CHAPTER 4

Characterization of MPS ability of *E. asburiae* PSI3 containing periplasmic invertases.

Chapter 4 *Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.*

4.1 Introduction

Rhizospheric microorganisms possessing the ability to solubilize the bound phosphate have been considered agriculturally important (Gyaneshwar et al., 2002; Khan et al., 2006). Most PSMs poorly solubilize bound soil phosphate by the acidification of the soil through secretion of organic acids (Khan et al., 2010, Archana et al 2012). Organic acids released in the soil decrease pH which leads to P release. Chelation properties of organic acid are also involved in the P release from soil. PSM secreting gluconic acid or 2-ketogluconic acid has been well characterized and these acids have been established as basis of mineral phosphate solubilizing (MPS) phenotype by gene cloning (Goldstein and Liu, 1987; Liu et al., 1992; Babu-Khan et al., 1995; Kim et al., 1997, 2003) as well as gene mutation (Gyaneshwar et al 1998). MPS mediated by Gram negative rhizobacteria has been extensively studied using glucose as a carbon source (Goldstein, 1995). Gluconic acid is the prominent organic acid produced by direct oxidation pathway via membrane bound quinoprotein dependent glucose dehydrogenase (GDH).GDH belongs to largest group of quinoproteins that requires cofactor 2,7,9-tricarboxyl-1-H-pyrollo-(2,3)-quinoline-4,5-dione (PQQ). Enzyme located on the surface of cytoplasmic membrane, which involves in first step of direct oxidation pathway in conversion of glucose to gluconic acid (Goldstein et al., 1993). Oxidation of aldose sugars contributes electrons directly to the respiratory electron transport system mediated by periplasmic GDH.

It is known that GDH mediated periplasmic oxidation of aldose sugars can contribute electrons directly to the respiratory electron transport pathway (Goldstein, 1995). The quinoprotein GDH controls the unique step in direct oxidation, where it

transfers electrons from aldose sugars to electron transport chain via two electrons, two proton oxidations mediated by the cofactor PQQ. In addition, protons generated from these oxidations can contribute directly tothe transmembrane proton motive force. GDH is an enzyme with diverse functions, one of which is its role in mineral phosphate solubilization owing to its ability in the production of organic acid such gluconic and 2-ketogluconic by direct oxidation of a wide range of aldosugars.GDH enzyme known to exhibit broad substrate in many organisms. GDH enzyme mechanism and properties are well characterized in various organisms (Goodwin and Anthony, 1998; Elias et al.,2004). Substrate range characterization of GDH has been reported in *E. asburiae* PSI3 (Sharma et al.,2005) and *Citrobacter* DHRSS (Patel et al., 2008).

Root exudates known to secrete substantial amount of reduced carbon compounds in their rhizosphere. Root exudates are utilized by the microorganism as their major carbon source which is responsible for root colonization. Apart from glucose and fructose, many different carbon sources were found in root exudates and sucrose is one of the most abundant sugar (Jeager et al., 1999). Sucrose has been detected in large amounts in the soil near the root tip and large numbers of bacteria occur near the root area, with the highest sucrose and tryptophan exudation. Cowpea root exudates also contain arabinose, ribose, glucose, and sucrose as the main constituents. Glucose and fructose were the major components in all growth stages of stonewool-grown tomato (Kamilova et al., 2006). Thus rhizobacteria demonstrating MPS ability using sucrose and fructose as carbon sources for P solubilization could be very effective in field conditions. For such sugars which are not glucose dehydrogenase (GDH) substrates, the organic acid secreted is of interest since organic

acids other than aldonic acids are expected. *Citrobactor* DHRSS uses sucrose as carbon source due to the presence of cytoplasmic invertase and solubilizes rock phosphatein buffered condition by secreting~49 mM acetic acid (Patel et al.,2008). Alternatively, presence of periplasmic invertase can produce glucose by hydrolyzing sucrose which be further used by glucose dehydrogenase to form gluconic acid. Hence, the present study investigates the potential of periplasmic invertases in conferring MPS ability of *E. asburiae* PSI3 on sucrose.

Chapter 4 *Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.*

4.2 Material and Methods

4.2.1 Bacterial strains, plasmids and growth condition

Table 4.1 bacterial strains and plasmid used in this study

Bacterial Strains	Genotype	Reference
E. coli JM109	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) e14- [F' traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17($r_K^- m_K^+$)*	Promega
E. coli DH5α	F$^-$*endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17*(r_K^- m_K^+), λ–	Sambrook 2001
E. coli DH10B	F$^-$*endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC)* λ$^-$	Invitrogen
E. coli S17.1	*thi pro hsdR recA RP4-2* (Tet::Mu) (Km::Tn7); Tmp^r	Simon et al., 1983
E. coli BL 21 (DE3)	F$^-$*ompT gal dcm lon hsdS$_B$(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	Sambrook 2001
E. asburiae PSI3	*Cajanus cajan*rhizosphere isolate	Gyaneshwar et al 1998
Plasmids		
pTTQ18	pUC18 *ori*, Amp^r , *tac*	Stark 1987

pTTQGm^r	pUC18 *ori*, Amp^r , Gm^r, *tac*	This study
pET22b(+)	T7 promoter, *pel*B, Amp^r	Novagen
pMal-p2	Amp^r,*tac, malE,*	NEB
pBluescript KS(+)	Amp^r	
pUZE3	Amp^r , *lac*, pUC ori, Zymomonas mobilis *invB* gene	Yanasi et al 1998
pRT23.04	pGEM-2 origin, Amp^r, *S.cerevisiae* invertase gene *suc*2	Roitsch ' and Lehale ,1988
pCNK1	pBluescript KS (+), Amp^r, *Anabaena* neutral invertase gene *invB*	This study
pCNK2	pJET2.1, Amp^r, constitutive *tac*, *Anabaena* neutral invertase gene *invB* with MBP signal sequence	This study
pCNK3	pET22b(+), Amp^r, *Zymomonas mobilis* invertase gene *invB*	This study
pCNK4	pBBR1MCS-2, Kan^r, *pelB-invB,*	This study
pCNK5	pTTQGm^r, Amp^r, Gm^r, *tac*, *suc*2	This study

4.2.2 Media and culture condition

E. coli, and *E. asburiae* PSI3 were routinely grown on Luria- Bertani (LB). For growth of plasmid bearing strains media were supplemented with kanamycin at 25 mg ml^{-1},Erythromycin (20 mg ml^{-1}) or ampicillin (50 mg ml^{-1}) (Sambrook and Russell, 2001).Antibiotic concentration was reduced to 1/4$_{th}$ concentration when grown in minimal media.

4.2.3 Description of plasmid used in this study

pTTQ18

pTTQ18 is a pUC18 based plasmid containing unique multiple cloning site andprovides direct selection of recombinant plasmids based on blue-white selection strategy. *LacZ*α gene in pTTQ18 is under the *tac* promoter which is strong and tightly regulated promoter in *Enterobactericeae* family (**Fig.**) (Stark, 1987).

pUZE3 and pRT23.04

pUZE3 contains extracellular invertase gene fragment of *Zymomonas mobilis* under lac promoter of pUC118 (Yanasi et al., 2002). pRT23.04 contains 6 kb genomic DNA fragment of *Saccharomyces cerevisiae*which consist of*suc*2 gene fragment in pGEM vector.

pET 22b (+)

pET 22b (+) is a periplasmic expression vector containing *pel* B signal sequence to import fusion protein in periplasm upstream of multiple cloning sites under control of T7 promoter.

pBBR1MCS-2

pBBR1MCS-2 is broad host range vector contains *Bordetella* origin of replication and kanamycine selection marker and unique multiple cloning sites under control of *lac* promoter. Direct selection of cloned gene based on blue white selection.

