *B. subtilis icd* mutant in early stationary phase accumulated ~15 fold higher intracellular citrate levels as compared to the wild type (Matsuno et al., 1999). *glt*A gene overexpression in *E. coli* increased the maximum cell dry weight by 23% and reduced acetate secretion (De Maeseneire et al., 2006).

Metabolic studies in *E. coli* demonstrated that high citric acid yields could be attained on glucose and depending on the host metabolism; glucose transport, flux though catabolic pathways and the regulatory mechanisms influenced by intracellular metabolite pools appear to facilitate the citrate accumulation (Elias, 2009). Overexpression of *Escherichia coli* citrate synthase (*glt*A) gene in *Pseudomonas fluorescens* ATCC 13525 yielded intracellular and extracellular citric acid levels during the stationary phase, respectively (Arch et al., 2009). It was also postulated that increasing CS activity in *P. fluorescens* for citric acid overproduction from glucose is a better strategy than *icd* mutation in *E. coli*, which reduces biomass and growth (Aoshima et al., 2003). The amount of citric acid produced by *P. fluorescens* overexpressing *E. coli cs* gene was similar to that secreted by the phosphate solubilizing *Bacillus coagulans* and *Citrobacter koseri* on glucose (Gyaneshwar et al., 1998). But the amount of citric acid secreted by this approach is insufficient for effective P solubilization in field condition. Hence further strategies are required to increase the citrate level.

3.1.4 Rational of the present study

Functional properties of nine sequence variants of *E. coli* CS at NADH binding site had had varied inhibition in kinetic parameters for catalysis (Fig. 3.5) (Stockell et al., 2003). In three cases, Y145A, R163L, and K167A, NADH inhibition has become extremely weak (**Table 3.2, Fig. 3.4**).

```
MADTKAKLTL NGDTAVELDV LKGTLGQDVI DIRTLGSKGV
FTFDPGFTST ASCESKITFI DGDEGILLHR GFPIDQLATD
SNYLEVCYIL LNGEKPTQEQ YDEFKTTVTR HTMIHEQITR

                    145pyrophosphate (NADH)
LFHAFRRDSH PMAVMCGITG ALAAFYHDSL DVNNPRHREI
    163 and 167 pyrophosphate (NADH)
AAFRLLSKMP TMAAMCYKYS IGQPFVYPRN DLSYAGNFLN
      207/208 NADH
MMFSTPCEPY EVNPILERAM DRILILHADH EQNASTSTVR
TAGSSGANPF ACIAAGIASL WGPAHGGANE AALKMLEEIS

306activesite
SVKHIPEFVR RAKDKNDSFR LMGFGHRVYK NYDPRATVMR
ETCHEVLKEL GTKDDLLEVA MELENIALND PYFIEKKLYP
  363 active site
NVDFYSGIIL KAMGIPSSMF TVIFAMARTV GWIAHWSEMH
SDGMKIARPR QLYTGYEKRD FKSDIKR 427
```

Figure 3.4: ***E. coli cs*** **protein sequence showing the regulatory variants**

Table 3.2: NADH binding and inhibition by variant citrate synthases (Stokell et al., 2003)

Variant	Kd (µM)	Ki (µM)	Maximum inhibition(%)
Wild type	1.6±0.1	2.8±0.4	100±10
R109L	1.16±.04	1.7±0.2	96±6
H110A	5.2±0.2	121±11	97±3
T111A	6.6±0.2	80±15	87±6
Y145F	>100	790±210	100±3
R163L	5.81±.04	400±80	77±8
K167A	4.1±0.2	630±130	100±10
Q128A	6.1±0.5	18±3	100±7
N189A	6.9±0.8	242±26	96±5
T204A	10.2±0.4	165±36	100±7

The present study demonstrates the effect of overexpression of NADH insensitive *cs* variants e.g., Y145F, R163L, and K167A on citric acid accumulation and secretion by *P. fluorescens* PfO-1.

3.2 WORK PLAN

The experimental plan of work includes the following-

3.2.1 Bacterial strains used in this study

Table 3.3: List of bacterial strains used.

Bacterial strains	Characteristics	Source/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
E. coli W620	*cs* mutant strain exhibiting glutamate auxotrophy, CGSC 4278 - *glnV44 gltA6 galK30 LAM-pyrD36 relA1 rpsL129 thi-1*; Str^r	*E. coli* Genetic Stock Center
P. fluorescens PfO-1	Wild type strain	
Pf (pAB8)	*P. fluorescens* PfO-1 with pAB8; Km^r	This sudy
Pf(pAB7)	*P. fluorescens* PfO-1 with pAB7; Km^r	This sudy
Pf (pR163L)	*P. fluorescens* PfO-1 with pR163L; Km^r	This sudy
Pf(pK167A)	*P. fluorescens* PfO-1 with pK167A; Km^r	This sudy
Pf(pY145F)	*P. fluorescens* PfO-1 with pY145F; Km^r	This sudy

Detailed characteristics of these strains and plasmids are given in **Section 2.1** Parent strains and the transformants of *E. coli* and *Pseudomonas fluorescens* were respectively grown at 37°C and 30°C with streptomycin and kanamycin as and when required, at final concentrations varying for rich and minimal media as described in **Section 2.2, Table 2.4**

3.2.2 Cloning and expression of NADH insensitive *E. coli cs* gene in *P. fluorescens* PfO-1

The strategy for construction of recombinant *P fluorescens* strain harbouring NADH insensitive *cs* gene are depicted in **Fig. 3.5**.

3.2.2.1 Construction of *Pseudomonas* stable plasmid containing *E. coli NADH* insensitive *cs* gene under *lac* promoter

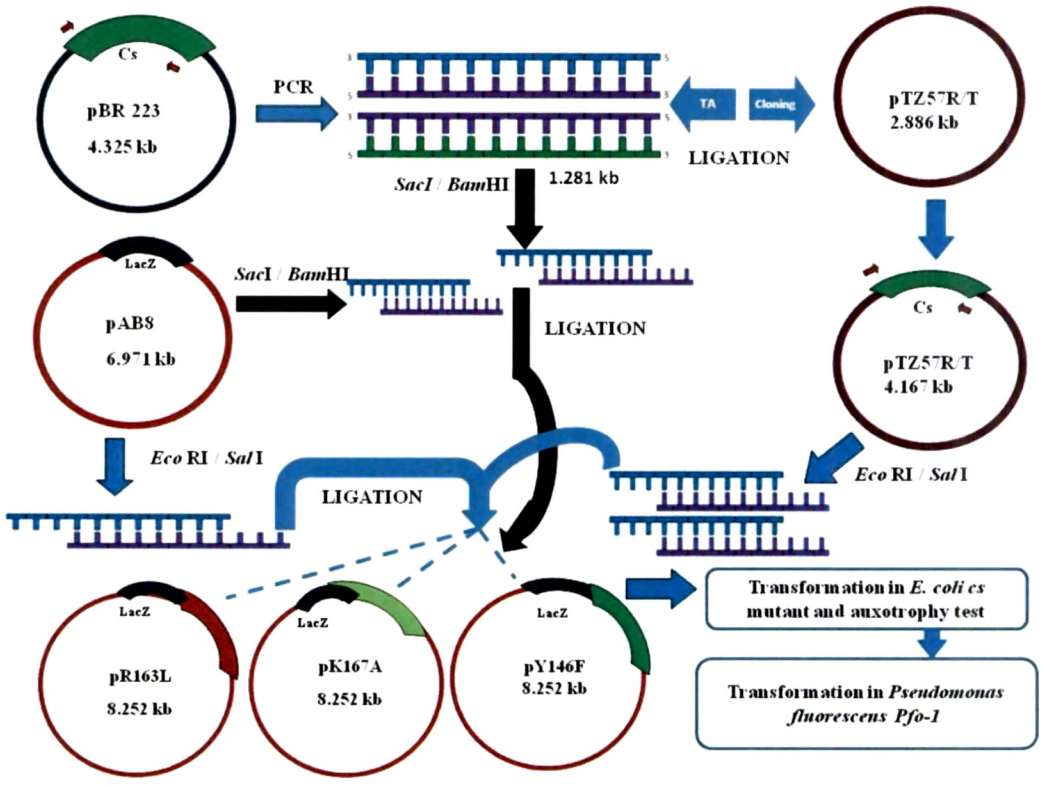

Figure 3.5: Schematic representation of construction of pseudomonad stable vectors containing NADH insensitive *E. coli cs* gene under *lac* promoter. Blue arrows indicate strategy1 and black arrow indicate strategy 2

3.2.2.1.1 Incorporation of NADH insensitive *E. coli cs* in pUCPM18:

PCR amplification of pBR322 plasmid containing NADH insensitive *E. coli cs* (*gltA*) gene are carried out using gene specific primer (Table 3.4) to get an amplification of 1281 bp each of *R163L, K167A, Y145F*

Table 3.4: NADH insensitive *cs* primers

Primer	Oligonucleotide Sequence(5'-3'end)	Tm/GC%
Ec*CS*ApaI SacIF	5'**CGAGCTC***GGGCCC*TTTTTCACGGAGGAAACC ACAATG GCT GAT ACA AAA GC	59.6°C, 52.2%
Ec*CS*KpnIBamHI R	**CG*GGATTC***C*GGATCC*G TTA ACG CTT GAT ATC GC	59.6°C, 46.2%

