CHAPTER 4

Characterization of MPS abilities of *E. asburiae* PSI3 containing periplasmic invertases
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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 (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., 1999). 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,
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases. 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 to the 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
Chapter 4 Characterization of MPS ability of *E. asburiae* PSI3 containing periplasmic invertases.

dehydrogenase (GDH) substrates, the organic acid secreted is of interest since organic acids other than aldonic acids are expected. *Citrobacter* DHRSS uses sucrose as carbon source due to the presence of cytoplasmic invertase and solubilizes rock phosphate in 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.
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

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>E. coli JM109</strong></td>
<td>endA1  glnV44 thi-1 relA1 gyrA96 recA1 mcrBC+ (lac-proAB) e14- [F’ traD36 proAB+ lacI’ lacZ M15] hsdR17(rKmK+)&lt;br&gt;<strong>E. coli DH5α</strong></td>
<td>Promega</td>
</tr>
<tr>
<td><strong>E. coli DH10B</strong></td>
<td>FendA1  glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Ph80lacZ M15 (lacZYA-argF)U169, hsdR17(rK- mK+), λ--&lt;br&gt;<strong>E. coli S17.1</strong></td>
<td>Sambrook and Russel, 2001</td>
</tr>
<tr>
<td><strong>E. coli BL21 (DE3)</strong></td>
<td>thi pro hsdR recA RP4-2 (Tet::Mu) (Km::Tn7); TmpI&lt;br&gt;<strong>E. coli PS13</strong></td>
<td>Invitrogen, 1983</td>
</tr>
<tr>
<td><strong>E. asburiae PS13</strong></td>
<td>F ompT gal dcm lon hsdS prim( Der- mK+) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])&lt;br&gt;<strong>Plasmids</strong></td>
<td>Sambrook and Russel, 2001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Cajanus cajan rhizosphere isolate</td>
<td>Gyaneshwar et al., 1998b</td>
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</table>
### Chapter 4 Characterization of MPS ability of *E. asburiae* PSI3 containing periplasmic invertases.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>pTTQ18</td>
<td>pUC18 ori, Amp&lt;sup&gt;+&lt;/sup&gt;, tac</td>
<td>Stark, 1987</td>
</tr>
<tr>
<td>pTTQGm&lt;sup&gt;T&lt;/sup&gt;</td>
<td>pUC18 ori, Amp&lt;sup&gt;+&lt;/sup&gt;, Gm&lt;sup&gt;T&lt;/sup&gt;, tac</td>
<td>This study</td>
</tr>
<tr>
<td>pET22b(+)</td>
<td>T7 promoter, pelB, Amp&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Novagen</td>
</tr>
<tr>
<td>pMal-p2</td>
<td>Amp&lt;sup&gt;T&lt;/sup&gt;, tac, malE</td>
<td>NEB</td>
</tr>
<tr>
<td>pBluescript KS(+)</td>
<td>Amp&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pUZE3</td>
<td>Amp&lt;sup&gt;T&lt;/sup&gt;, lac, pUC ori, Zymomonas mobilis invB gene</td>
<td>Yanasi et al., 1998</td>
</tr>
<tr>
<td>pRT23.04</td>
<td>pGEM-2 origin, Amp&lt;sup&gt;T&lt;/sup&gt;, <em>S.cerevisiae</em> invertase gene suc2</td>
<td>Roitsch and Lehale, 1988</td>
</tr>
<tr>
<td>pCNK1</td>
<td>pBluescript KS (+), Amp&lt;sup&gt;T&lt;/sup&gt;, <em>Anabaena</em> neutral invertase gene invB</td>
<td>This study</td>
</tr>
<tr>
<td>pCNK2</td>
<td>pJET2.1, Amp&lt;sup&gt;T&lt;/sup&gt;, constitutive tac, <em>Anabaena</em> neutral invertase gene invB with MBP signal sequence</td>
<td>This study</td>
</tr>
<tr>
<td>pCNK3</td>
<td>pET22b(+), Amp&lt;sup&gt;T&lt;/sup&gt;, <em>Zymomonas mobilis</em> invertase gene invB</td>
<td>This study</td>
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<tr>
<td>pCNK4</td>
<td>pBBR1MCS-2, Kan&lt;sup&gt;T&lt;/sup&gt;, <em>pelB-invB</em>,</td>
<td>This study</td>
</tr>
<tr>
<td>pCNK5</td>
<td>pTTQGm&lt;sup&gt;T&lt;/sup&gt;, Amp&lt;sup&gt;T&lt;/sup&gt;, Gm&lt;sup&gt;T&lt;/sup&gt;, tac, suc2</td>
<td>This study</td>
</tr>
</tbody>
</table>

#### 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<sup>-1</sup>, Erythromycin (20 mg ml<sup>-1</sup>) or ampicillin (50 mg ml<sup>-1</sup>) (Sambrook and
Chapter 4 Characterization of MPS ability of *E. asburiae* PSI3 containing periplasmic invertases.

