CHAPTER 6

Phosphate mobilization ability of *E. asburiae* PSI3 containing extracellular *Uromyces viciae-fabae* invertase (*inv1*), *C. braakii phytase* (*appA*) and *Vitreoscilla* haemoglobin (*vgb*) genes

Chapter 6 *Phosphate mobilization ability of E. asburiae PSI3 containing extracellular Uromyces viciae-fabae invertase (inv1), C. braakii phytase (appA) and Vitreoscilla haemoglobin (vgb) genes.*

6.1 Introduction

Availability of organic P for the plants or microorganism requires mineralization of substrate (bound form) by phosphatase enzymes. In soils, phytases (monoester phosphatases) contribute upto 60% of P derived from organic phosphates (Bunemann, 2008). Phytates in soil are generally complexed with either cations to form precipitates of salt or adsorbed to the soil components (Turner et al., 2002). Generally phytates are not available to attack by enzymes in soil due to formation of insoluble precipitate like Al or Fe- phytate or adsorbed to mineral or clay particle of soils. Formation of Ca-phytate was demonstrated by potentiometric titration method above pH 5 (Martin and Evans, 1986). Calcite rich soils adsorb large amount of IHP to form Ca-phytate precipitate (Celi et al., 2000). All major phytases *viz* HAPs, BAPs, and PAPs demonstrate hydrolysis of Ca^{+2}, Mg^{+2} and Mn^{+2} phytates but unable to solubilize precipitate of Al^{+3}, Fe^{+2}, Fe^{+3}, Cu^{+2}, and Zn^{+2} ions. Presence of these precipitates also inhibits solubilization of Ca^{+2}- phytates. At high molar ratio of Ca^{2+}: IP6 (6:6) leads to precipitation of insoluble Ca-IHP, Ca^{2+} which ultimately leads to the reduction in 50% enzymatic dephosphorylation of Na-phytae even at pH-4.5 (Dao, 2003). At lower pH below 4 all the molar ratio of Ca ions and IHP become soluble (Grynspan and Cheryan, 1983). At pH-4.0, Ca-IHP remains soluble and dephosphorylation is not affected by Al and Fe complexes. However, organic acids like citrate, oxalate and malate solubilize phytate salts and facilitate the release of P with the help of enzyme hydrolysis. Phytate adsorbed on aluminum precipitate are resistant to all three forms of phytases. P from these complex phyates can be released in presence of phytase enzymes with organic acid like citrate, oxalate and malate (Tang et al., 2006).

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Phytase producing rhizobacterial strains which secrete gluconic, acetic and small amount of pyruvic acids are able to solubilize all three forms of insoluble phytate *viz* Fe^{+2}-IHP, Fe^{+3}-IHP and Al^{+3}-IHP. Amongst these acids, gluconic acid is more efficient as compared to other organic acid as 5mM is sufficient to release P from bound phytate (Patel, 2010). Plant study with natural isolates which produces gluconic acid improved plant growth with respect to shoot weight, dry shoot/root ratio and P content in synthetic media supplied with insoluble phytate as sole P source. In synthetic medium, plants also secret acids which also contribute in P acquisition. Improved plant growth and shoot P (mg per dry weight) shown by plant due to P acquisition from phytate-P (Richardson et al., 2000; 2001a, b).

Inoculation of PSM in soil showed inconsistent results of enhancement of P content in the plant as well as growth promotion. These variations in field were attributed to differences in the composition and properties of soils along with nature of soil microflora and type of crops (Kucey et al., 1989). These inconsistencies arise due to inability of these PSMs to release P in the soil. Most of the PSMs isolated from the soil under unbuffered condition where as alkaline vertisols soil having rich in Calcium phosphate complexes having very strong buffering capacity which limit its ability to solubilize P in soil condition (Gyaneshwar et al., 1998b).In case of acidic alfisol, Al and Fe complexes of P make condition difficult for P solubilization (Srivastava et al., 2007). These organisms generally show P solubilization ability on CaP due to lowering of pH in under unbuffered condition. In buffered condition, these PSMs are not able to overcome the buffering strength due secretion of insufficient amount of organic acids.