SacI ApaI: CGAGCTC*GGGCCC* ***KpnI BamHI***: GG**GGTACC**CGGATCCG

RBS: 5'CACG**GAGGA**ATCAACTT **3'**

Cloning of the isolated *cs* gene was carried out in broad host range cloning vector pUCPM18 having kanamycin resistance gene using two strategies. In one strategy the amplified 1281 bp gene was directly cloned into pUCPM18 km^r vector in SacI/BamHI site under *lac* promoter. In another strategy 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 and restriction enzyme digestion and subcloned into pUCPM18 kan^r vector in *EcoRI/SalI* restriction site (**Fig.3.6**)

3.2.2.1.2 Functional confirmation of *CS* expressed from pR163L, pK167A and pY145F

E. coli W620, which exhibited glutamate auxotrophy due to mutation in *cs* gene, was used to determine the functionality of the *cs* gene. pAB7, pR163L, pK167A and pY145F along with the respective controls pAB8 plasmids were transformed into *E. coli* W620 (**Table 2.1, Section 2.2**). The transformants were selected on agar plates with streptomycin and kanamycin (doses as recommended in **Section 2.2**) and confirmed the presence of

respective plasmids. Subsequently these were subjected to auxotrophy complementation studies (**Section 2.6**).

3.2.2.1.3 Development of *P. fluorescens* PfO-1 harboring NADH insensitive *E. coli cs* gene

The recombinant plasmids pR163L, pK167A and pY145F along with control pAB8 and plasmid containg wild type *cs* gene pAB7 were transformed by elecroporation in *P. fluorescens* PfO-1 (**Section 5.2.2**). The transformants were selected on pseudomonas agar plate containing kanamycin and were confirmed by fluorescence and restriction enzyme digestion of the isolated plasmids from the respective strains (**Section 2.4.5**).

3.2.3 Effect of NADH insensitive *E. coli cs* gene expression on the physiology and glucose metabolismof of *P. fluorescens* PfO-1.

P. fluorescens PfO-1 transformants were subjected to physiological experiments involving growth and organic acid production profiles on M9 minimal medium with 100mM glucose as carbon source. The samples withdrawn at regular interval were analyzed for O.D.600nm, pH, and extracellular glucose (**Section 2.7.2**). Stationary phase cultures harvested at the time of pH drop were subjected to organic acid estimation using HPLC (**Section 2.7.3**). The physiological parameters were calculated as in section 2.7.3. The enzyme assays were performed as described in 2.8, with *CS*, G-6-PDH,ICDH PYC being assayed in both mid-log to late-log and stationary phase cultures while ICL and GDH are being assayed in the mid log and stationary phase cultures respectively.

3.3 RESULTS

3.3.1 Construction of *Pseudomonas* stable plasmid containing NADH insensitive *E. coli cs* gene under *lac* promoter

The plasmids pR163L. pK167A and pY145F containing *E. coli NADH insensitive cs* gene *r163l, k16a* and *y146f* respectively under *lac* promoter of pUCPM18 plasmid with km^r gene were constructed in a two step cloning procedure schematically represented and discussed in section (**Fig. 3.6**). All plasmids were confirmed based on restriction digestion pattern (**Fig. 3.7-3.14**)) and PCR (**Fig. 3.15**).The plasmids pAB8 and pAB7 were taken as a control for all the experiments.

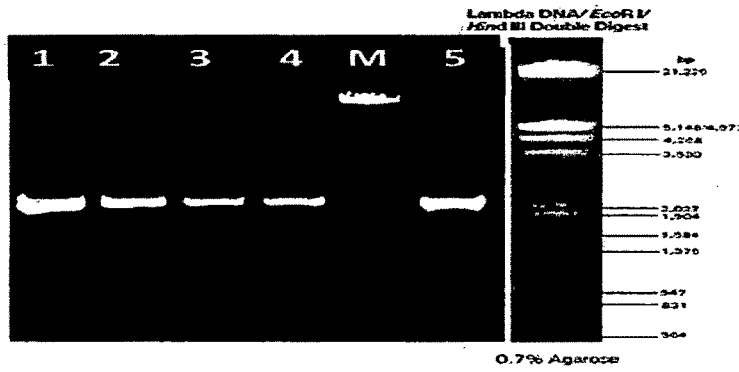

Figure 3.6: PCR amplification of NADH insensitive *cs* gene. *R163L* (Lane1), *K167A (Lane2), Y145F* (Lane3); pCC*gltA* (Lane4), pES*gltA* (Lane5) each of 1281 bp

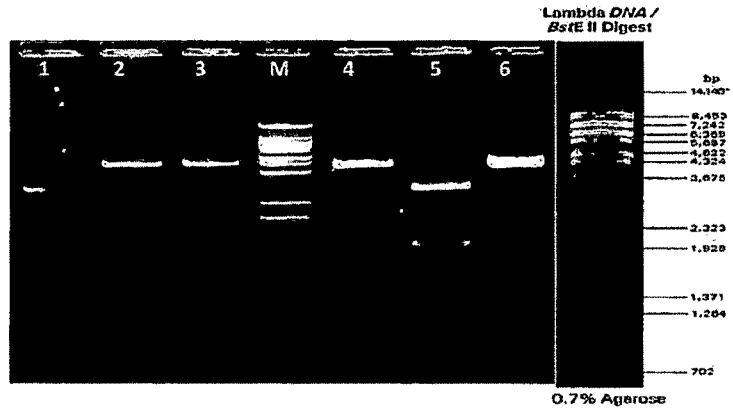

Figure 3.7: Restriction digestion pattern of pTZ57R/T *Y146F* clone with *Sac*I. Lane1, 5: plasmid with Y145F gene in right orientation (2886 bp and 1281 bp).Lane 2, 3, 4, 6: plasmid with *Y145F* in opposite orientation (4167 bp). Lane M: Molecular weight marker (MWM)-Lamda DNA cut with *BstE*II

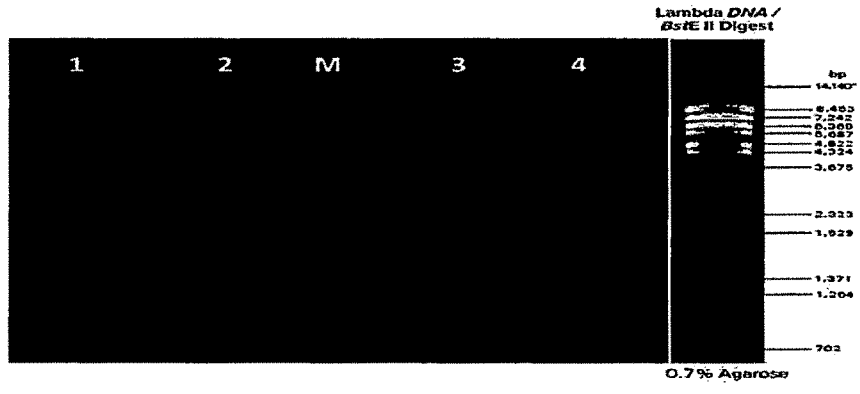

Figure 3.8: Restriction digestion pattern of pTZ57R/T *K167A* clone . Lane1-Lane4: pTZ57R/T *K167A* plasmid digested with *KpnI*(2886 bp and 1281 bp) . Lane M: Molecular weight marker(MWM)-Lamda DNA cut with *BstE*II

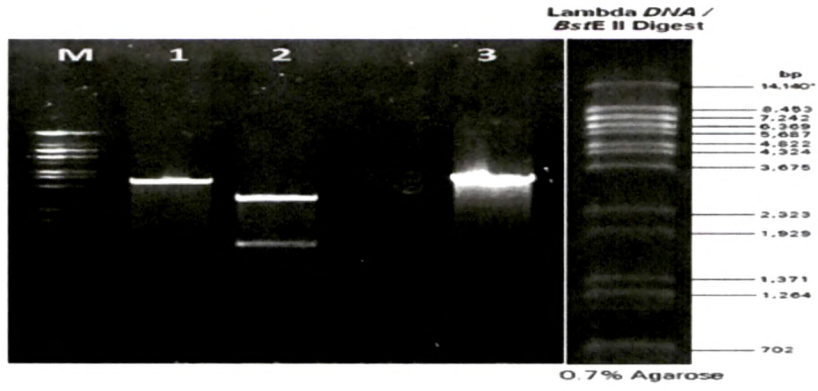

Figure 3.9: Restriction digestion pattern of pTZ57R/T *R146L*clone with *KpnI*. Lane1, 3: plasmid containing gene in opposite orientation (4167 bp). Lane 2: plasmid containing gene in right orientation (2886 bp and 1281 bp). Lane M: Molecular weight marker(MWM)-Lamda DNA cut with *BstE*II

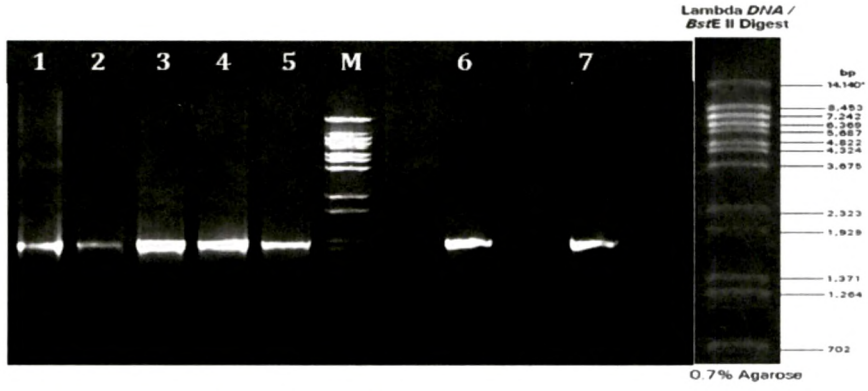

Figure 3.10: PCR amplification of TA clones. Lane1, 2: *R163L*; Lane3, 4:*Y145F*; Lane5, 6: *K167A* Lane7: wild type *cs* gene each gene of 1281 bp; Lane M: Molecular weight marker (MWM)-Lamda DNA cut with *BstE*II.