Russell, 2001). Antibiotic concentration was reduced to 1/4th 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 and provides direct selection of recombinant plasmids based on blue-white selection strategy. *LacZα* gene in pTTQ18 is under the *tac* promoter which is strong and tightly regulated promoter in *Enterobactericeae* family (Fig. 4.1) (Stark, 1987).

pUZE3 and pRT23.04

pUZE3 contains extracellular invertase gene fragment of *Zymomonas mobilis* under *lac* promoter of pUC118 (Yanasi et al., 1998). pRT23.04 contains 6 kb genomic DNA fragment of *Saccharomyces cerevisiae* which consist of *suc2* 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.
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

**Fig. 4.1** Map of plasmids used in study

*Genetic manipulation of Enterobacter asburiae PSI3 for enhanced Phosphorus nutrition*
4.2.4 Cloning of *Anabaena* neutral inverate (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 (invB) from *Anabaena* PCC 7120 genomic DNA was PCR amplified using *Pfu* DNA polymerase, gene specific forward primer and reverse primers. BamHI and HindIII RE sites were incorporated in forward and reverse primers, respectively. The PCR conditions were: initial denaturation at 94°C for 1 min followed 30 cycles each consisting 94°C for 15 s, 62°C for 30 s, 72°C for 90 s and followed by final extension at 72°C for 5 min. The amplified ~1.45 kb PCR product was purified using Pure Link gel extraction kit (Invitrogen, USA), blunt ended cloned in *EcoRV* 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 (malE) gene was PCR amplified from pMal-p2 plasmid with the help of gene specific primer. *malE* signal sequence was PCR amplified using gene specific forward primer

5’GCAGGAATCGATTTCACACAGGAACAGCGATG AAAATAAAAACAGGATGC

3’ and reverse primer MBP RP5’ GCTTCTGCATTTTGATTTTG 3’. The PCR
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

conditions were: initial denaturation at 94°C for 1 min followed 30 cycles each consisting 94°C for 15 s, 52°C for 15 s, 72°C for 30 s and followed by final extension at 72°C for 5 min. To make gene expression under control of tac constitutive promoter second PCR was performed with

Forward primer
Tact15'TCGTATAATGGATCGAATTGTGAGCGGAATCGATTTTCACACAGGA AA3’ Reverse primer MBP RP
5’ GCTTCTGCATTTCGATTTTG 3’. The PCR conditions were: initial denaturation at 94°C for 1 min followed 30 cycles each consisting 94°C for 15 s, 55°C for 15 s, 72°C for 30 s and followed by final extension at 72°C for 5 min. PCR amplicon of ~200bp was obtained. Neutral invertase was PCR amplified using

Forward primer INV FP 5’ CAAAAATCGAAATGCAGAGC 3’
Reverse primer INV RP 5’ CCCAAGCTTGGGAAAAATTATCGAGATA 3’

and pCNK1 as template which gives ~1.45 kb PCR amplicon. The PCR conditions were: initial denaturation at 94°C for 1 min followed 30 cycles each consisting 94°C for 15 s, 55°C for 30 s, 72°C for 90 s and followed by final extension at 72°C for 5 min. Finally both the PCR product were join by doing recombinant PCR using Tac1 as forward primer and INV RPa as reverse primer using equimolar concentration of both the PCR product. The PCR conditions were: initial denaturation at 94°C for 1 min followed 30 cycles each consisting 94°C for 15 s, 55°C for 30 s, 72°C for 120 s and followed by final extension at 72°C for 5 min. Finally ~1.65 kb PCR amplicon was obtained and cloned in TA cloning vector pTZ57R/T (Fermantas). Screening of positive
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