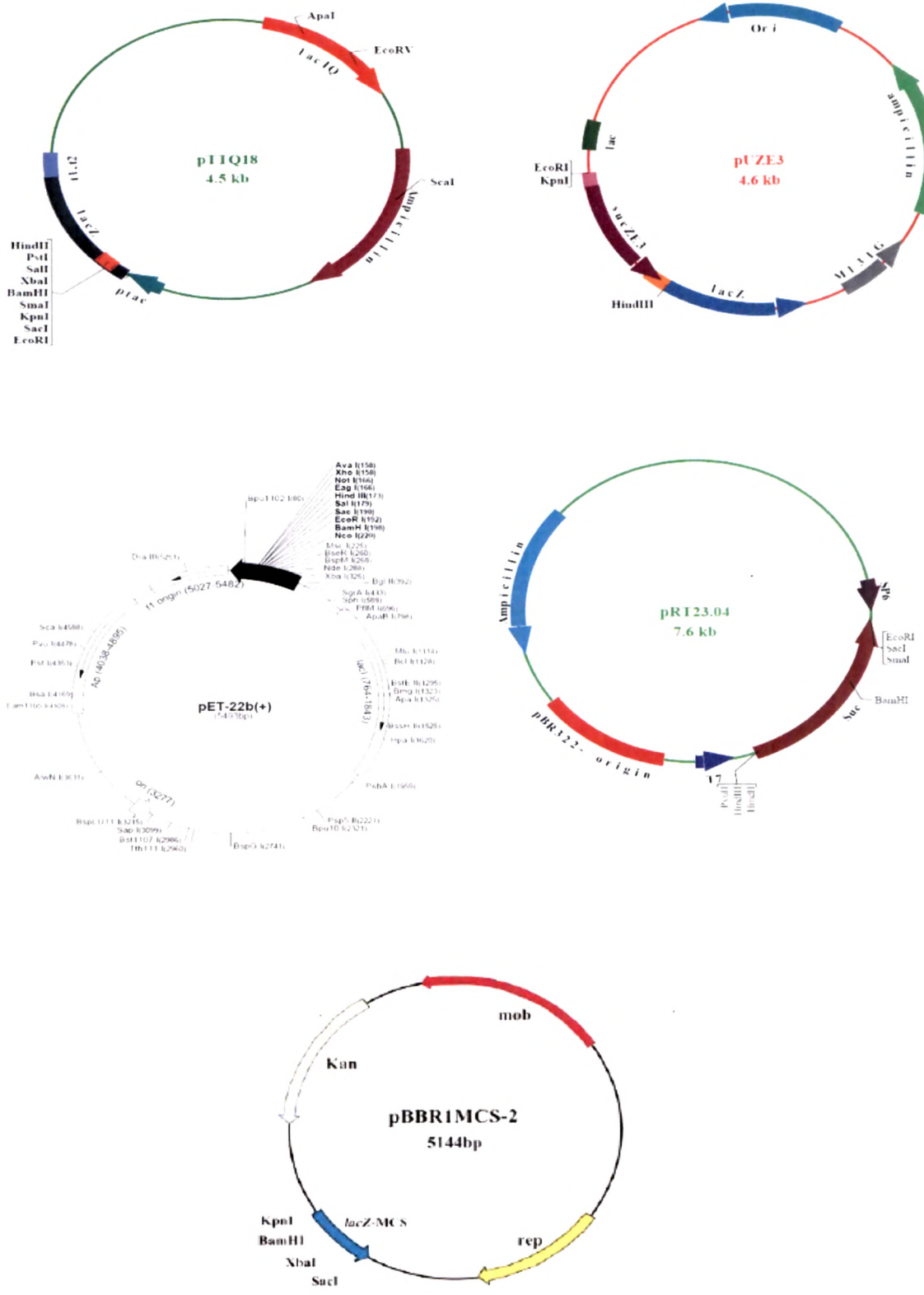


Fig. 4.1 Map of plasmids used in study

4.2.4 Cloning of*Anabaena* neutral invertase (*invB*) in pBluescript KS (+)

Sequence of Neutral invertase (*invB*) gene was obtained from gene bank (Accession No. AJ311089)to design gene specific forward and reverse primers. The template genomic DNA of *Anabaena* PCC 7120 was obtained from Prof. S.K. Apte (BARC, Mumbai).Neutral invertase (*inv*B) from *Anabaena* PCC 7120 genomic DNA was PCR amplified using *Pfu* DNA polymerase, gene specific forward primer and reverse primers. *Bam*HI and *Hind*III RE sites were incorporated in forward and reverse primers, respectively. The PCR conditions were: initial denaturation at 94^0C for 1 min followed 30 cycles each consisting 94^0C for 15 s, 62^0C for 30 s, 72^0C for 90 s and followed by final extension at 72^0C for 5 min. The amplified ~1.45 kb PCR product was purified using Pure Link gel extraction kit (Invitrogen, USA), blunt ended cloned in *Eco*RV site of pBluescript KS(+)with the help of T4 DNA ligase and transformed in *E. coli* JM109 competent cells.

Screening of positive transformant was done by blue white screening on LA plates containing 50 µg/ml Ampicillin and 25µl X-gal (40mg/ml). White colonies were picked and further confirmed by RE digestions.

4.2.5 Construction of pCNK2 to express neutral invertase (*invB*) in periplasm under the control of constitutive *tac* promoter

Periplasmic signal sequence of maltose binding protein (*mal*E) gene was PCR amplified from pMal-p2 plasmid with the help of gene specific primer. *mal*E signal sequence was PCR amplified using gene specific forward primer 5'*GCGGAATCGATTTTCACACAGGAAACAGCGA*TG AAAATAAAAACAGGTGC 3' and reverse primer MBP RP5' GCTTCTGCATTTCGATTTTG 3'. The PCR conditions were: initial denaturation at 94^0C for 1 min followed 30 cycles each

consisting 94^{0}C for 15 s, 52^{0}C for 15 s, 72^{0}C for 30 s and followed by final extention at 72^{0}C for 5 min. To make gene expression under control of *tac* constitutive promoter second PCR was performed with

Forward primer

Tac15'TCGTATAATGGATCGAATTGTGAGCGGAATCGATTTTCACACAGGA AA3' Reverse primer MBP RP

5' GCTTCTGCATTTCGATTTTG 3'.The PCR conditions were: initial denaturation at 94^{0}C for 1 min followed 30 cycles each consisting 94^{0}C for 15 s, 55^{0}C for 15 s, 72^{0}C for 30 s and followed by final extention at 72^{0}C for 5 min. PCR amplicon of ~200bp was obtained. Neutral invertase was PCR amplified using

Forward primer INV FP 5' CAAAATCGAAATGCAGAAGC 3'

Reverse primer INV RP 5' CCCAAGCTTGGGAAAAATTTATCGAGATA 3'

and pCNK1 as template which gives ~1.45 kb PCR amplicon. The PCR conditions were: initial denaturation at 94^{0}C for 1 min followed 30 cycles each consisting 94^{0}C for 15 s, 55^{0}C for 30 s, 72^{0}C for 90 s and followed by final extention at 72^{0}C for 5 min. Finally both the PCR product were join by doing recombinant PCR using Tac1 as forward primer and INV RPas reverse primer using equimolar concentration of both the PCR product. The PCR conditions were: initial denaturation at 94^{0}C for 1 min followed 30 cycles each consisting 94^{0}C for 15 s, 55^{0}C for 30 s, 72^{0}C for 120 s and followed by final extention at 72^{0}C for 5 min. Finally ~1.65 kb PCR amplicon was obtained and cloned in TA cloning vector pTZ57R/T (Fermantas). Screening of positive transformant was done by blue white screening on LA plates containing 50

µg/ml Ampicillin and 25µl X-gal (40mg/ml). Positive transformant were confirmed by RE digestion and PCR amplification.

4.2.6 Cloning and expression of *Zymomonas mobilis* acidic invertase in broad host range expression vector pBBR1MCS-2

Acidic invertase of *Zymomonas mobilis* is encoded by gene *invB*. 1.5 kb *suc*ZE3 was isolated from plasmid pUZE3 by *Eco*RI and *Hind*III digestion. Isolated fragment was further subcloned in the periplasmic expression vector pET 22b (+) to obtained pCNK4. pCNK4 was further digested with *Xba*I and *Hind*III which released 1.6kb fragment. 1.6 kb fragment contains periplasmic leader peptide *pel*B and *suc*ZE3. Gel eluted fragment was end filled and cloned in EcoRV digested pBBR1MCS-2 under control of *lac* promoter to obtained pCNK5. Right orientation clone was confirmed by RE's digestion.

4.2.7 Cloning and expression of *Saccharomyces cerevisiae*periplasmic invertase gene *suc2* in expression vector pTTQGm^r

Periplasmic invertase of *Saccharomyces cerevisiae* was PCR amplified by gene specific forward and reverse primers. *Suc*2 gene sequence was obtained from Gene Bank (Accession No. ID Z46921_41) and specific primers were designed from the sequence Sequences of *suc*2 primer given below

Forword primer- 5'GGAATTCC*AGGAGG*CTAACGTATATGATGCTT 3'

Reverse primer- 5' CCGCTCGAGCTCCCTCTATTTTACTTCCCTTAC 3'

Underline sequence represent restriction enzyme site *Eco*RI and *Sac*I respectively. Italic sequence showed ribosome binding site (RBS). *suc*2 gene was PCR amplified from plasmid pRT23.03 which contains periplasmic invertase genomic DNA fragment of *Saccharomyces cerevisiae*. The PCR conditions were: initial denaturation

at 94^0C for 1 min followed 30 cycles each consisting 94^0C for 15 s, 48^0C for 30 s, 72^0C for 90 s and followed by final extension at 72^0C for 5 min. PCR amplification of *suc*2 gene was done according to above program which gives 1.6 kb amplicon. PCR product and expression vector pTTQGm^r were digested with *Eco*RI and *Sac*I. Purification and ligation was done using the similar method mention above. Positive clones were confirmed by RE digestion.