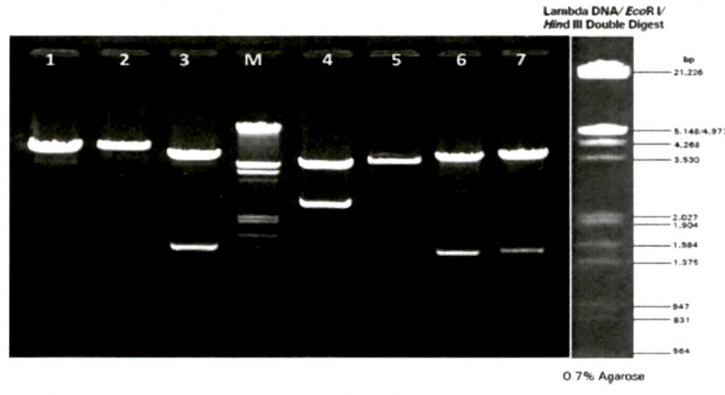

Figure 3.11: Restriction digestion pattern for pY145F. Lane1: pY145F linearizes with SacI (8252bp); Lane2: pY145F linearizes with BamHI (8252 bp); Lane3: pY145F digested with *EcoRI-SalI* (6971bp,1281bp); Lane4: pY145F digested with *EcoRI-BglII*(6971bp,2918 bp);Lane5: pAB8 digested with *EcoRI-BglII*

(5334bp,1637 bp); Lane6:pY145F digested with ApaI (6971 bp,1281 bp); Lane6: pY145F digested with *KpnI* (6971 bp,1281 bp); LaneM:MWM with Lamda DNA *EcoRI*/HindIII double digest.

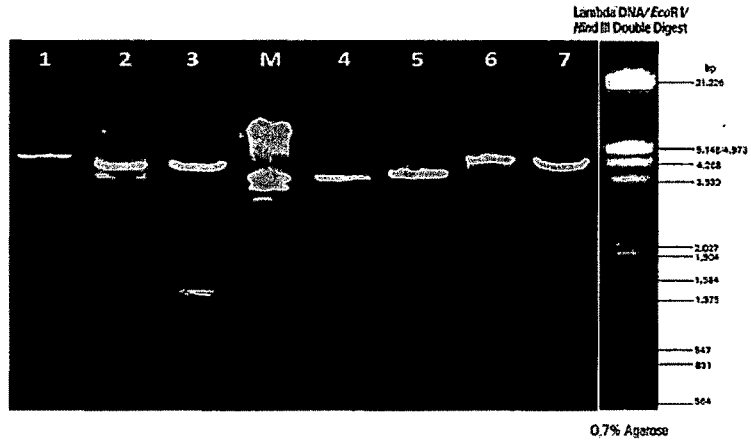

Figure 3.12: Restriction digestion pattern for pK167A. Lane1: pK167A plasmid linearized with BamHI (8252bp); Lane2; pK167A digested with ApaI (6971bp, 1281bp); Lane3: pK167A digested with *EcoRI-SalI*(6971bp,1281bp);Lane4: pK167A digested with *EcoRI-BglII* (5334bp,2918 bp); lane5:pAB8 digested with *EcoRI-BglII* (5550bp,1421bp); lane6: pK167A plasmid linearized with SacI (8252bp); Lane7: pK167A plasmid linearized with *EcoRI* (8252bp); laneM: MWM lamda DNA *EcoRI*/HindIII double digest

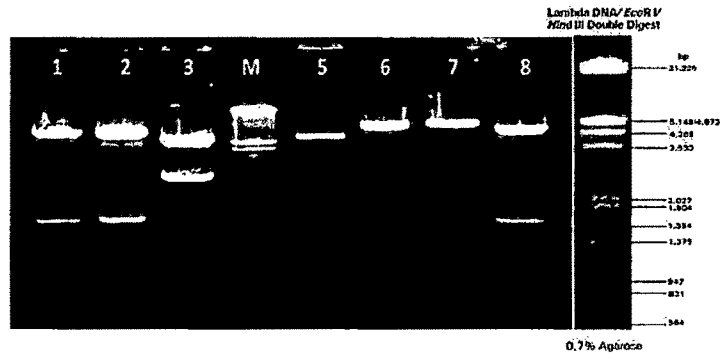

Figure 3.13: Restriction digestion pattern for pR163L. Lane1: pR163L digested with *KpnI* (6971bp,1281bp); Lane2: pR163L digested with *EcoRI-SalI* (6971bp,1281bp); Lane3: pR163L digested with *EcoRI-BglII*(5334bp,2918 bp); Lane5: pAB8 digested with *EcoRI-BglII* (5550bp,1421bp); lane6: pR163L linearized withApaI(8252bp); lane7: pR163L linearized with SacI(8252bp) ; Lane8: pR163L digested with *EcoRI* (6971bp,1281bp); laneM: MWM lamda DNA *EcoRI*/HindIII double digest

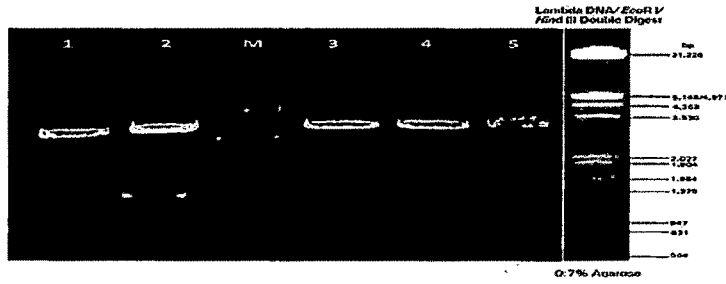

Figure 3.14: Restriction digestion pattern for pUCPM18 Km^r containing NADH insensitive *cs* gene digested with EcoRI-BamHI (StrategyII). Lane1,Lane2: pY145F (6971bp,1281bp); Lane3: pR163L ((6971bp,1281bp); Lane4: pK167A (6971bp,1281bp); Lane5: pAB8 (6971bp,1281bp);LaneM: MWM containing LamdaDNA EcoRI-HindIII double digest.

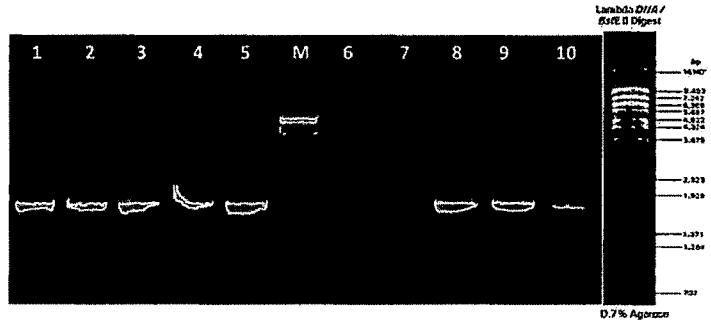

Figure 3.15: PCR amplification of NADH insensitive gene cloned in pUCPM18 km^r vector. Lane1-Lane3: pUCPM18 Km^r containing Y145F gene; Lane4,5: pUCPM18 Km^r containing R163L gene; Lane6-Lane10:pUCPM18 Km^r containing K167 gene;each gene of 1281bp;LaneM: MWM containing lamda DNA cut with BstEII.

3.3.2 *E. coli cs* mutant complementation

E. coli cs mutant strain harboring pAB7, pR163L, K167A and pY145F could grow on M9 minimal medium containing glucose as carbon source o when induced with 0.1mM IPTG without glutamate supplementation unlike the controls pAB8 (**Fig.3.16**).