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In most of the PSMs, gluconic acid is the major organic acid responsible for the P solubilization (Goldstein, 1995). In Gram negative bacteria, PQQ dependent GDH converte glucose into gluconic acid via direct oxidation pathway. PSMs with broad substrate range GDH are more efficient PSMs (Sharma et al., 2005, Patel et al., 2008). Survival and physiology of the rhizobacteria in the rhizosphere is mainly determined by the availability of the nutrients. Nature and amount of root exudates vary with plant genotype, nutritional status, plant age and mycorrhizal infection (Marschner et al., 2004). Approximately 50–100 mg C g^{-1} soil was estimated to be secreted by plant roots daily (Trofymow et al., 1987; Iijima et al., 2000). Many different carbon sources are found in the root exudates and sucrose is one of the common sugars. High levels of sucrose and bacteria were found at the root tips of annual grass *Avena barbata* (Jaeger *et al.*, 1999). Rice plants in hydroponic conditions secreted upto 270 µmol glucoše/g root dry weight (Bacilio-Jim´enez et al., 2003). Glucose, fructose and xylose were the major sugars detected in the exudates of stonewool-grown tomato whereas glucose and fructose were the major components in all growth stages of tomato (Kamilova et al., 2006).

Under oxygen limiting condition VHb improves growth, metabolite and protein synthesis (Frey and Kallio, 2003). Production and improvement of recombinant protein and metabolite can be optimize by introducing *vgb* gene in host. VHb is encoded by *vgb* gene has been expressed successfully in numerous organisms for growth improvement or metabolite production (Stark et al., 2011). Heterologous expression of *vgb* gene in *Streptomyces aureofaciens* increased yield of chlortetracycline 40-60% more than wild type culture under low dissolve oxygen condition (Meng et al.,2002). In similar report, *Saccharopolyspora*

erythraea showed 60% increase in erythromycin production after genome integration of *vgb* gene (Brunker et al., 1998). Similar effects of *vgb* gene expression were observed in case of *Streptomyces cinnamonensis* and *Acremonium chrysogenum* for antibiotic production (DeModena et al., 1993; Wen et al., 2001). Oxygen is essential in degradation of many soil or water contaminants using microorganism due to low dissolved oxygen level. *vgb* gene has been extensively used to overcome this problem by enabling the bacteria with better growth and improved degradation of organic contaminants under hypoxic condition. (Liu et al., 1996; Patel et al., 2000; Nasr et al., 2001; Urgun-Demirtas et al., 2003; 2004; So et al., 2004). 2,4-Dinitrotoluene degradation has also been done successfully by *vgb* gene incorporation in *Burkholderia* sp in continuous flow sand column reactor (So et al., 2004).

Extensive physiological studies in *E. coli* demonstrated that the heterologous expression of *vgb* gene increases natural resistance to nitrosative stress and oxidative stress (Frey et al., 2002; Anand et al., 2010). These stresses can damage nucleic acids, proteins and cellular membrane when level of free radical increased beyond capacity of cell's mechanism of elimination (Farr and Kogoma, 1991). Cells expressing *vgb* gene minimally affected by these free radicals as compared to plasmid controls which clearly indicates the positive effect on cell survival (Frey et al., 2002; Anand et al., 2010). In the rhizosphere, microbial metabolism could be regulated by multiple stress conditions including microaerbic conditions. Present study demonstrates the P mobilization ability *E. asburiae* PSI3 overeexpressing *vgb, inv* and *appA* genes.