4.2.8 Sucrose utilization study in *E. coli*

Sucrose utilization study was done in minimal medium containing sucrose and raffinose as a carbon source. Composition of Minimal medium used in this study has mentioned in material and methods section. *E. coli* DH5α and BL21 (DE3) transformants were used for sucrose utilization study.

4.2.9 Invertase activity

Invertase activity was performed according to protocol mention in Material and Method section.

Enzyme activity measured with induced and uninduced conditions. For induction, 0.2mM IPTG was used. One unit of invertase is defined by the amount of enzyme that catalyzes the production of 1μmol of glucose per minute under assay condition. Specific activity is defined as units per milligram of protein.

4.2.10 Glucose dehydrogenase activity

Glucose dehydrogenase activity was performed according to protocol mention in Material and Method section

4.2.11 Invertase activity staining

Detection of invertase activity on 10%SDS PAGE under semi native condition was based on Gabrial and Wang method (1969). Bacterial cell extract were prepared

by sonication or lysis by lysozyme. Periplasmic fraction was obtained by chloroform treatment (Kutsu et al 1984). Different fractions of cell free extract were suspended in loading buffer without SDS and were run on SDS-PAGE without prior heating (Laemmli, 1970). After electrophoresis, gels were briefly rinsed in distilled water and incubated in acidic sucrose solution (0.1 M sucrose, 0.1 M NaOAc pH 5.0) for 60 min at 37^0C. Following three wash steps in distilled water for 5 min, gels were subjected to 0.1M idoacetamide treatment for 5 min followed by three water wash to prevent background staining. The activity staining gel was developed by boiling in 0.5 M NaOH containing 0.1%2,3,5-triphenyltetrazoliumchloride, giving rise to pink bands at positions of invertase activity.

4.2.12 Physiological study

E. asburiae PSI3 used in this study was isolated from the rhizosphere of *Cajanus cajan* (Pigeon pea) (Gyaneshwar et al 1998). *E. asburiae* PSI3 was grown on Tris rock phosphate (TRP) minimal medium for growth, acidification and P solubilization monitoring. TRP medium used in this study consisted of 50-100mM Tris buffer pH-8.0, Glucose 50mM, Sucrose 50 and 100mM or mixture of both as and when required, 1mg/ml Rock phosphate. Senegal Rock Phosphate used in this study consist of (%) silica 5-6, P_2O_5 36-37, CaO 49-51, fluorine 3-4, insoluble acid 5-6, $CO_2$1-2, Fe_2O_3 0.6-0.8, sulphate 0.3-0.6, and Al_2O_3 0.6-0.8. Macronutrient contains (1L) NH_4Cl 5 g, KCl 5g, NaCl 2.5g, and $CaCl_2$ 15 mg and micronutrients $FeSO_4 \cdot 7H_2O$ 3.5 mg, $ZnSO_4 \cdot 7H_2O$ 0.16 mg, $CuSO_4 \cdot 5H_2O$ 0.08 mg, H_3BO_3 0.5 mg, $CaCl_2 \cdot 2H_2O$ 0.03 mg, and $MnSO_4 \cdot 4H_2O$ 0.4 mg, respectively.

E. coli and *E. asburiae* PSI3 were grown at 37^0C and 200 rpm. Aliquots were drawn at 12 hr. interval to till pH drop below 5. Cell density were measured at 600 nm

and pH monitored as indicator for medium acidification. Culture were centrifuged at 9000×g for 5 min and culture supernatant were taken to measure P release in medium (Ames, 1968).

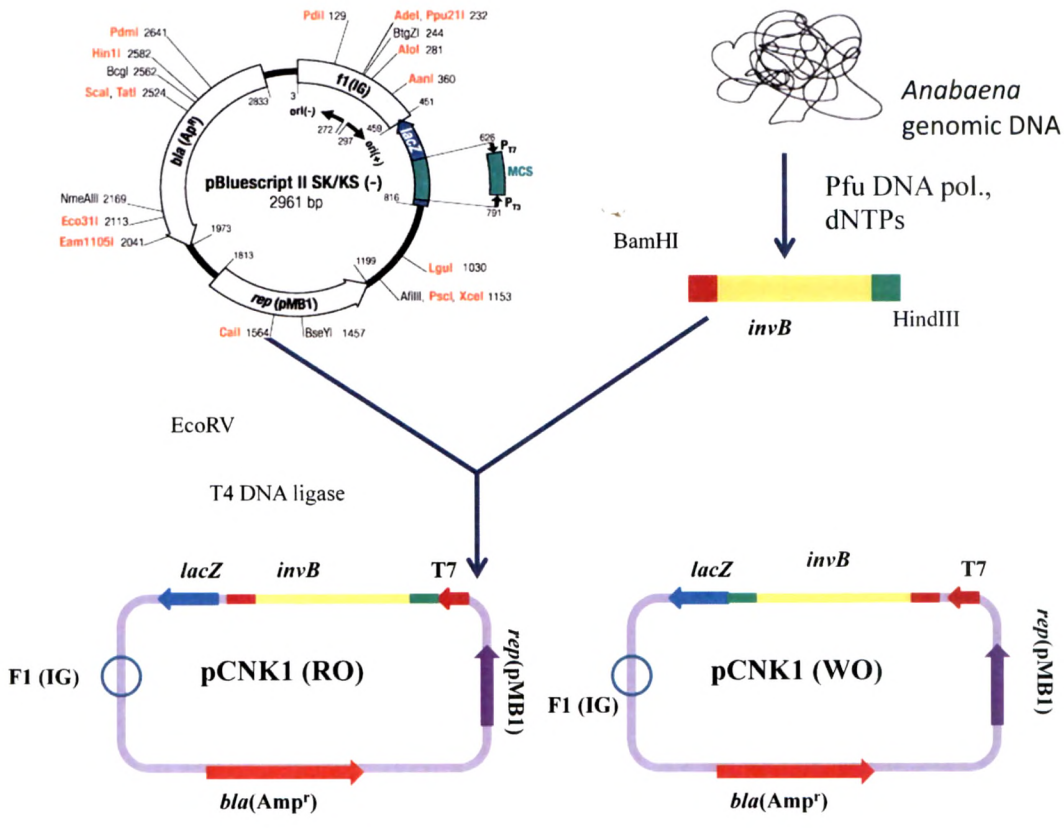
4.2.13 Analytical method

All the physiological experiments used an initial cell density of ~0.025 OD at 600nm as monitored spectrophotometrically (path length of 1cm; Shimadzu UV-1700 spectrophotometer). Growth was monitored as increase in absorbance at 600nm and pH of the medium was monitored at 12h time intervals. When pH of the medium reached below 5 sample (2 ml) were taken from flask were centrifuged at 9,200 *g* for 10 min at 4 °C and the culture supernatants were used to estimate organic acid and P release in the medium. For HPLC analysis, the culture supernatant was passed through 0.2μm nylon membranes (Pall Life Sciences, India) and the secreted organic acids were quantified using RP C-18 column on UFLC (Shimadzu corporation, Japan). The column was operated at room temperature using mobile phase of 0.02% orthophosphoric acid at a flow rate of 1.0 ml min^{-1} and the column effluents were monitored using a UV detector at 210 nm. Standards of organic acids (Sigma) were prepared in double distilled water, filtered using 0.2μm nylon membranes and were subjected to chromatography under similar conditions for determining the individual retention time. Comparison of peak area with external standards was used for quantification. The statistical analysis of all the parameters has been done using Graph Pad Prism (version 5.0) software.

4.3 Results

4.3.1 Cloning of *Anabaena* PCC 7120 neutral invertase (*inv*B) gene in cloning vector pBluescript KS(+)

*inv*B was PCR amplified from genomic DNA of *Anabaena* PCC 7120 using gene specific primers with the help of *Pfu* polymerase. ~1.45 kb PCR was blunt ended cloned in cloning vector pBluescript KS (+) under *Eco*RV RE site to obtained pCNK1 (RO) and pCNK1 (WO) (**Fig.4.1**). pCNK1 (RO) give ~4.4 kb linearize plasmid with *Bam*HI digestion but in case of pCNK1 (WO) it give ~1.45kb release of *inv*B (**Fig. 4.2**).