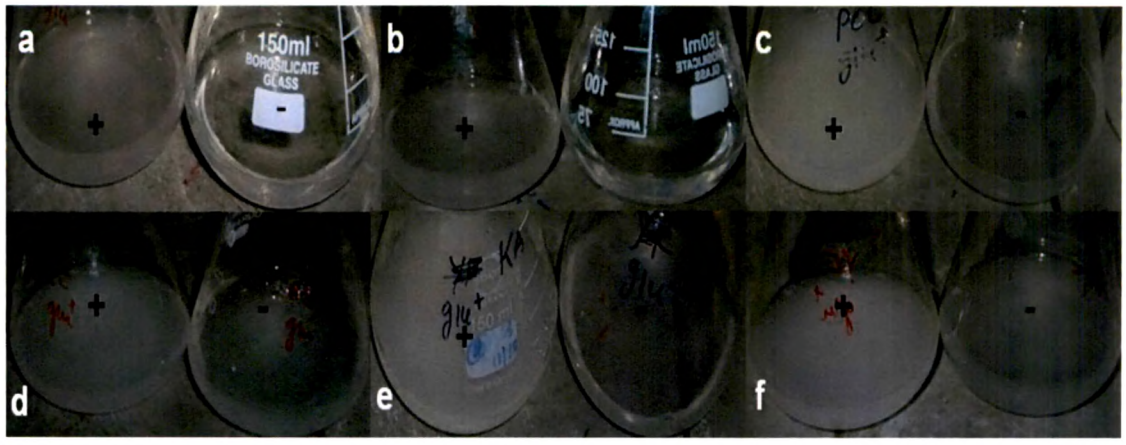

Figure 3.16: Complementation of *E. coli* W620 mutant phenotype by wild type and NADH insensitive *cs* plasmids. a: *E. coli* W620-deletion mutant of *cs* gene b: *E. coli* W620 with pAB8 plasmid c: *E. coli* W620 with pAB7 plasmid d: *E. coli* W620 with pR163L plasmid e: *E. coli* W620 with pK167 plasmid f: *E. coli* W620 with pY145F plasmid. All plasmid bearing strains are induced with 0.1mM IPTG. Growth is monitored on M9 medium with 0.2% glucose (Section) +/– at the corners of each image indicates absence and presence of 340µg/ml glutamate in the medium, respectively.

3.3.3 Partial sequencing of pY145F plasmid

Partial sequencing of the PCR product amplified from pY146F plasmid when analysed using NCBI BLAST (Basic Local Alignment Seaech Tool) and Ribososmal Database Project (RDP) II, online homology search programs, revealed maximum identity (99%) to *E. coli* citrate synthase (GenBank Accession number AAA23892) (**Fig. 3.17-3.18**).

```
>YFPCRPRODUCT_YFFOR_S702
CNCNNANCGGCACCCTGAACGGGGATACAGCTGTTGAACTGGATGTGCTGAAAGGCACGC
TGGGTCAAGATGTTATTGATATCCGTACTCTCGGTTCAAAAGGTGTGTTCACCTTTGACC
CAGGCTTCACTTCAACCGCATCCTGCGAATCTAAAATTACTTTTATTGATGGTGATGAAG
GTATTTTGCTGCACCGCGGTTTCCCGATCGATCAGCTGGCGACCGATTCTAACTACCTGG
AAGTTTGTTACATCCTGCTGAATGGTGAAAAACCGACTCAGGAACAGTATGACGAATTTA
AAACTACGGTGACCCGTCATACCATGATCCACGAGCAGATTACCCGTCTGTTCCATGCTT
TCCGTCGCGACTCGCATCCAATGGCAGTCATGTGTGGTATTACCGGCGCGCTGGCGGCGT
TCTTTCACGACTCGCTGGATGTTAACAATCCTCGTCACCGTGAAATTGCCGCGTTCCGCC
TGCTGTCGAAAATGCCGACCATGGCCGCGATGTGTTACAAGTATTCCATTGGTCAGCCAT
TTGTTTACCAGCGCAACGATCTCTCCTACGCCGGTAACTTCCTGAATATGATGTTCTCCA
CGCCGTGCGAACCGTATGAAGTTAATCCGATTCTGGAACGTGCTATGGACCGTATTCTGA
TCCTGCACGCTGACCATGAACAGAACGCCTCTACCTCCACCGTGCGTACCGCTGGCTCTT
```

CGGGTGCGAACCCGTTTGCCTGTATCGCAGCAGGTATTGCTTCACTGTGGGGACCTGCGC
ACGGCGGTGCTAACGAAGCGGCGCTGAAAATGCTGGAAGAAATCAGCTCCGTTAAACACA
TTCCGGAATTTGTTCGTCGTGCGAAAGACAAAAATGATTCTTTCCGCCTGATGGGCTTCG
GTCACCGCGTGTACAAAATTACGACCCGCGCGCCACCGTAATGCGTGAAACCTGCCATGA
AGTGCTGAAAGAGCTGGGCACGAANNTGACCTGCTGNAGT

Figure 3.17: Partial sequence of *E. coli* NADH insensitive *cs* gene

```
>gb|U00096.2| Escherichia coli str. K-12 substr. MG1655, complete genome
Length=4639675

 Features in this part of subject sequence:
   citrate synthase

 Score = 1777 bits (962),  Expect = 0.0
 Identities = 971/975 (99%), Gaps = 1/975 (0%)
 Strand=Plus/Minus

Query  4       CACCCTGAACGGGGATACAGCTGTTGAACTGGATGTGCTGAAAGGCACGCTGGGTCAAGA  63
               |||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753668  CACCCTCAACGGGGATACAGCTGTTGAACTGGATGTGCTGAAAGGCACGCTGGGTCAAGA  753609

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Query  364     CTCGCATCCAATGGCAGTCATGTGTGGTATTACCGGCGCGCTGGCGGCGTTCTTTCACGA  423
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Query  424     CTCGCTGGATGTTAACAATCCTCGTCACCGTGAAATTGCCGCGTTCCGCCTGCTGTCGAA  483
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Sbjct  753248  CTCGCTGGATGTTAACAATCCTCGTCACCGTGAAATTGCCGCGTTCCGCCTGCTGTCGAA  753189

Query  484     AATGCCGACCATGGCCGCGATGTGTTACAAGTATTCCATTGGTCAGCCATTTGTTTACCA  543
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Sbjct  753188  AATGCCGACCATGGCCGCGATGTGTTACAAGTATTCCATTGGTCAGCCATTTGTTTACCC  753129

Query  544     GCGCAACGATCTCTCCTACGCCGGTAACTTCCTGAATATGATGTTCTCCACGCCGTGCGA  603
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Query  604     ACCGTATGAAGTTAATCCGATTCTGGAACGTGCTATGGACCGTATTCTGATCCTGCACGC  663
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Sbjct  753068  ACCGTATGAAGTTAATCCGATTCTGGAACGTGCTATGGACCGTATTCTGATCCTGCACGC  753009
```

Figure 3.18 : NCBI BLAST analysis of partial cs sequence

Pairwise alignment of the sequence with original *E. coli* K12 NADH sensitive *cs* sequence from database using EBI parwise alignment tool, revealed a mutation of tyrosine residues in 146 amino acid position to phenylalanine (**Fig. 3.19**).

```
PCR              1 --------------CNCNNANCGGCACCCTGAACGGGGATACAGCTGTTG    36
                                 ..|..|.| .|||||||.||||||||||||||||||
ORIGINAL         1 atggctgatacaaaagcaaaac-tcaccctcaacggggatacagctgttg    49

PCR             37 AACTGGATGTGCTGAAAGGCACGCTGGGTCAAGATGTTATTGATATCCGT    86
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
ORIGINAL        50 aactggatgtgctgaaaggcacgctgggtcaagatgttattgatatccgt    99

PCR             87 ACTCTCGGTTCAAAAGGTGTGTTCACCTTTGACCCAGGCTTCACTTCAAC   136
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
ORIGINAL       100 actctcggttcaaaaggtgtgttcacctttgacccaggcttcacttcaac   149

PCR            137 CGCATCCTGCGAATCTAAAATTACTTTTATTGATGGTGATGAAGGTATTT   186
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
ORIGINAL       150 cgcatcctgcgaatctaaaattacttttattgatggtgatgaaggtattt   199

PCR            187 TGCTGCACCGCGGTTTCCCGATCGATCAGCTGGCGACCGATTCTAACTAC   236
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
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PCR            237 CTGGAAGTTTGTTACATCCTGCTGAATGGTGAAAAACCGACTCAGGAACA   286
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ORIGINAL       250 ctggaagtttgttacatcctgctgaatggtgaaaaaccgactcaggaaca   299

PCR            287 GTATGACGAATTTAAAACTACGGTGACCCGTCATACCATGATCCACGAGC   336
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ORIGINAL       300 gtatgacgaatttaaaactacggtgacccgtcataccatgatccacgagc   349

PCR            337 AGATTACCCGTCTGTTCCATGCTTTCCGTCGCGACTCGCATCCAATGGCA   386
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ORIGINAL       350 agattacccgtctgttccatgctttccgtcgcgactcgcatccaatggca   399

PCR            387 GTCATGTGTGGTATTACCGGCGCGCTGGCGGCGTTCTTTCACGACTCGCT   436
                   ||||||||||||||||||||||||||||||||||.||||||||||||||
ORIGINAL       400 gtcatgtgtggtattaccggcgcgctggcggcgttctatcacgactcgct   449
```

Figure 3.19: EBI pairwise alignment of NADH insensitive and wild type *cs* gene showing the position of mutation

3.3.4 Heterologous overexpression of *E. coli NADH insensitive cs* gene in *P. fluorescens* PfO-1

pY146F plasmid transformed in *P. fluorescens* PfO-1 by electrporation. The transformants showed resistance against gentamycin. Restriction enzyme digestion of the isolated plasmid revealed the authenticity of the plasmid (**Fig.3.20**).