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6.2 Material and Methods

6.2.1 Bacterial strains, plasmids and growth conditions

Table 6.1 Bacterial strains and plasmids used in this study

Bacterial Strains	Genotype	Reference
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. asburiae PSI3	*Cajanus cajan* rhizosphere isolate	Gyaneshwar et al 1998
Plasmids		
pUC16-vhb	Amp^r, pUC16 backbone	Khosala et al 1987
pTTQ18	Amp^r, *tac* promoter	Stark 1987
pCNK11	Amp^r, pTTQ18, *vgb*	This study
pCNK15	Amp^r, pTTQ18,Gm^r, *vgb*	This study
pCNK21	Amp^r, Gm^r, pTTQ18, *vgb*, *app*A, *tac*	This study
pCNK22	Amp^r, pTTQ18, Gm^r, *vgb*, *app*A , *Uinv, tac*	This study

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6.2.2 Construction of *Vitreoscilla* heamoglobin gene *vgb* in expression vector pTTQGm^r

Step I:- Plasmid pUC16-vhb was digested with *Sal*I and *Hind*III which gives 1.3 kb release of *vgb* gene with its oxygen sensitive promoter. 1.3kb VHB fragment was gel purified and cloned in gel purified pTTQ18 digested with *Sal*I and *Hind*III to obtained pCNK11. Clone was confirmed by RE analysis using *Sal*I, *Eco*RI, *Hind*III.

Step II:- *E. asburiae* PSI3 is naturally resistant to ampicillin. Hence, aminoglycoside-(3)-N-acetyltransferase III (*aacC3*) gene from pGM160 (*Hind*III fragment) was cloned in *Hind*III site of pCNK11 to obtain pCNK15. Recombinant plasmid was confirmed by growing them on gentamycin containing LA plates and restriction digestion analysis.

6.2.3 Construction of *Vitreoscilla* heamoglobin gene *vgb* and *C. braakii* phytase gene *app*A in expression vector pTTQGm^r

Plasmid pCNK10 was digested with *Kpn*I and *Bam*HI enzymes which give 1.3kb *app*A fragment. *app*A fragment was gel eluted and cloned in gel eluted pCNK15 digested with *Kpn*I and *Bam*HI to obtained pCNK21. Clone was confirmed by RE digestion and PCR amplification using gene specific primers. Functionality of phytase gene *app*A was confirmed by enzyme assay.

6.2.3 Construction of *Vitreoscilla* heamoglobin gene (*vgb*), *Uromycities fabae* invertase (*Uinv*) and *C. brakii* phytase gene (*app*A) in expression vector pTTQGm^r

Cloning of *Uinv* was done in pCNK21 using Sequence and Ligation Independent Cloning (SLIC) method (Li, MZ and Elledge, J., 2008). To amplify *Uinv* gene, specific primers were designed which had 20 nucleotide homology to pCNK21 in both forward and reverse

primers. *Uinv* was PCR amplified from plasmid KS:*inv*. PCR amplification was done using Phusion high fidelity DNA polymerase to avoid any mutation due to its high fidelity. ~2.3 kb PCR amplicon was obtained and purified using PCR clean up kit (Invitrogen). pCNK21 was digested with *Eco*RI and gel eluted using Pure Link gel elution kit (Invitrogen). Both vector and PCR product was quantified with the help of Nanovue (GE Helthcare). 1µg vector and insert were treated separately with T4 DNA polymerase at room temperature for 30 minute. Reaction was stopped by addition of one tenth of reaction volume 10mM dCTP and kept on ice. T4 DNA polymerase treated 100ng vector and 100ng PCR were mixed together in 1X ligase buffer in 10 µl reaction volume. Reaction mixture was incubated at 37^0C for 30 min. 5 µl reaction mixture was transformed in DH10B competent cell. Positive clones were confirmed by RE digestion. Functionality of the *E. coli* BL21 (DE3) transformant was confirmed by growing on sucrose minimal media plate and invertase enzyme assay.