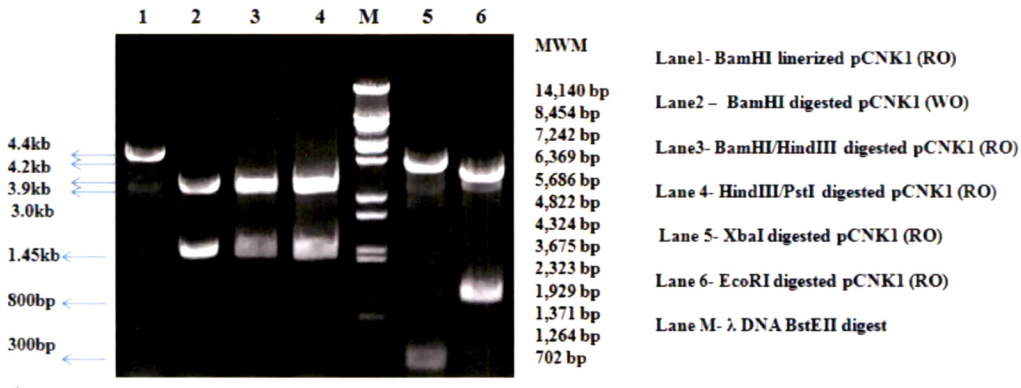


Fig. 4.2 Restriction digestion pattern of pCNK1(RO) and pCNK1 (WO)

4.3.2 Cloning and expression of PCC 7120 neutral invertase (*inv*B) with *malE* signal sequence in periplasmic under control of constitutive *tac* promoter.

135 bp signal sequence of maltose binding protein (*malE*) was PCR amplified from pMal-p2 vector with gene specific primers. To express signal sequence under control of constitutive *tac* promoter, second PCR was done with modified *tac* promoter sequence having abolish repressor binding site as forward primer and *malE* signal sequence reverse primer this give 185 bp PCR product (**Fig.4.5**). 1.4kb *inv*B gene was PCR amplified from pCNK1. Cloning of periplasmic neutral invertase under constitutive *tac* promoter (*tac**) was achieved by recombinant PCR of *tac*malE* signal sequence with neutral invertase which yielded 1.6 kb PCR product. Final PCR product was cloned in TA cloning vector. Positive clone was confirmed by RE digestion with *Sac*I and *Hind*III which release the complete insert of 1.6kb.*Eco*RI digestion gives the 500bp release due to presence of one RE site in both gene and vector. Similarly *Xba*I digestion gives 200bp release.

Functionality of pCNK2 was confirmed by checking *E. coli* (pCNK2) growth on M9 minimal medium agar plate containing sucrose as carbon source in absence of

IPTG (**Fig. 4.6**). *E. coli* does not grow in sucrose minimal medium but *E. coli* (pCNK2) possessing invertase allows the growth on sucrose as sole carbon source. Growth on sucrose in the absence of IPTG indicates constitutive expression of *tac* promoter. In liquid minimal medium, *E. coli* cells grew till O.D. 0.3 and showed cell lysis on further growth. Lethality prevented further studies in *E. asburiae* PSI3.

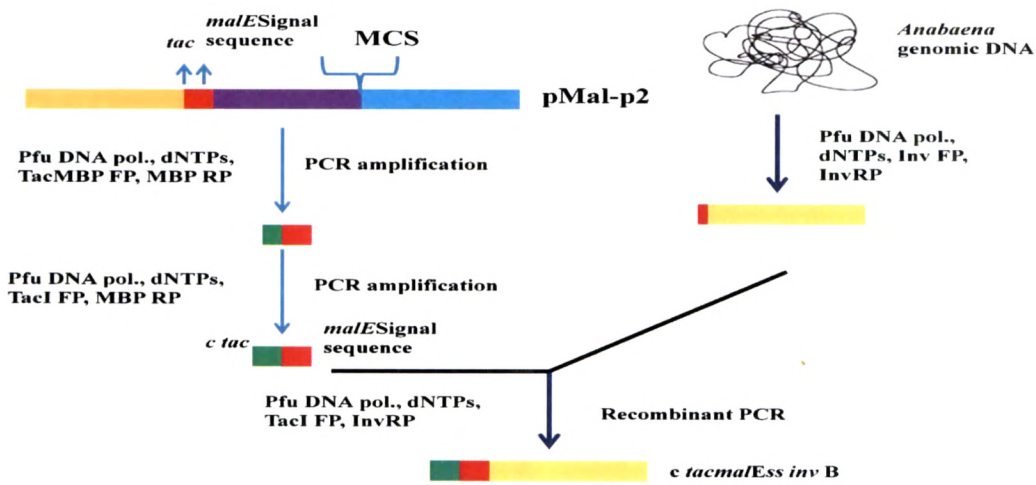


Fig. 4.4 Schematic representation of recombinant PCR strategy of *tac*SS-invB*.

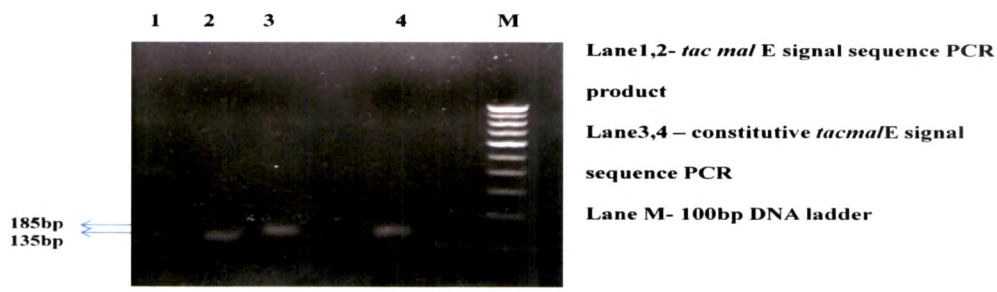


Fig. 4.5 Recombinant PCR of constitutive *tac* promoter with *malE* signal sequence.

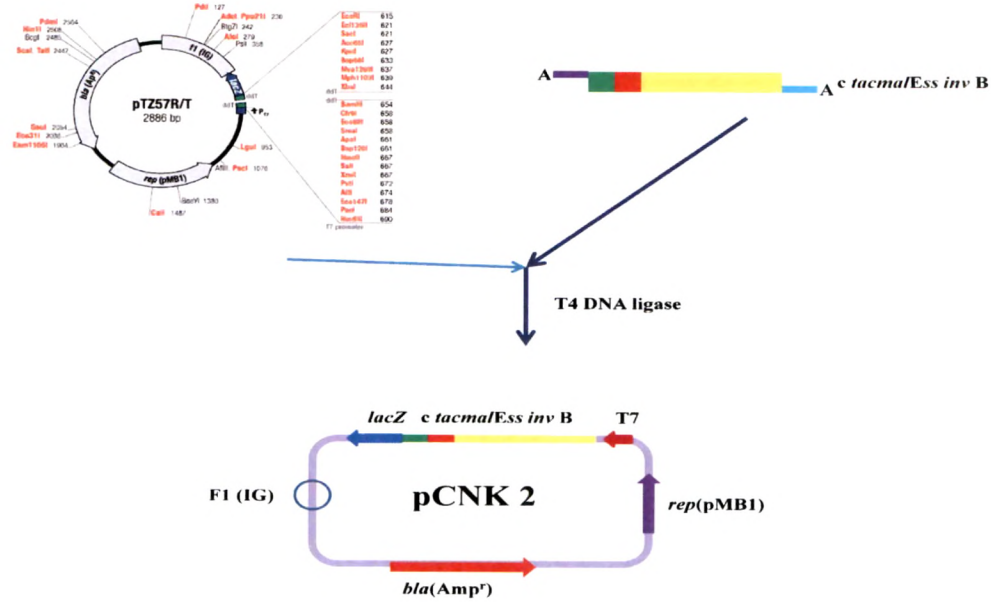


Fig. 4.6 Schematic representation of cloning strategy of pCNK2

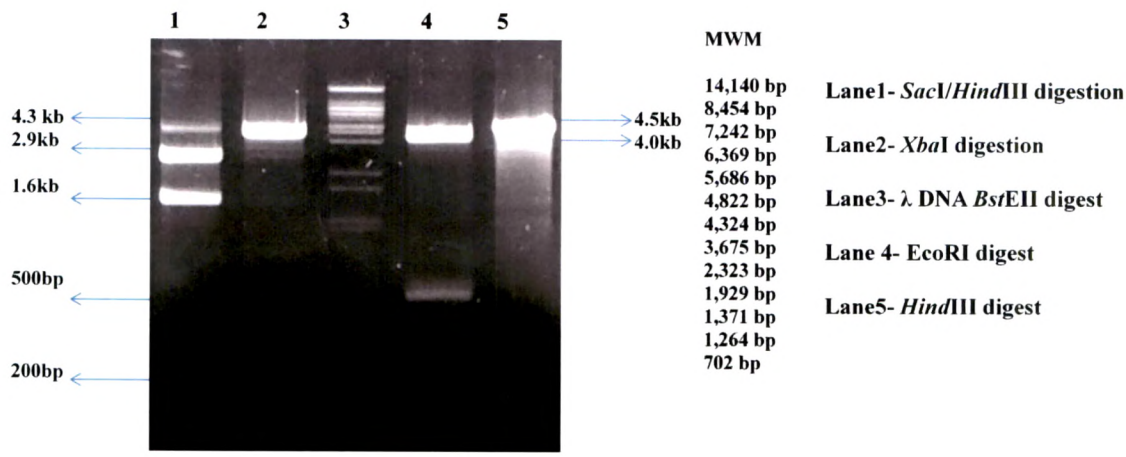


Fig. 4.7 Restriction digestion pattern of pCNK2.