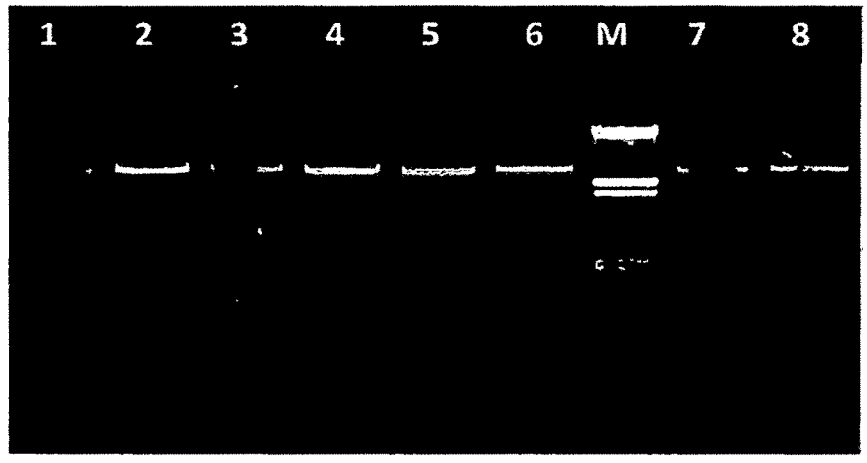


Figure 3.20: EcoRI-BamHI restriction digestion pattern for pUCPM18 Km^r containing *E. coli* NADH insensitive *cs* gene from *P. fluorescens* PfO-1 and *P. fluorescens* ATCC13525 transformants. Lane1: *P. fluorescens* PfO-1 with pR163L plasmid (6971,1281bp);Lane2: *P. fluorescens* PfO-1 with pAB8 plasmid (6971 bp); Lane3: *P. fluorescens* ATCC13525 pR163L (6971 ,1281 bp);Lane4: *P. fluorescens* ATCC13525 with pAB (6971 bp);Lane5: *P. fluorescens* PfO-1 with pAB7 (6971,1281 bp);Lane6: *P. fluorescens* ATCC13525 with pAB7(6971, 1281 bp);Lane7: *P. fluorescens* ATCC13525 with Y146F(6971, 1281 bp); Lane8: *P. fluorescens* PfO-1 with Y146F (6971,1281 bp); LaneM: MWM with Lamda DNA *EcoRI*/HindIII double digest

3.3.5 Biochemical effects of *E. coli cs* gene overexpression in *P. fluorescens* PfO-1

3.3.5.1 Alterations in citrate synthase activity

P. fluorescens PfO-1 harbouring pY145F showed maximum *CS* activity of 424.6±16.1U and 333.4±8.5U in the mid log and stationary phase, respectively, on M9 minimal medium in the presence of 100 mM glucose which is about 4.7 and 5.6 fold higher than that in the control Pf(pAB8), that showed 90.3±6.7U and 60±14.7 CS activity respectively. Also Pf(pY145F) showed the highest *cs* activity amongst all the other variants which is 2 fold,1.7 fold and 1.96 fold higher as compared to the wild type *cs* bearing strain *Pf*(pAB7) and other two NADH insensitive *cs* bearing strain *Pf* (pR163L) and *Pf* (K167A) respectively in the mid log phase (**Fig.3.21**).

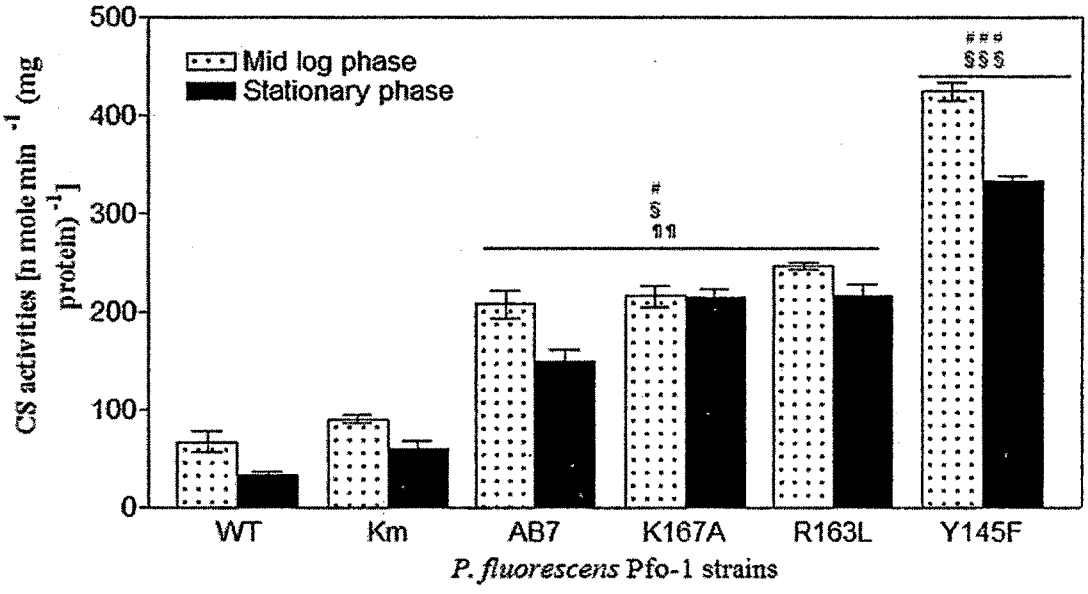
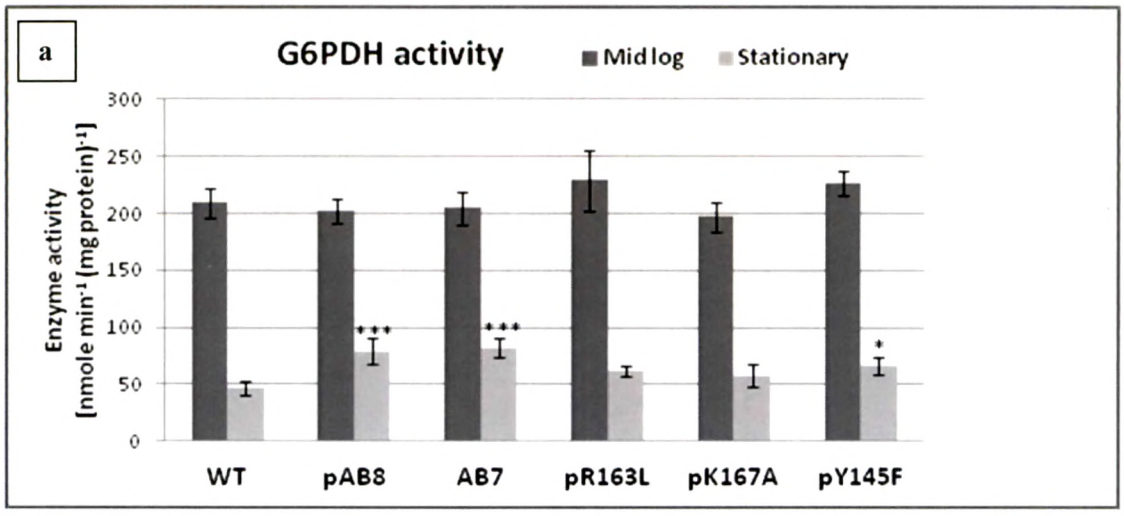
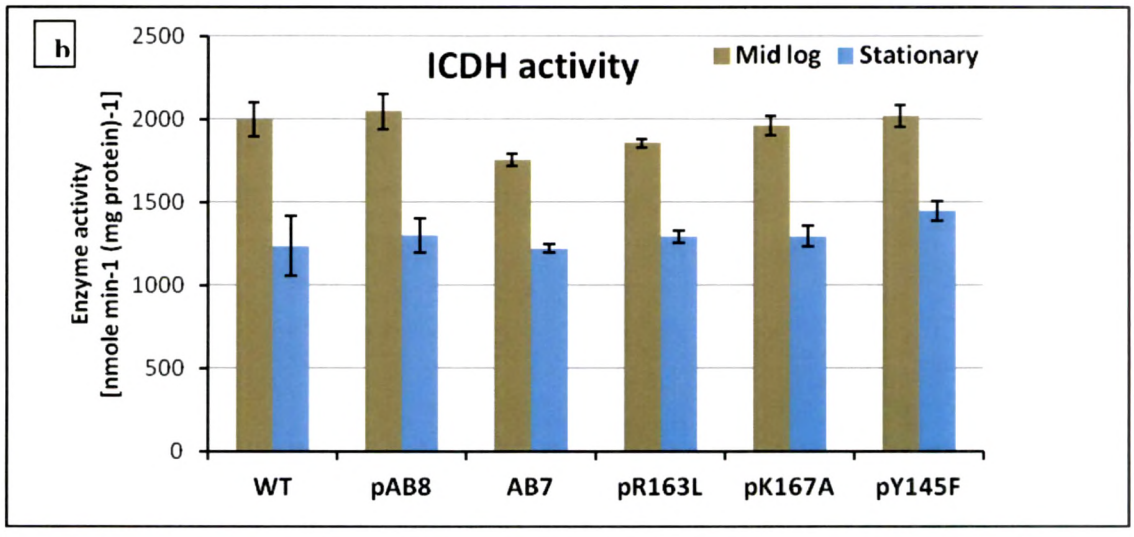