6.2.4 Physiological experiments

6.2.5 Expression of *vgb* gene under oxygen sensitive promoter

vgb gene expression study was done in *E. coli* BL21 (DE3) containing pCNK15, pCNK21 and pCNK22. Cells were grown in LB medium till O.D. 600_{600} reaches 0.2-0.3, then 10mM CCl_4 was used to induce oxygen sensitive promoter. Cells were finally grown till growth reaches stationary phase. Cell were harvested and washed with normal saline. Cells were then lysed with lysozyme and loading dye was added to the sample followed by boiling. Finally sample was loaded on 15% SDS PAGE to analyze expression of *vgb* gene.

6.2.6 Development of *Vitreoscilla* haemoglobin (*vgb*), *appA-vgb* and *Uinv-appA-vgb* expressing *E. asburiae* PSI3.

The plasmid pCNK15 expressing *vgb* of *Vitreoscilla* sp. from oxygen sensitive promoter, pCNK21, pCNK22 and pTTQGm^r were incorporated in *E. asburiae* PSI3 by transformation. The transformants were confirmed by plasmid isolation and RE digestion.

6.2.7 Growth and MPS phenotype of *E. asburiae* PSI3 transformant.

MPS ability of *E. asburiae* PSI3 transformants was monitored on 100mM Tris buffered RP agar plates or Tris buffered phytate plates as and when required. The growth and pH profile of all the transformants was checked on TRP liquid medium or Tris phytate minimal medium (mention as in Material and Methods) and the culture supernatants of the samples withdrawn at regular intervals till the time of pH drop (media pH<5) were analyzed for O.D. 600_{600}, pH and organic acid production .

6.2.8 Analytical methods

All the physiological experiments used an initial cell density of ~0.025 O.D. 600 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; samples (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.

The column was operated at room temperature using mobile phase of 5mM H_2SO_4 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 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.

6.2.9 Data analysis

Physiological experiments were done in six independent triplicates. Data are expressed in mean and standard deviation. Differences in mean values were determined using general analysis of varience (ANOVA) and linear regression analysis was done using Graph Pad Prism5.0.

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6.3 Results

6.3.1 Construction of *vgb* gene with its native oxygen sensitive promoter in expression vector pTTQGm^r

1.3 kb genomic DNA fragment of *Vitreoscilla* sp. containing *vgb* gene with native oxygen sensive promoter was excised out from plasmid pUC-vhb16 with the help of *Sal*I and *Hind*III. 1.3 kb fragment was cloned in pTTQ18 under *Sal*I and *Hind*III to obtain pCNK11. Clone was confirmed by *Eco*RI/*Hind*III and *Sal*I/*Hind*III digestion which gives 1.3kb *vgb* gene. 1.6 kb Gm^r was incorporated in pCNK11 under *Hind*III for selection in *E. asburiae* PSI3.