4.3.3 Cloning of *inv*B with *pel*B signal sequence in pBBR1MCS-2

Plasmid pUZ3 containing *Zymomonas mobilis* extracellular invertase (*inv*B) was isolated by digestion with *Eco*RI and *Hind*III. Double digestion showed ~1.6 kb fragment containing *inv*B. *inv*B fragment was gel eluted and ligated in periplasmic expression vector pET22b(+) under *Eco*RI and *Hind*III to obtained pCNK3.

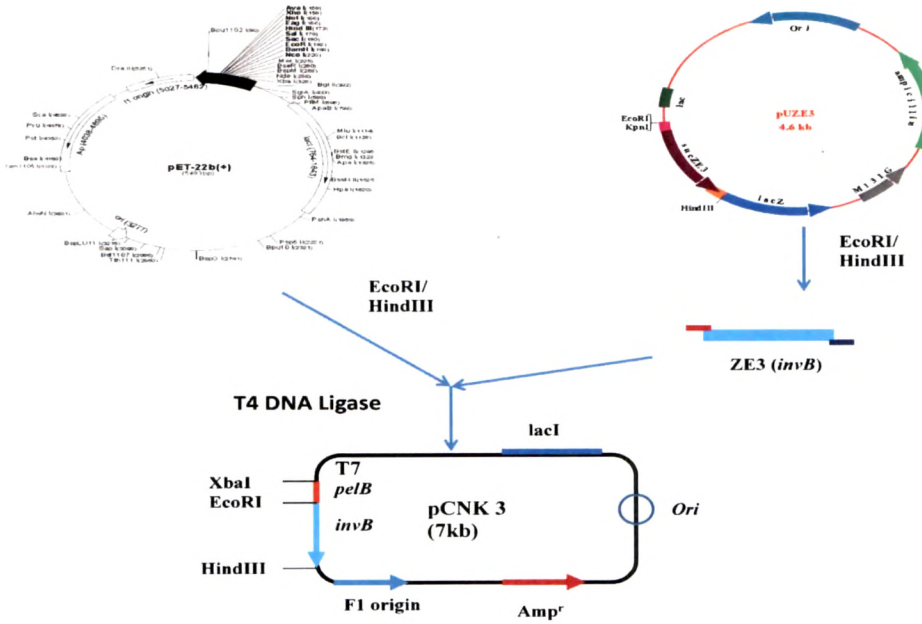
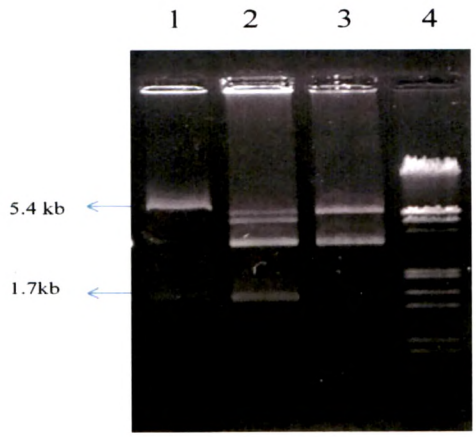


Fig. 4.8 Schematic representation of cloning strategy of pCNK3



Lane 1,2 - 1.6kb release *invB*with*pelB*by *Xba*I and *Hind*III double digestion.

Lane4- λ DNA/*EcoR*I/*Hind*III double digest

Fig. 4.9 Restriction digestion pattern of pCNK3.

*inv*B along with *pel*B sequence was isolated from pCNK3 digestion with *Xba*I and *Hind*III which gives ~1.7kb release. 1.7kb fragment was blunt ended by Klenow fragment. pBBR1MCS-2 was digested with *Eco*RV followed by gel purification. 1.7 kb fragment was cloned in pBBR1MCS-2 to obtained pCNK4. Right orientation clones were confirmed by 1.7kb release with *Xba*I digestion (**Fig. 4.11**).

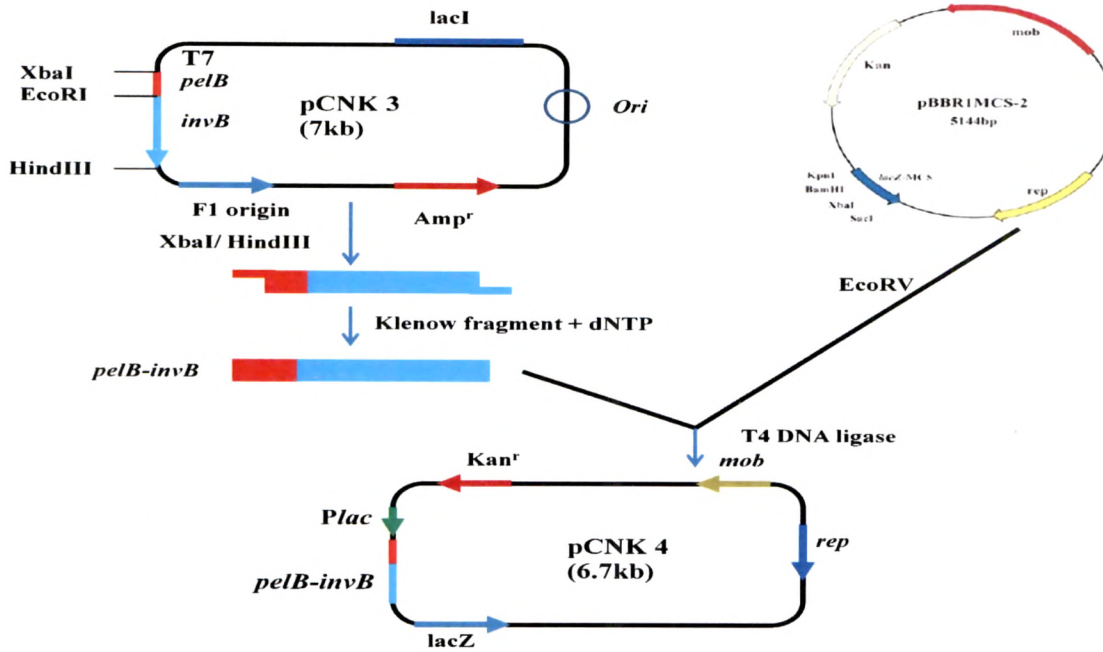


Fig.4.10 Schematic representation of cloning strategy of pCNK4.

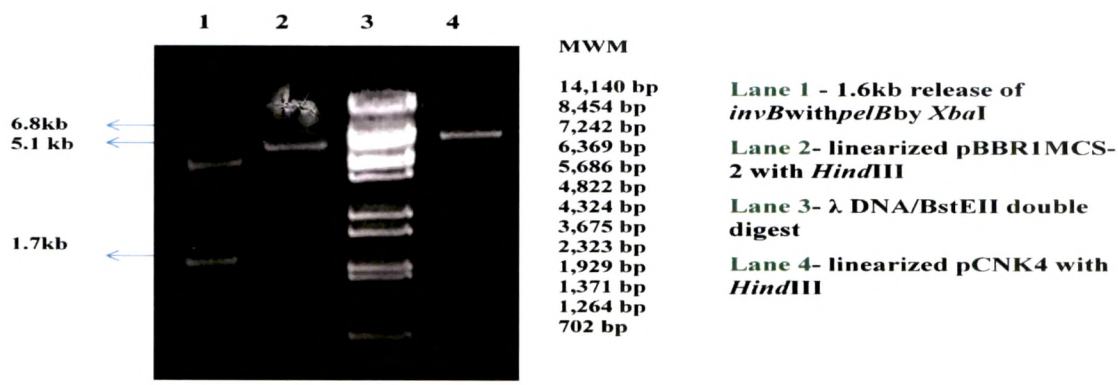


Fig. 4.11: Restriction digestion pattern of pCNK4.