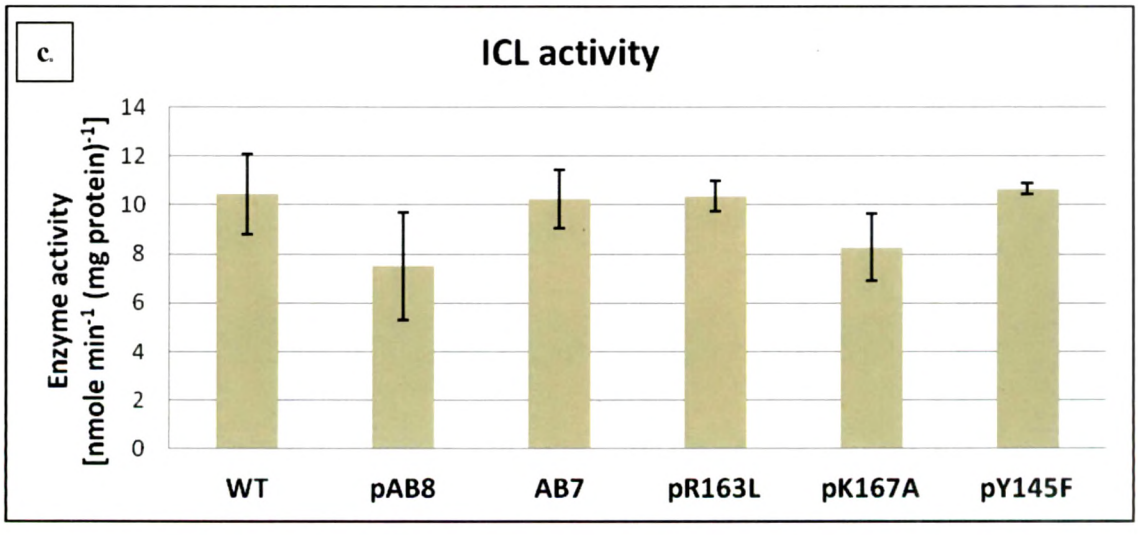
Figure 3.21: Citrate synthase activity of *P. fluorescens* PfO-1 transformants. The activity have been estimated using wild type, *Pf*(pAB7), *Pf*(pAB8), *Pf*(pK16A), *Pf*(pR163L) and *Pf*(pY146F) cultures grown on M9 minimal medium with 100mM glucose from mid log phase and stationary phase cultures. Values are represented in the units of nmoles/min/mg total protein. The values are depicted as Mean ± S.E.M of 4 independent observations. # Comparison of parameters with respective Wild type, § with respect to Vector control pAB8, ¶ comparision between parameter of pY146F with pAB7,pR163L and pK167A.###, §§§,¶¶¶P<0.001; .##, §§,¶¶P<0.01; .#, §,¶P<0.05

3.3.5.2 Alterations in G-6-PDH, ICDH, ICL, PYC, and GDH activities

The effect of overexpression is also being monitored at the level of key enzymes of glucose catabolism in both mid log and stationary phase of growth. In *Pf* (pY146F) the periplasmic GDH activity increased by 2.1,1.4 and 1.26 fold as compared to the wild type, *Pf* (pAB8) and *Pf* (pAB7), respectively, in the stationary phase. There is a significant alteration in GDH activity found in vector control *Pf*(pAB8) as compared to the wild type strain. Similarly a significant increase in PYC activity (2.4, 2.1 and 0.15 fold in the mid log and 6.5, 4.1, 1.7 old in the stationary phase) was observed as compared to the respective controls. ICL and ICDH activities in both mid log and stationary phase and G6PDH activities in the stationary phase cultures remained unaltered. However in the mid log phase an altered G6PDH activities had increase in activity in *Pf* (pAB8) and *Pf* (pAB7) and significant decrease in *Pf*(pY146F) were monitored as compared to the wild type strain (**Fig. 3.22**).


a
G6PDH activity
Mid log
Stationary
Enzyme activity
[nmole min-1 (mg protein)-1]
300
250
200
150
100
50
0
***
***
*
WT
pAB8
AB7
pR163L
pK167A
pY145F


b
ICDH activity
Mid log
Stationary
Enzyme activity
[nmole min-1 (mg protein)-1]
2500
2000
1500
1000
500
0
WT
pAB8
AB7
pR163L
pK167A
pY145F


c.
ICL activity
Enzyme activity
[nmole min-1 (mg protein)-1]
14
12
10
8
6
4
2
0
WT
pAB8
AB7
pR163L
pK167A
pY145F

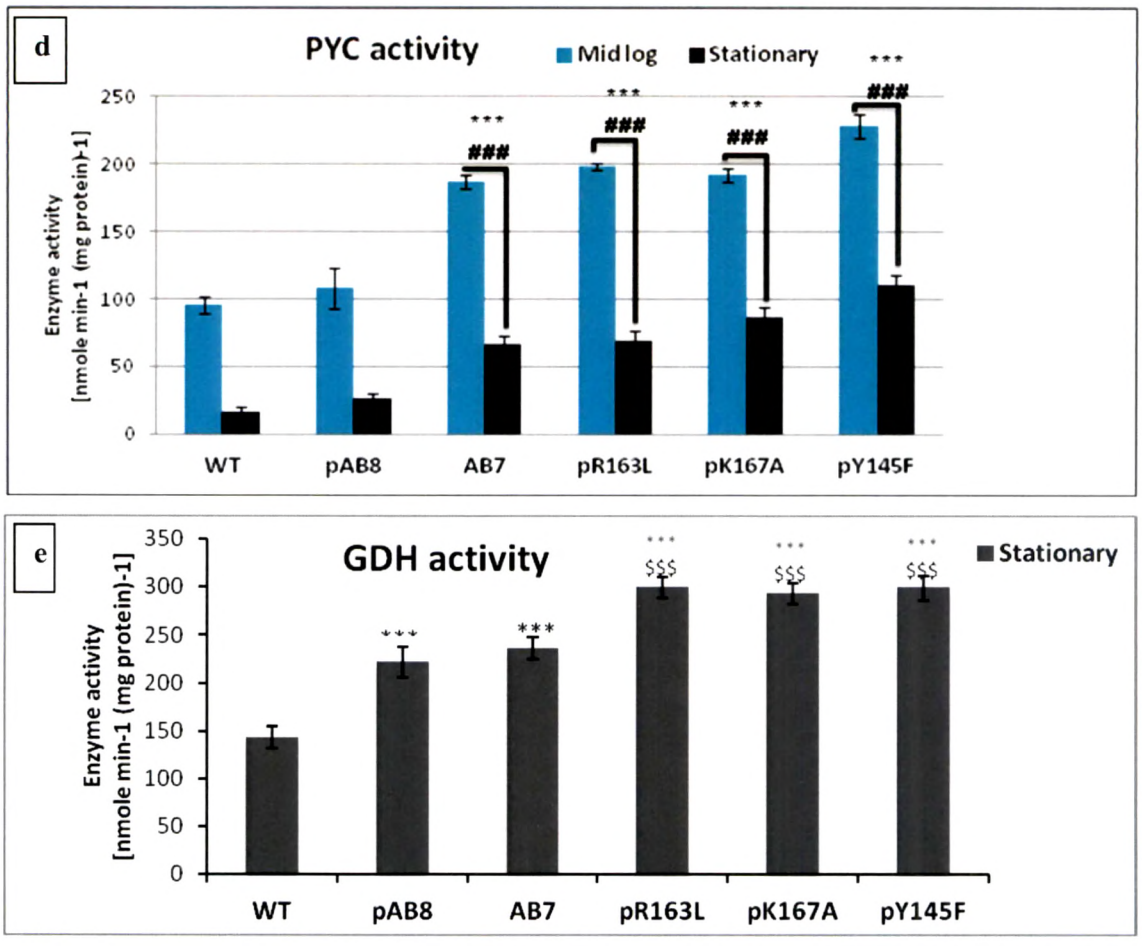


Figure 3.22: Activities of enzymes G-6-PDH (a) , ICDH (b), ICL (c), PYC (d), and GDH e) in *P. fluorescens* PfO-1 *cs* transformants. The activities have been estimated using wild type, *Pf*(pAB7), *Pf* (pAB8), *Pf* (pK16A), *Pf* (pR163L) and *Pf* (pY146F) cultures grown on M9 minimal medium with 100mM glucose. All the enzyme activities were estimated from mid log phase and stationary phase cultures except ICL and GDH which were estimated only in mid log and stationary phase respectively. All the enzyme activities are represented in the units of nmoles/min/mg total protein, . The values are depicted as Mean ± S.E.M of 4 (N=4) independent observations. *Comparison of parameters with respective Wild type; $ Comparison with respect to pAB8 and ,# comparision between parameters of pY146F with pAB7, pR163L and pK167A.***,$$$,###P<0.001; **,$$,##P<0.01; *,$,#P<0.05

3.3.5.3 Organic acid secretion

The NADH insensitve *cs* overexpression caused a significant alterations in both intracellular and extracellular citric acid lvels and yields. Intracellular citric acid levels in *Pf*(pY146F) increased by 5.7, 6.9 and 2.6 fold while there is a 5, 7.9 and 2.9 fold increase in

The NADH insensitve *cs* overexpression also caused quantitative changes in secretion of gluconic, pyruvic and acetic acid levels. Stationary phase culture supernatents of *Pf* (pY145F) showed 4.4, 3.8 and 1.6 fold increased level of gluconic acid compared to wild type strain, *Pf*(pAB8) and *Pf*(pAB7), respectively (**Fig. 3.24**). On the other hand, pyruvic acid levels in the extracellular medium decreased significantly by 2.54 , 2 and 1.4 fold while acetic acid level increased by 2.9, 2.5 and 1.46 fold, respectively, compared to the controls.