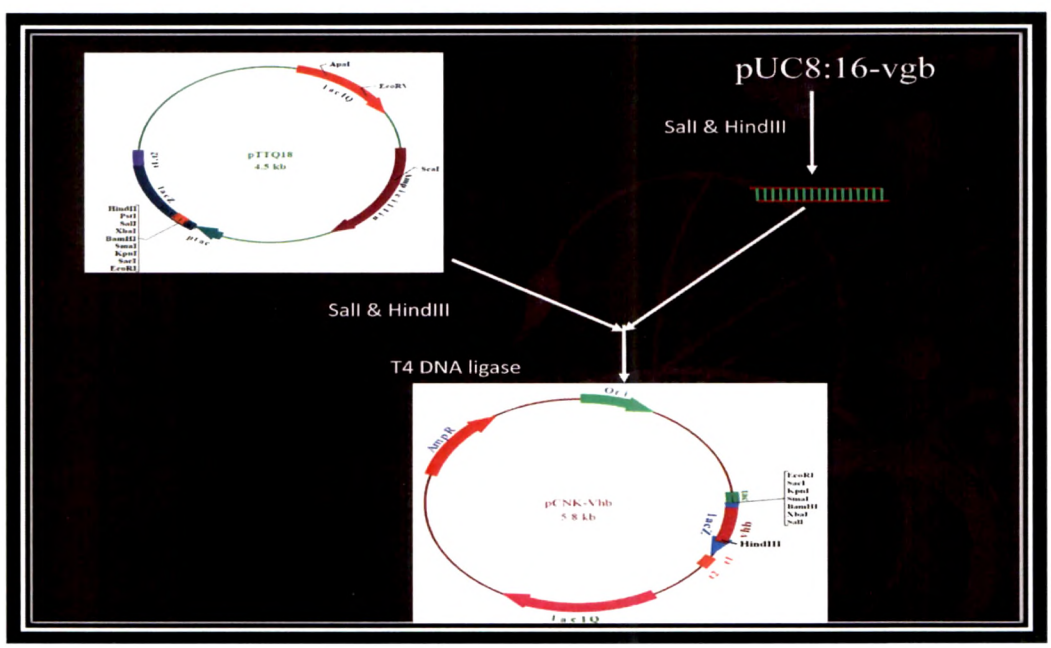

Fig.6.1: Schematic representation of cloning of *vgb* gene in pTTQ18 (pCNK11)

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Positive clone were confirmed by *Eco*RI/*Hind*III and *Sal*I/*Hind*III digestion which gives 1.3kb *vgb* and 1.6 kb *aac*C3 gene and further confirmed by monitoring growth on LA containing gentamycin.

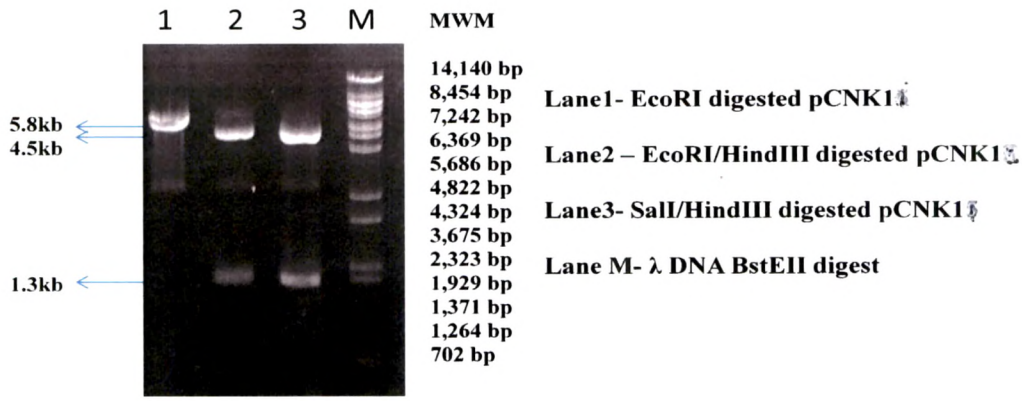

Fig.6.2 Restriction digestion pattern of pCNK11.

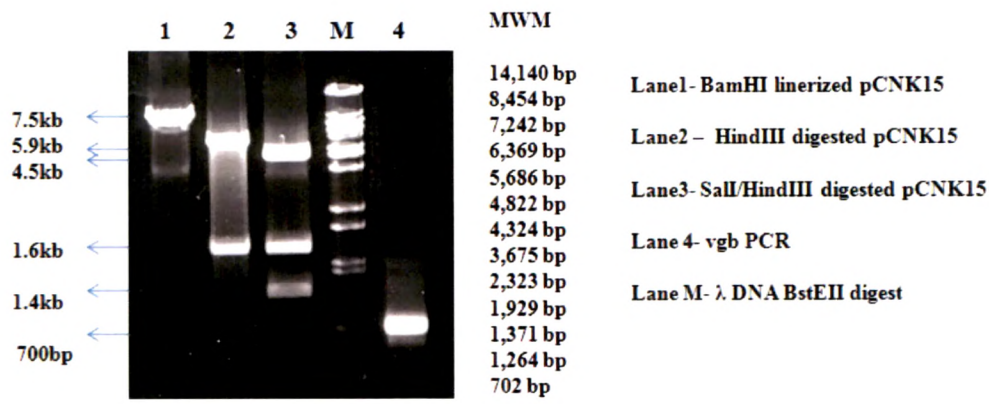

Fig.6.3 Restriction digestion pattern of pCNK15.