4.3.4 Cloning of *S. cerevisiae* extracellular invertase *suc*2 in pTTQGm^r

1.6 kb *suc*2 was cloned in pTTQGm^r under *Eco*RI and *Sac*I. Positive clone was confirmed by different RE's digestion. XbaI digestion gives 780bp due presence of RE site in gene and vector. *Eco*RI/*Bam*HI digestion gives release of 900bp and vector backbone of5.8kb.*Bam*HI/*Hind*III double digestion gives the 1.6 kb gentamycine release, ~750 bp from *suc*2 gene and vector backbone of 5.4kb.

*Eco*RI/*Pst*I double digestion gives release of 1.6 kb *suc*2 gene and 6.1kb vector backbone (**Fig. 4.13**).

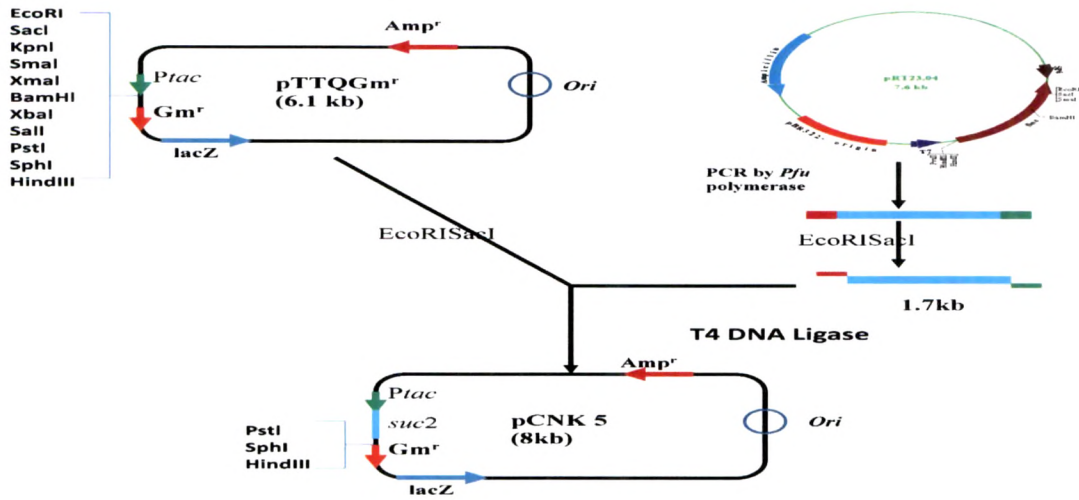


Fig.4.12 Schematic representation of cloning of *suc*2 gene in pTTQGm^r.

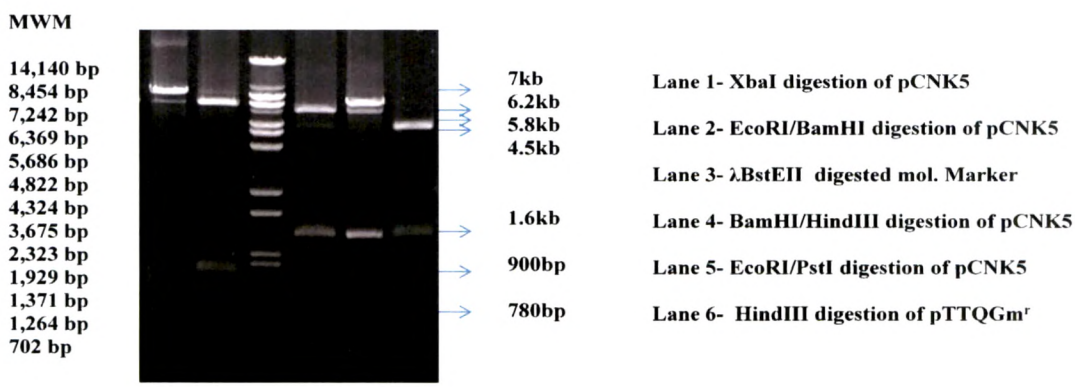


Fig. 4.13 Restriction digestion pattern of pCNK5.

4.3.5 Different substrate utilization of *E. coli* containing invertase gene.

Functionality of pCNK3 pCNK4 and pCNK5 was confirmed by checking *E. coli* (pCNK3), *E. coli* (pCNK4) and (pCNK5) growth on liquid M9 minimal medium

and agar plate containing sucrose as carbon source (**Fig.4.14**).

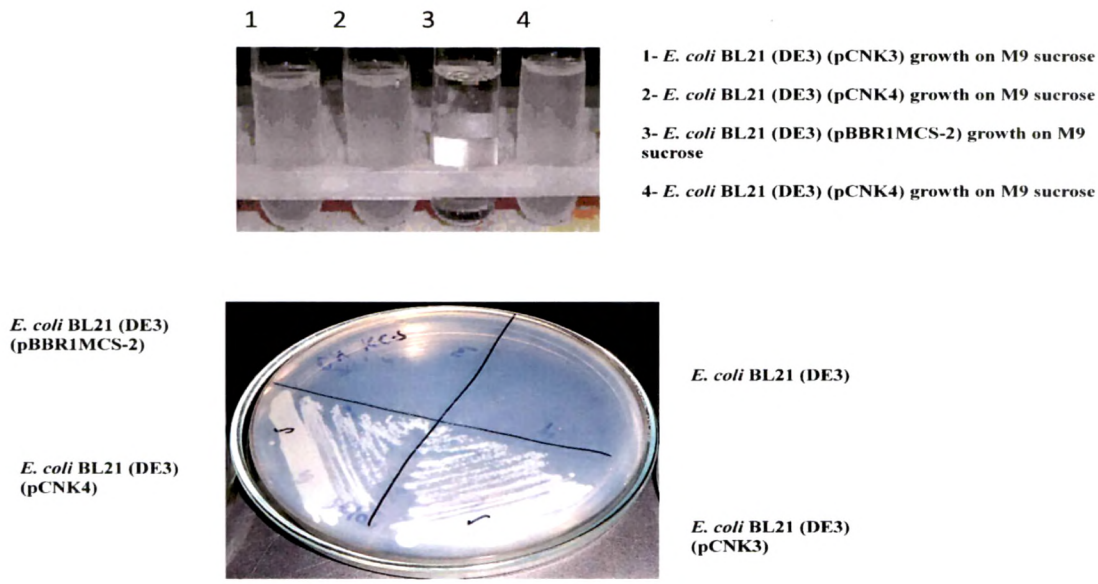


Fig. 4.14 Growth of *E. coli* transformant pCNK3 and pCNK4 on M9 minimal medium containing sucrose as carbon source.

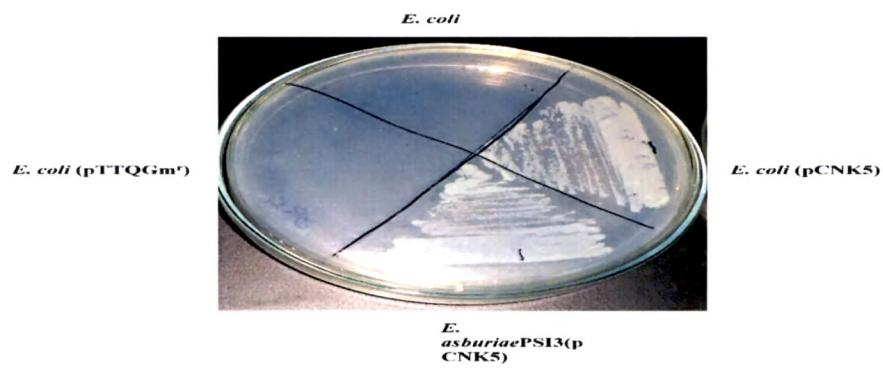
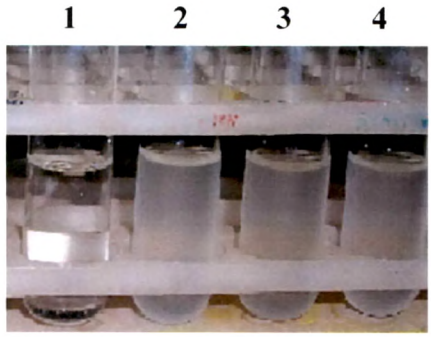


Fig. 4.15 Growth of *E. coli* (pCNK5) on M9 minimal medium agar plate with sucrose as c source.