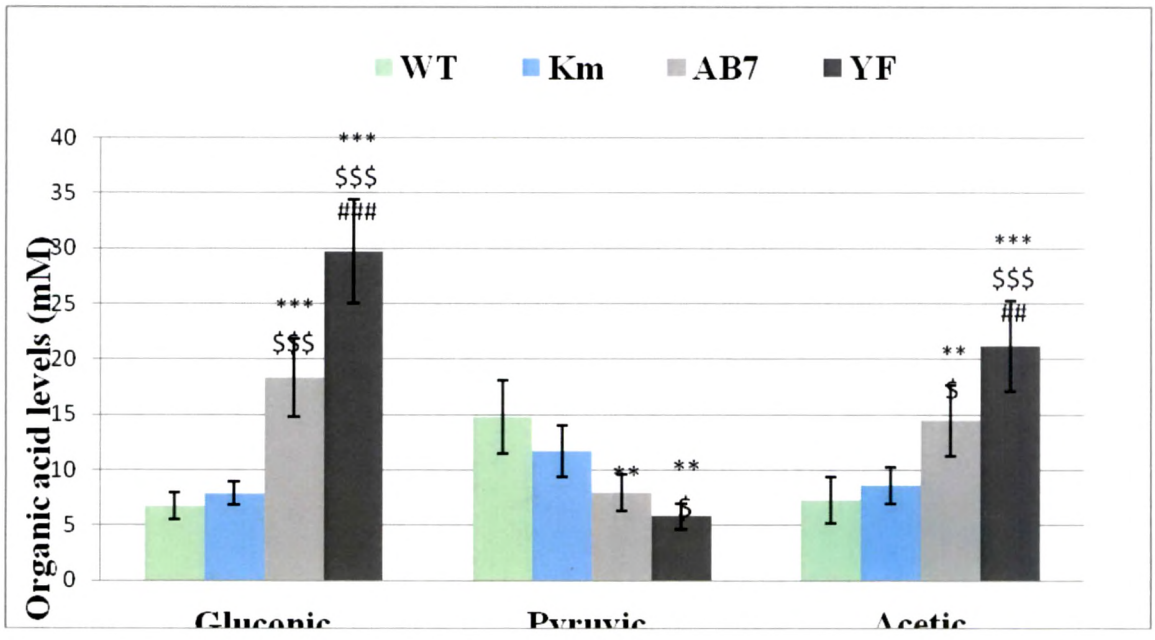


Figure 3.24:Organic acid secretion from *P. fluorescens* Pf0-1 NADH insensitive *cs* transformants. Gluconic, pyruvic and acetic acid levels in mM of Wild type, pAB8,pAB7,pYF designated as respective colours in the graph. Parameters estimated from stationary phase cultures grown on M9 medium with 100mM glucose.Results are expressed as Mean ±S.E.M of 4 independent observations. * comparision of parameters with wilt type control; $ comparision of parameters with vector control pAB8,# comparision between parameters of AB7 and YF.***,$$$,###P<0.001; **,$$,##P<0.01; *,$,#P<0.05

3.3.6 Effect of NADH insensitive *E. coli* cs overexpression on growth, biomass and glucose utilization of *P. fluorescens* PfO-1

In the presence of excess glucose, increase in *CS* activity did not significantly affect the growth profile and acidification of the medium within 30 h. However, there is a significant enhancement of pH drop in case of Y146F compared to wild type *cs* bearing strain and other control strain. Specific growth rate, specific total glucose utilization rate and total amount of glucose utilized after 30h remained unaffected. The amount of glucose consumed intracellularly is reduced by 1.49 fold in *Pf* (pY146F) as compared to *Pf* (pAB8). The increase in *CS* activity in *Pf*(pY146F) strain improved the biomass yield by 3.2, 2.38 and 2 fold compared to WT, *Pf* (pAB8) and *Pf* (pAB7), respectively (**Table 3.5, Fig. 3.25**).

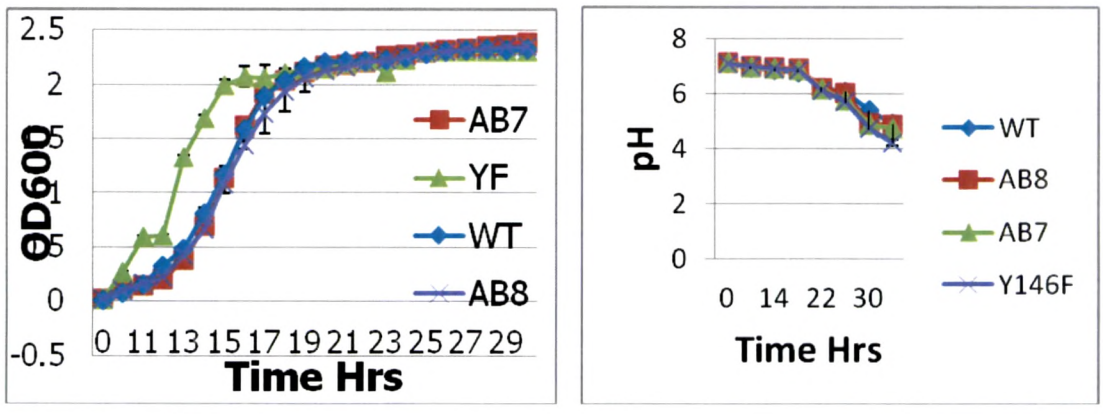


Figure 3.25: Growth and pH profiles of *P. fluorescens* PfO-1 *cs* transformants on M9 minimal medium with 100mM glucose. The values plotted represent the Mean±S.D of 4-6 independent observations.

Table 3.5: Physiological variables and metabolic data from *P. fluorescens* Pf0-1 *cs* transformants grown on M9 medium with 100mM glucose

Bacterial strain	Sp. Growth rate $\mu(h^{-1})^{\dagger}$	Total glucose utilized(mM)‡	Glucose consumed (mM) ‡	Biomass yield Ydcw/Glc† ($g\ g^{-1}$)	Sp. Glucose utilization rate QGlc† [g (g dcw)$^{-1}$ h^{-1}]
WT	0.42±.03	50.45±7.08	45.94±6.62	0.12±0.02	4.5±0.55
Pf (pAB8)	0.59 ± 0.04	69.88 ± 9.4	62.22 ± 8.5	0.16 ± 0.03	7.20 ± 1.3

Pf(pAB7)	0.65 ± 0.04	65.86 ± 4.86	48.6 ± 3.57	0.19± 0.04	6.35 ± 1.56
Pf(pY145F)	0.61±0.06	71.46±6.7	41.71±5.75$$$	0.38±0.01$$$	6.8±1.03

. The results are expressed as Mean ± S.E.M of readings from 4-6 independent observations. † Biomass yield (Ydcw/Glc), specific growth rate (μ) and specific glucose consumption rate (QGlc) were determined from the mid-log phase of each experiment. ‡ Total glucose consumed and glucose utilized were determined at the time of pH drop (30h) for *Pf* (Y146F), *Pf* (pAB7) and *Pf* (pAB8) *Comparison of parameters with respective wild type; $ Comparison of parameters with *Pf* (pAB8) and # comparision between parameters of pY146F and pAB7. .***,$$$,###P<0.001; **,$$,##P<0.01; *,$,#P<0.05

3.4 DISCUSSION

Present study demonstrates the effect of overexpression of NADH insensitive *E. coli cs* in metabolically distinct *P. fluorescens* PfO-1. Three *E. coli* NADH insensitive *cs* R163L, K167A and Y145F when constitutively overexpressed under *lac* promoter in *P. fluorescens* PfO-1, a maximum of 5.6 fold and 2 fold overexpression was obtained in *Pf* (Y145F) as compared to the control and strain bearing the wild type *cs* gene. Our data is supported by a study of Duckworth et al., (2003) which showed maximum weakening of NADH binding in case of Y145F. The study carried out in *E. coli* K12 strain showed Ki value of 790 ±210 μM for Y145F as against wild type *cs* with a Ki value of 2.8 ±0.4 μM for 100 percent inhibition by NADH (**Table.3.2**) which clearly indicates weakening of NADH binding to the active site and enhancement of *CS* activity.