6.3.2 Construction of coexpressing *vgb* and *appA* gene in pTTQGm^r

To study the simultaneous expression of *vgb* and *app*A genes, pCNK21 was constructed. 1.3 kb fragment of *C. braakii* phytase expressing gene *appA* was excised out

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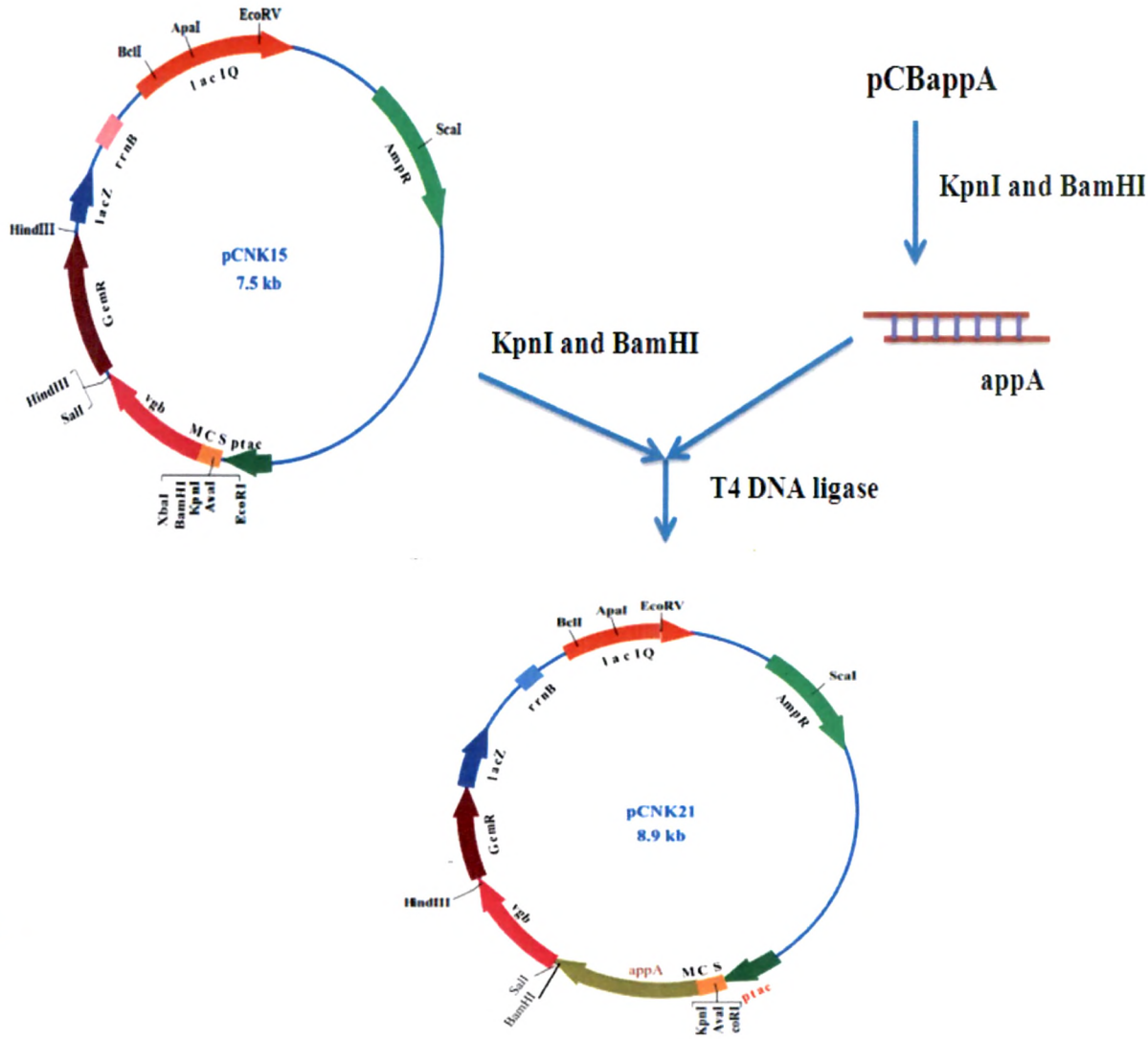