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 sucZE3 was isolated from plasmid pUZE3 by EcoRI and HindIII digestion. Isolated fragment was further subcloned in the periplasmic expression vector pET 22b (+) to obtained pCNK4. pCNK4 was further digested with XbaI and HindIII which released 1.6kb fragment. 1.6 kb fragment contains periplasmic leader peptide pe/B and sucZE3. 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-str

Periplasmic invertase of Saccharomyces cerevisiae was PCR amplified by gene specific forward and reverse primers. Suc2 gene sequence was obtained from Gene Bank (Accession No. ID Z46921_41) and specific primers were designed from the sequence Sequences of suc2 primer given below

Forward primer- 5’GGAAATTCCAGGGCTAAACGTATATGATGCTT 3’
Reverse primer- 5’ CCGCTCGAGCTCCCTCTATTTTTACTTCCCTTAC 3’

Underline sequence represent restriction enzyme site EcoRI and SacI respectively. Italic sequence showed ribosome binding site (RBS). suc2 gene was PCR amplified

Genetic manipulation of Enterobacter asburiae PSI3 for enhance Phosphorus nutrition
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

from plasmid pRT23.03 which contains periplasmic invertase genomic DNA fragment of Saccharomyces cerevisiae. The PCR conditions were: initial denaturation at 94°C for 1 min followed 30 cycles each consisting 94°C for 15 s, 48°C for 30 s, 72°C for 90 s and followed by final extension at 72°C for 5 min. PCR amplification of suc2 gene was done according to above program which gives 1.6 kb amplicon. PCR product and expression vector pTTQGm' were digested with EcoRI and SacI. 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 (Ames 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°C. Following three wash steps in distilled water for 5 min, gels were subjected to 0.1M iodoacetamide 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., 1998b). 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, P2O5 36-37, CaO 49-51, fluorine 3-4, insoluble acid 5-6, CO21-2, Fe2O3 0.6-0.8, sulphate 0.3-0.6, and Al2O3 0.6-0.8. Macronutrient contains (1L) NH4Cl 5 g, KCl 5g, NaCl 2.5g, and CaCl2 15 mg and micronutrients FeSO4· H2O 3.5 mg, ZnSO4· 7H2O 0.16 mg, CuSO4· 5H2O 0.08 mg, H3BO3 0.5 mg, CaCl2·2H2O 0.03 mg, and MnSO4· 4H2O 0.4 mg, respectively.
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

*E. coli* and *E. asburiae* PSI3 were grown at 37°C 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, 1964).

**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.

Genetic manipulation of Enterobacter asburiae PSI3 for enhance Phosphorus nutrition
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

4.3 Results

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

invB 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 EcoRV RE site to obtained pCNK1 (RO) and pCNK1 (WO) (Fig. 4.1). pCNK1 (RO) give ~4.4 kb linearize plasmid with BamHI digestion but in case of pCNK1 (WO) it give ~1.45kb release of invB (Fig. 4.2).
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

4.3.2 Cloning and expression of PCC 7120 neutral invertase (invB) 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 invB 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 SacI and HindIII which release the complete insert of 1.6kb. EcoRI digestion gives the 500bp release due to presence of one RE site in both gene and vector. Similarly XbaI 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.
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

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

Fig. 4.6 Schematic representation of cloning strategy of pCNK2

Fig. 4.7 Restriction digestion pattern of pCNK2.

4.3.3 Cloning of invB with pelB signal sequence in pBBR1MCS-2

Plasmid pUZ3 containing Zymomonas mobilis extracellular invertase (invB) was isolated by digestion with EcoRI and HindIII. Double digestion showed ~1.6 kb fragment containing invB. invB fragment was gel eluted and ligated in periplasmic expression vector pET22b(+) under EcoRI and HindIII to obtained pCNK3.
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

**Fig. 4.8** Schematic representation of cloning strategy of pCNK3

\[ \text{invB along with pelB sequence was isolated from pCNK3 digestion with XbaI and HindIII which gives } \sim 1.7 \text{kb release. 1.7kb fragment was blunt ended by Klenow fragment. Plasmid pBBR1MCS-2 was digested with EcoRV followed by gel purification. 1.7 kb fragment was cloned in pBBR1MCS-2 to obtained pCNK4. Right orientation clones were confirmed by 1.7kb release with XbaI digestion (Fig. 4.11).} \]
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

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 suc2 in pTTQGm<sup>r</sup>

1.6 kb suc2 was cloned in pTTQGm<sup>r</sup> under EcoRI and SacI. Positive clone was confirmed by different RE’s digestion. XbaI digestion gives 780bp due presence of RE site in gene and vector. EcoRI/BamHI digestion gives release of 900bp and vector backbone of5.8kb. BamHI/HindIII double digestion gives the
Chapter 4 *Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.*

1.6 kb gentamycin release, ~750 bp from *suc2* gene and vector backbone of 5.4kb. *EcoRI/PstI* double digestion gives release of 1.6 kb *suc2* gene and 6.1kb vector backbone (Fig. 4.13).