In our earlier work when *E. coli* wild type *cs* gene was constitutively overexpressed under *lac* promoter in *P. fluorescens* ATCC 13525 a 2 fold enhanced activity was observed compared to the control strain (Buch et al., 2009). In the present study, as a consequence of 5.6 fold increase in CS activity in *Pf* (pY145F) there is a 6.9 fold elevated intracellular citrate level which is accompanied by 51.6 and 29.6 fold enhancement of extracellular citrate levels and yields. Remarkably the high citrate accumulation in *Pf* (pY145F) had no effect on growth. This pattern of accumulation of intracellular citric acid was similar to *P. fluorescens* ATCC13525 overexpressing *E. coli* wild type *cs* gene in which 2 fold increase in CS activity lead to 2 fold elevated intracellular citrate level and 26 fold enhancement of

extracellular citrate yield (Buch et al., 2009). In another study *icd* mutant of *E. coli* K and B strains resulted in an increase of ~3.8 and 2.5 fold CS activity and enhanced citrate accumulation but unlike our study, in this case citrate accumulation had a negative effect on growth of the *E. coli* strains (Aoshima et al., 2003). Overexpression of mitochondrial CS genes also resulted in increased citrate efflux in cultured carrot cells (Koyamaet al. 1999), Arabidopsis (Koyama et al. 2000), and canola (Anoop et al. 2003) plants

Increase in intracellular citrate level and yield by 1.9 and 2.39 fold, respectively, in *Pf* (pY145F) compared to *Pf* (pAB7) does not lead to similar increase in extracellular citrate levels. Citric acid being the substrate of central carbon metabolism must be transported into and out of the cell for efficient bioactivity. Therefore low level of extracellular citrate can be attributed to weak efflux transport mechanism in *P. fluorescens*. The transport of citric acid in gram negative bacteria is mediated by hydroxycarboxylate transporters which are a family of secondary transporter. These transporters are either H^+ or Na^+ symporters or they catalyze exchange between two substrates. (Lolkema and Sobczak, 2005). From database it is clear that the citrate transport in fluorescent pseudomonads is H^+ dependent. The low efficiency of the native citrate transport can be attributed to the low intracellular proton concentration compared to the outer membrane proton concentration. The active transport system for citrate excretion also appears to be the main rate-determining factor in citrate overproduction by yeasts (Anastassiadis and Rehm, 2005) while in addition to citrate export, transport of sugar and ammonia into the cell are also crucial for citric acid production by *A. niger* (Papagianni, 2007).

Increased gluconic acid levels with simultaneous reduction in pyruvic acid levels could be explained by increased PYC activity in *Pf* (pY145F), which could probably divert pyruvate flux towards increased OAA biosynthesis to meet the increased CS activity. Even in *A. niger* the enhancement of anaplerotic reactions replenishing TCA cycle intermediates predisposes the cells to form high amounts of citric acid (Legisa and Mattey, 2007). However, *cs* overexpression in *A. niger* did not affect PYC activity (Ruijter et al., 2000). Enhancement of biosynthetic reactions due to shortage of TCA cycle intermediates was also observed in citric acid accumulating *E. coli* K and B strains in the form of increased glyoxylate pathway (Aoshima et al., 2003; Kabir and Shimizu, 2004). However, similar

increase in flux through glyoxylate shunt was not apparent in *Pf* (pY145F) as evident from very low and unaltered ICL activity detected in *Pf* (pAB8) and *Pf* (pY145F). Low ICL activity was consistent with earlier reports in *P. fluorescens* ATCC13525 and *P. indigofera* in which ICL contributed negligibly to glucose metabolism (Buch et al., 2009; Diaz-Perez et al., 2007).

The phosphoenolpyruvate carboxylase reaction and the glyoxylate shunt are utilized for the supply of oxaloacetate to the TCA cycle. The glyoxylate shunt contributes to supplying oxaloacetate via glyoxylate, succinate, fumarate and malate by using isocitrate in the TCA cycle and acetyl-CoA which is produced by acetate catabolism. Our experimental results using the *cs* overexpression strain suggest that *P. fluorescens* PfO-1 might use the phosphoenolpyruvate carboxylase rather than the glyoxylate shunt reaction for maintaining intracellular oxaloacetate levels to adapt to the higher OAA demand which is supported by the increase in acetate production in *Pf* (pY145F) compared to the control. Enhanced CS activity in *Pf* (PY145F) also increased the periplasmic glucose oxidation which is reflected by increase in GDH activity and gluconic acid production. Moreover, significant decrease in glucose consumption without affecting the glucose utilization suggested the involvement of direct oxidation pathway for carbon flux distribution in *P. fluorescens.* The increased carbon flow through glycolysis led to increased protein synthesis that is reflected to increased biomass.The citrate induced oligosaccharide synthesis was reported in *Agrobacterium* sp. ATCC 31749 (Ruffing et al., 2011)

The central carbon metabolism network gets to the heterologous overexpression of NADH insensitive *E. coli cs* in *P. fluorescens* PfO-1 (**Fig. 3.26**). The conditions created in the present work include: improvement of glucose uptake, improvement of CS activity and citrate production compared to the earlier report by Buch et al. (2003), suppression of pyruvate secretion and enhanced acetate production, increased direct oxidation of glucose leading to more gluconate production.

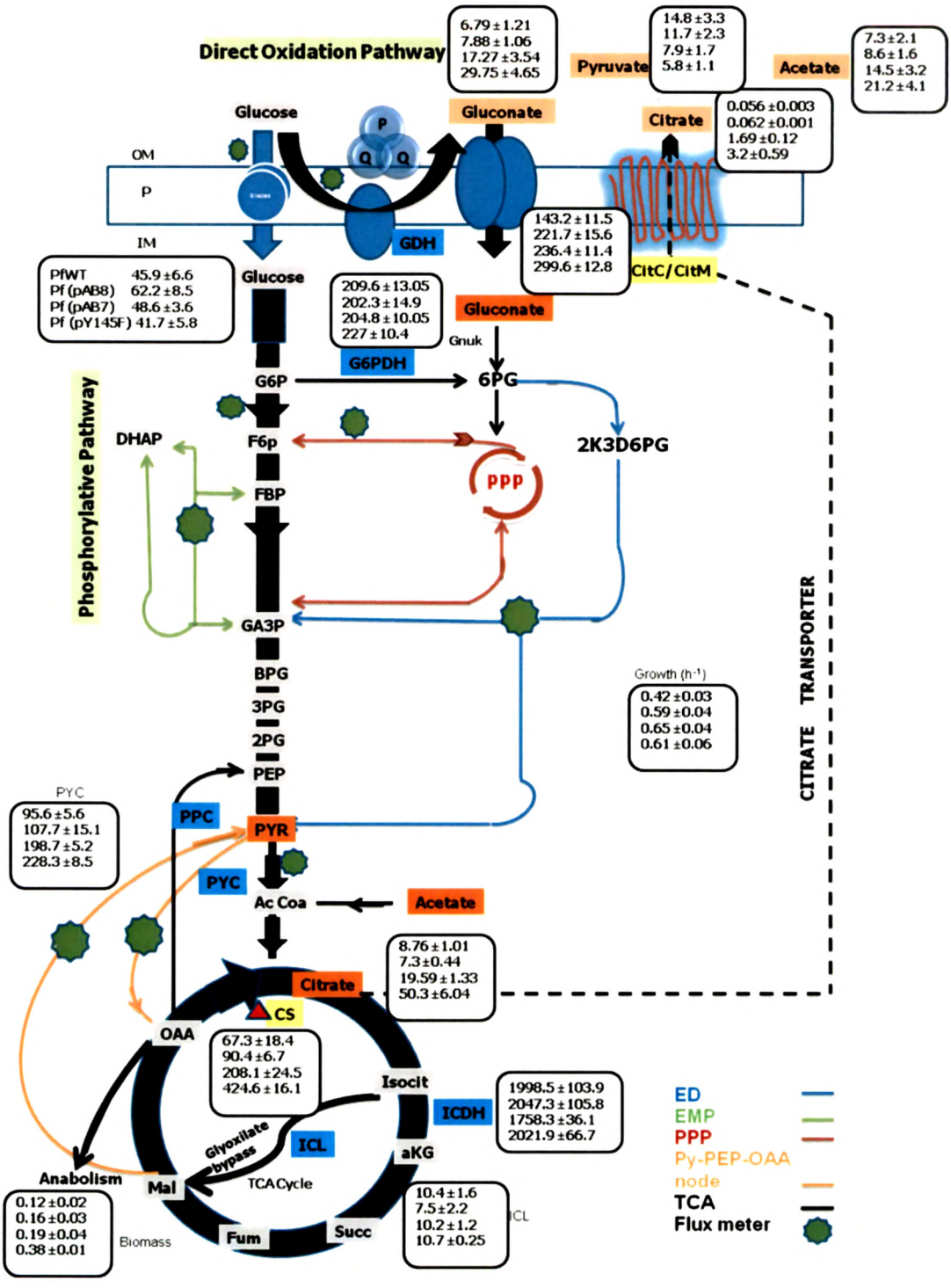


Figure 3.26: Key metabolic fluctuations in *P. fluorescens* PfO-1 overexpressing NADH insensitive *E. coli* CS