Schematic representation of pCNK21 cloning strategy

from pCBAppA by *Kpn*I and *Bam*HI and cloned in pCNK15 under control of *tac* promoter. Clone was confirmed by digestion with *Kpn*I/*Bam*HI and *Eco*RI/*Bam*HI which gives 1.3kb *appA* fragment and 7.3 kb vector backbone. *Eco*RI/*Hind*III digestion released of 2.6kb containing *vgb* along with *appA*, 1.6kb Gm^r and 4.5kb vector backbone. *Hind*III alone released 1.6kb Gm^r and 7kb vector backbone. Functionality of the *appA* gene was confirmed by enzyme activity.

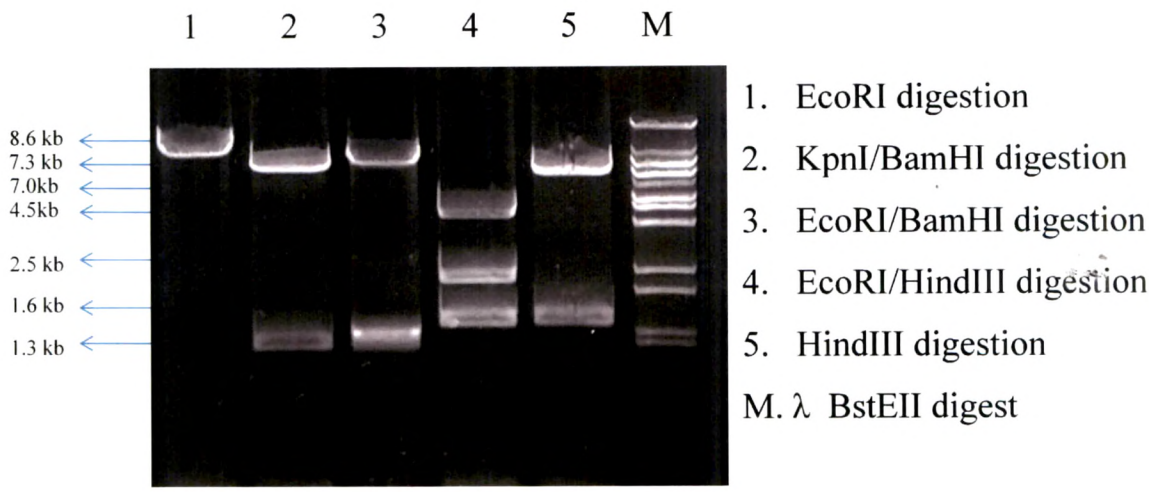