![Schematic representation of cloning of suc2 gene in pTTQGm'].(Image)

**Fig. 4.12** Schematic representation of cloning of *suc2* gene in pTTQGm'.

![Restriction digestion pattern of pCNK5](Image)

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

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

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 suc2 (Fig.).

Fig. 4.17: Invertase enzyme detection on semi native PAGE
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

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').

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

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.

![E. u. VC vs E. u. pCNK4](image)

**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.

![Growth and pH profile](image)

**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.

*Genetic manipulation of Enterobacter asburiae PSI3 for enhance Phosphorus nutrition*
Chapter 4 Characterization of MPS ability of *E. asburiae* PSI3 containing periplasmic invertases.

**Fig. 4.20:** MPS ability of *E. a.* PSI3 (pTTQGm') 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') and *E. a.* PSI3 (pCNK5) in TRP containing 75mM Tris and 50mM sucrose.

*Genetic manipulation of Enterobacter asburiae PSI3 for enhance Phosphorus nutrition*
Chapter 4 Characterization of MPS ability of *E. asburiae PSI3* containing periplasmic invertases.

**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.

<table>
<thead>
<tr>
<th>Plasmids of the <em>E. asburiae PSI3</em> transformant</th>
<th>Final O.D</th>
<th>Tris (pH-8.0) + sucrose + glucose (mM)</th>
<th>Gluconic acid (mM)</th>
<th>Pi (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBBR-MCS-2</td>
<td>0.21±0.01</td>
<td>75 +75 0</td>
<td>ND</td>
<td>UD</td>
</tr>
<tr>
<td>pCNK4</td>
<td>0.31±0.03</td>
<td>75 +75 0</td>
<td>21.65±0.94</td>
<td>0.180±0.043</td>
</tr>
<tr>
<td>pTTQGm′</td>
<td>0.24±0.02</td>
<td>100 +50 0</td>
<td>ND</td>
<td>UD</td>
</tr>
<tr>
<td>pCNK5</td>
<td>0.415±0.03</td>
<td>100 +50 0</td>
<td>8.3</td>
<td>UD</td>
</tr>
<tr>
<td>pTTQGm′</td>
<td>0.316±0.007</td>
<td>75 +50 0</td>
<td>ND</td>
<td>UD</td>
</tr>
<tr>
<td>pCNK5</td>
<td>0.44±0.003</td>
<td>75 +50 0</td>
<td>22</td>
<td>0.438±0.073</td>
</tr>
<tr>
<td>pTTQGm′</td>
<td>0.34±0.008</td>
<td>100 +50 +25</td>
<td>9.4</td>
<td>UD</td>
</tr>
<tr>
<td>pCNK5</td>
<td>0.52±0.02</td>
<td>100 +50 +25</td>
<td>34</td>
<td>0.479±0.081</td>
</tr>
</tbody>
</table>

UD- Undetected; ND – Not Determined.
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

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 sources 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

*Genetic manipulation of Enterobacter asburiae PSI3 for enhance Phosphorus nutrition*
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 (Vásquez-Bahena et al., 2006). However, *Z. mobilis invB* expression under *tac* promoter reduced invertase activity by ~22% as compared to *lac* promoter into *E. coli* (Yanasi et al., 1998). However, *suc2* 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 CaHPO4 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 hydroxyapatite 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 FePO4 and nitrate as nitrogen source (Reyes et al., 1999b). However, when FePO4 replaced by hydroxyapatite, it produced 90 mM gluconic and 0.28 mM citric acid. Sucrose utilizing fungi also showed better phenotype. Similarly, *Penicillium oxalicum* CBPS-Tsa produced
Chapter 4 Characterization of MPS ability of E. asburiae PSI3 containing periplasmic invertases.

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$_2$O$_5$ (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).