1- *E. coli* (pTTQGm^r) growth on M9 sucrose

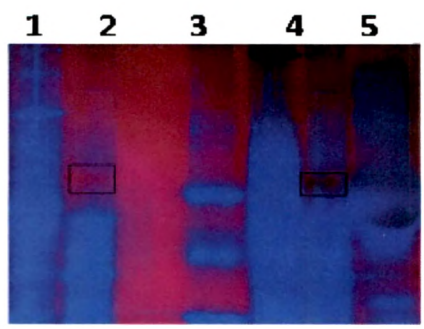
2- *E. coli* (pCNK5) growth on M9 sucrose

3- *E. coli* (pCNK5) growth on M9 raffinose

4- *E. coli* (pCNK5) growth on M9 glucose

Fig. 4.16 Utilization of different sugars by *E. coli* (pCNK5).

Organism containing invertase gene can specifically recognize sucrose as substrate but it can also utilize raffinose as substrate due to its structural similarity with sucrose. *E. coli* having invertase gene could utilize sucrose and raffinose in 20:1 ratio. Hence, the *E. coli* (pCNK5) transformant was easily grown on M9 sucrose and raffinose media as shown in (**Fig.4.16**). Positive transformants showed growth on M9 raffinose and sucrose while vector control fails to grow on these sugars. *E. coli* (pCNK5) transformant give invertase activity on semi native PAGE as expected 60 kDa band corresponding to *suc*2 (**Fig**.).



Lane 1 – IPTG induced *E. asburiae* PSI3 (pTTQGm^r)

Lane 2 and 6 – IPTG induced *E. asburiae*PSI3 (pCNK5) (60kD protein)

Lane 4- Mid Range Mol. Wt. Marker (29-100kD)

Lane 5- Uninduced *E. asburiae* PSI3 (pCNK5)

Lane 7- WT *E. asburiae* PSI3

Fig. 4.17: Invertase enzyme detection on semi native PAGE

In uninduced condition, no activity was detected in *E. coli* and *E. asburiae* PSI3 transformants. In induced condition periplasmic invertase activity of *E. a.* PSI3 (pCNK4) and *E. coli* (pCNK4) were 0.438±0.031U and 0.403±0.049 U respectively where as no activity was detected in uninduced condition. Periplasmic invertase activity in *E. coli* (pCNK5) and *E. asburiae* PSI3 (pCNK5) was 18.4±0.023 mU and 29.3±0.036 mU, respectively. GDH activity was similar in *E. asburiae* PSI3 (pCNK5) and *E. asburiae* PSI3 (pTTQGm^r).

4.3.6 Effect of overexpression of periplasmic invertases on mineral phosphate solubilization of *E. asburiae* PSI3

E. asburiae PSI3 (pCNK4) showed acidification and mineral phosphate solubilization ability on TRP plates containing 75mM sucrose and 75mM Tris buffer pH-8.0 while *E. asburiae* PSI3 and *E. asburiae* PSI3 (pBBR1MCS-2) did not show acidification and mineral phosphate solubilization ability in similar condition (**Fig.4.18**). In liquid TRP medium, growth of *E. asburiae* PSI3 (pCNK4) reached up to 0.4 O.D. and acidified the medium to pH5.8 in 96 hr (**Fig. 4.19**). Pi release was found to be 0.180± 0.041 mM. *E. asburiae* PSI3 did not secrete gluconic acid when sucrose provided as a sole carbon source thus did not show acidification of medium while *E. asburiae* PSI3 (pCNK4) produced18.65 mM gluconic acid. In case of 50 mM sucrose and 100 mM Tris (pH-8.0) buffering condition, *E. asburiae* PSI3 (pCNK5) showed growth up to 0.4 O.D. but failed to showed MPS ability. 50mM sucrose and 75 mM Tris buffer pH-8.0 its showed growth up to 0.4 O.D. and showed pH drop of medium to 4.5 where as vector control showed similar growth pattern but failed to drop pH below 7.0 (**Fig. 4.20, 4.21**). *E. asburiae* PSI3 (pCNK5) produced 22 mM gluconic acid and the Pi release was found to be 0.438+0.073 mM. When 25mM

glucose and 50mM sucrose with 100mM Tris buffered pH-8.0 TRP medium were used, *E. asburiae* PSI3 (pCNK5) showed similar growth and pH drop pattern with improved MPS ability. Pi release was 0.479+0.081 mM and produced 34mM of gluconic acid.

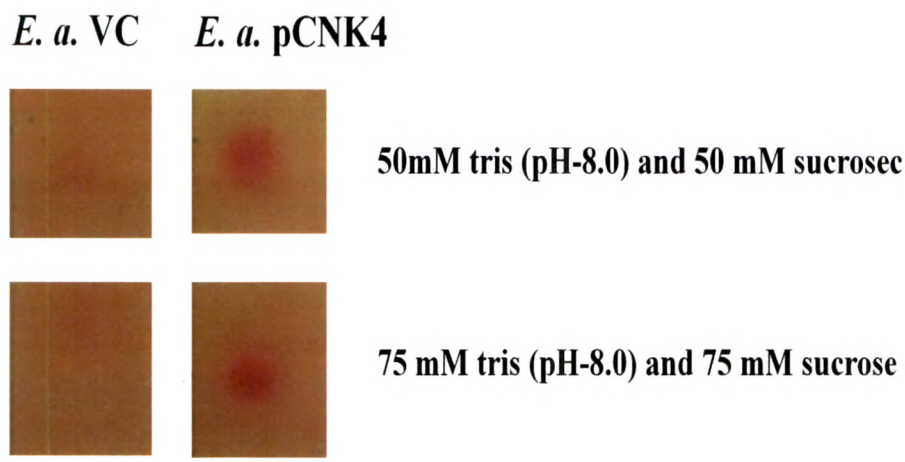


Fig.4.18: MPS ability of *E. asburiae* PSI3 VC (pTTQGm) and *E. asburiae* PSI3 (pCNK4) in TRP agar medium with sucrose as carbon source.

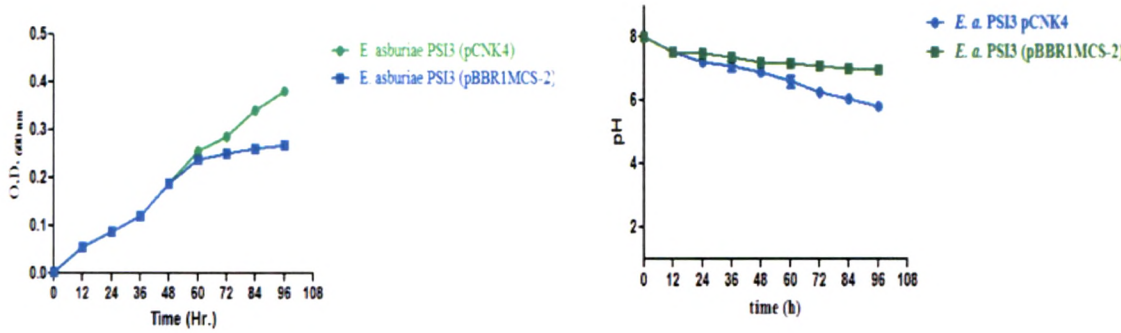


Fig.4.19: Growth and pH profile of *E. a.* PSI3 (pBBR1MCS-2) and *E. a.* PSI3 (pCNK4) in TRP containing 75mM Tris and 75mM sucrose.

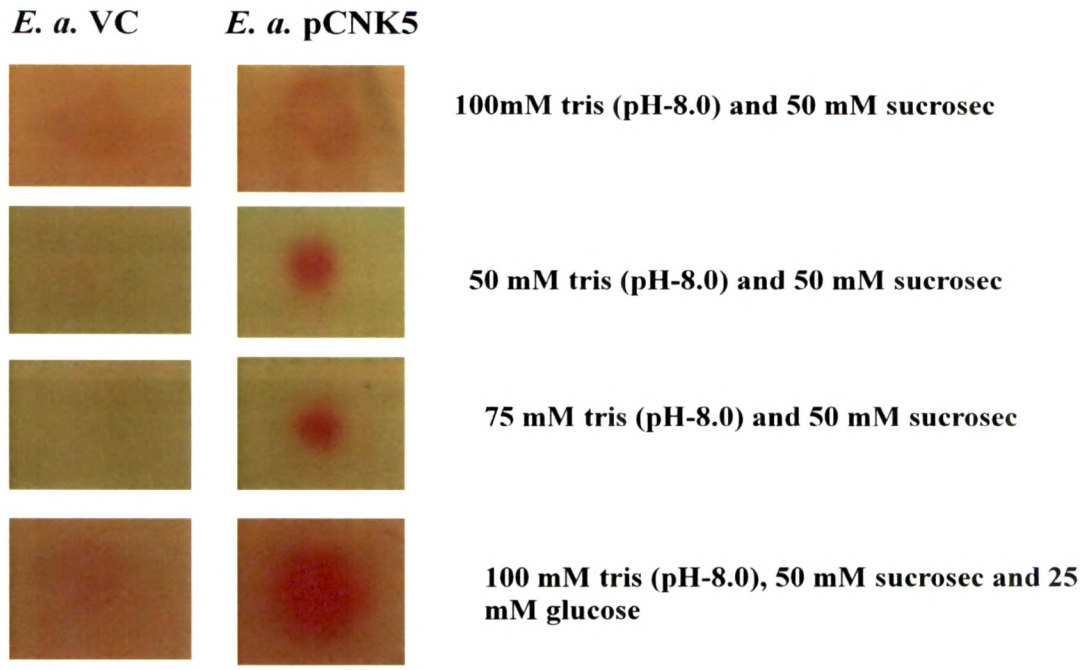


Fig. 4.20: MPS ability of *E. a.* PSI3 (pTTQGm^r) and *E. a.* PSI3 (pCNK5) in TRP containing varied Tris and carbon source.

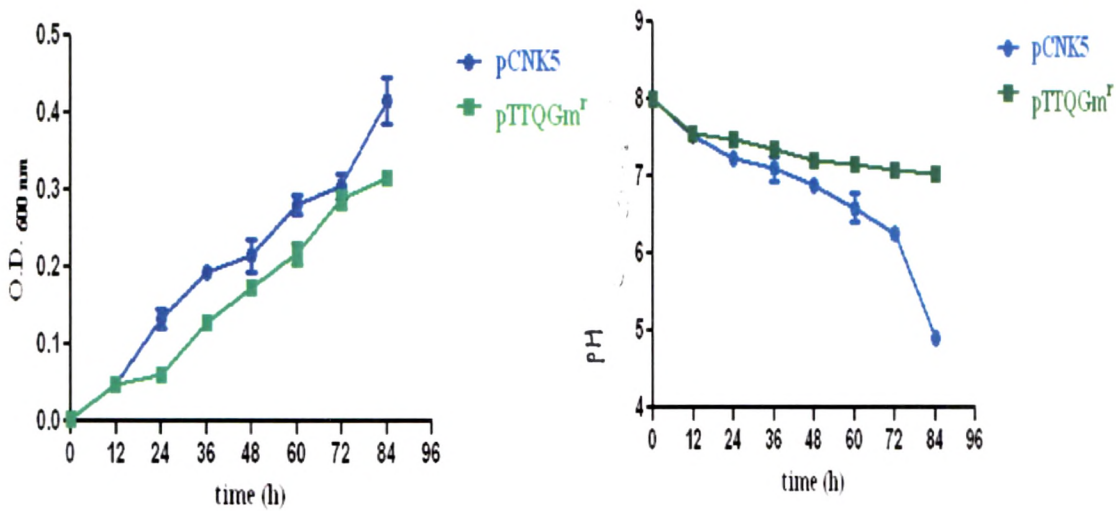


Fig. 4.21: Growth and pH profile of *E. a.* PSI3 (pTTQGm^r) and *E. a.* PSI3 (pCNK5) in TRP containing 75mM Tris and 50mM sucrose.

Table 4.2: Amount of Organic acid secretion and P release by *E. asburiae* PSI3 (pTTQGm), *E. asburiae* PSI3 (pCNK4) and *E. asburiae* PSI3 (pCNK5) in TRP medium containing different amount of sucrose and glucose.

Plasmids of the *E. asburiae* PSI3 transformant	**Final O.D**	**Tris (pH-8.0) + sucrose + glucose (mM)**	**Gluconic acid (mM)**	**Pi (mM)**
pBBR-MCS-2	0.21±0.01	75 +7 5 + 0	ND	UD
pCNK4	0.31±0.03	75 + 75 + 0	21.65±0.94	0.180±0.043
pTTQGm^r		100 + 50 + 0	ND	UD
pCNK5		100 + 50 + 0	8.3	UD
pTTQGm^r		75 + 50 + 0	ND	UD
pCNK5		75 + 50 + 0	22	0.438± 0.073
pTTQGm^r		100 + 50 +25	9.4	UD
pCNK5		100 + 50 +25	34	0.479±0.081

UD- Undetected; ND – Not Determined.

4.4 Discussion

Most PSMs release P from calcium phosphate complexes in the presence of glucose at very high levels (~100mM). Since root exudates contain a variety of carbon source present in very low amounts (~10-100μM), the PSM ability could be limited by the availability of carbon sources. Thus, PSM are required to release P in the presence of low amount of carbon sources. But, the levels of carbon sources required to demonstrate PSM ability in laboratory may not correspond to the root exudates as laboratory conditions differ from the rhizosphere which could be similar to steady state-steady flow conditions. Earlier studies demonstrated that a mixture of aldose sugars solubilize rock phosphate by *E. asburiae* PSI3 under buffered (alkaline vertisol mimicking) conditions, required only 15 mM amounts of each sugar whereas independently each sugar is required at 75mM. This MPS ability is mainly due to oxidation of aldosugars to their corresponding acids. High levels of sucrose and bacteria were found at the root tips of annual grass *Avena barbata* (Jaeger *et al.*, 1999). *Peucedanum alsaticum* and *Peucedanum cervaria* belong to umbelliferi family roots produces sucrose as a major carbon source which is available to root associating microorganisms (Hadacek and Kraus, 2002). Thus, presence of fructose and sucrose in the root exudates will contribute towards organic acid secretion.

Present study describes the effects of incorporation of three different periplasmic invertases in *E. asburiae* PSI3. Overexpression of *Anabaena* neutral invertase (*invB*) in *E. coli* periplasm under constitutive *tac* promoter caused cell lysis while overexpression of periplasmic *Z. mobilis* invertase (*pelB-invB*) and *S. cerevisiae suc2* genes grew normally. Reasons for these effects are not very clear. Expression of

Chapter 4 *Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.*

Z. mobilis periplasmic *invB* gene under *lac* promoter in *E. asburiae* PSI3 showed 0.438U enzyme activity. *Z. mobilis* periplasmic *invB* expression from pET vector in *E. coli* BL21 (DE3) showed 1.7U under 10l fermentation process (V'asquez-Bahena et al., 2006). However, *Z. mobilis invB* oxpression under *tac* promoter reduced invertase activity by ~22% as compared to *lac* promoter into *E. coli* (Yanasi et al., 1998). However, *suc*2 gene expression in *E. asburiae* PSI3 under *tac* promoter enhanced periplasmic invertase activity to 29.3 mU while 14mU external invertase activity was found in wild type yeast expressing from its own promoter (Rothe and Lehle, 1998).

E. asburiae PSI3 containing periplasmic invertase produced high amount of gluconic acid leading to good MPS ability under buffered condition which further improved when sucrose and glucose were used together. Many organisms had been reported to show MPS ability on sucrose as carbon source including fungi and bacteria. *Penicillium bilaii* solubilize $CaHPO_4$ in presence of sucrose as carbon source by producing 10 mM each citric and oxalic acid (Cunningham and Kuiack, 1992). *Penicillium rugulosum* showed better MPS ability on sucrose as compared to glucose or maltose as carbon source in the presence of hydroxyapetite as P source. *P. rugulosum* secretes gluconic and citric acid in presence of sucrose as carbon source (Reyes et al., 1999a). Mps++ mutant strain of *Penicillium rugulosum* produced 14.3 mM citric and 7.7 mM gluconic acids in presence of $FePO_4$ and nitrate as nitrogen source (Reyes et al., 1999b). However, when $FePO_4$ replaced by hydroxyapatite, it produced 90 mM gluconic and 0.28 mM citric acid. Sucrose utilizing fungi also showed better phenotype. Similarly, *Penicillium oxallicum* CBPS-Tsa produced 563mg/L P in medium when 5g/L sucrose and CaP was provided and P release was

enhanced to 824 mg/L in presence of sodium nitrate as nitrogen (Kim et al., 2003). On sucrose, *Aspergillus aculeatus* solubilized Sonari rock phosphate and released 4.4 mg/100ml P_2O_5 (Narsian and Patel, 2000). *Aspergillus niger* BHUAS01 and *Penicillium citrinum* solubilized tricalcium phosphate in presence of sucrose and released 421µg/ml P after 21 day incubation (Yadav et al., 2010; 2011).

Among bacteria *Azotobacter chroochocum* isolated from wheat rhizosphere and *Citrobacter* DHRSS isolated from sugar cane showed MPS ability (Kumar and Narula, 1999; Patel et al., 2008). *Citrobacter* DHRSS showed MPS ability on sucrose produces 49 mM acetic acid and released 170µM P in the medium (Patel et al., 2008).