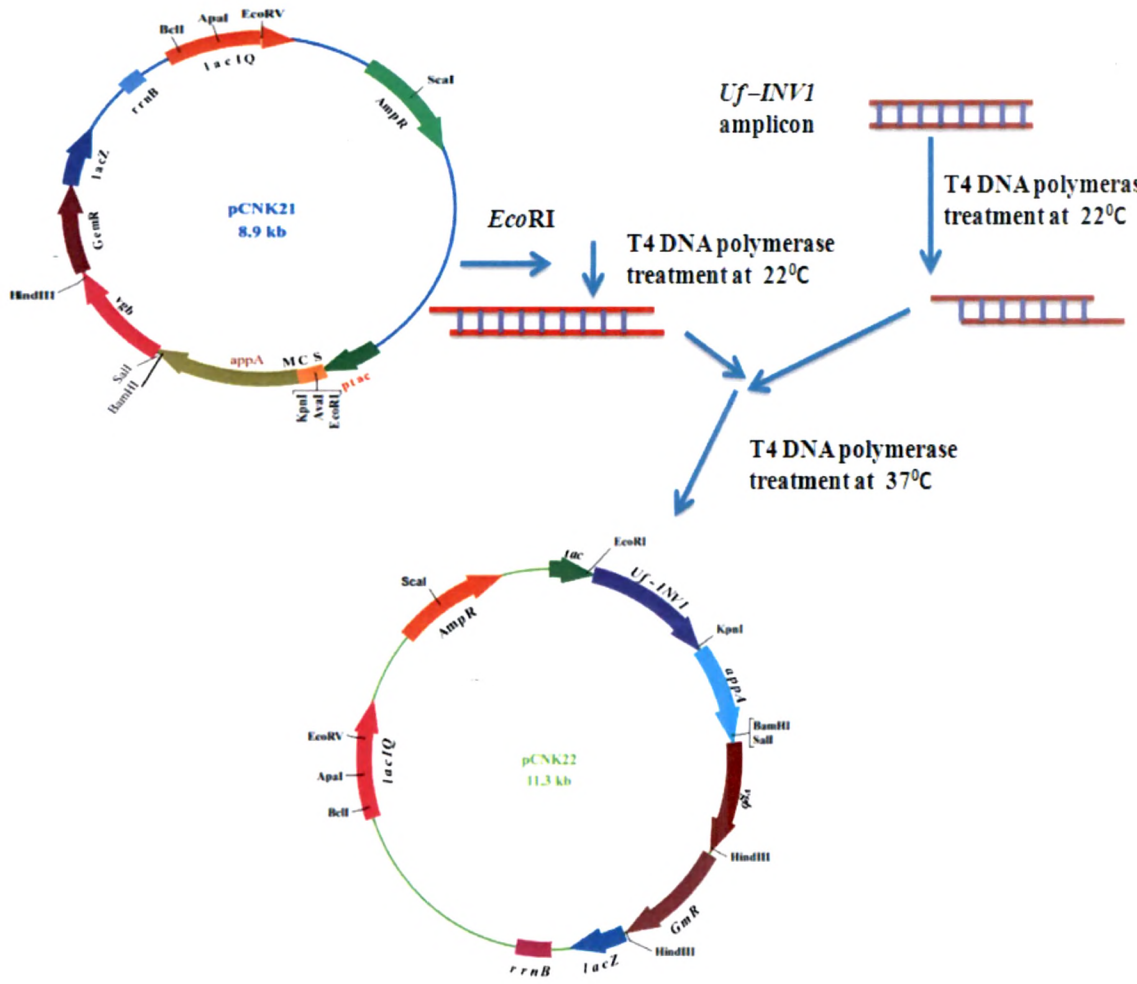


Fig.6.4 Restriction digestion pattern of pCNK21.

6.3.3 Construction of *Uinv, appA* and *vgb* gene cluster in pTTQGm^r

2.3 kb PCR amplicon of *Uinv* was obtained using gene specific primers having 20 nucleotide homology sequences with vector pCNK21. PCR product was ligated to the pCNK21. Positive clones were confirmed by *Hind*III digestion which gives 5.7, 3.9, and 1.6 kb fragments where as vector give release of 1.6 kb gentamycine with *Hind*III digestion and



Schematic representation of pCNK22 cloning strategy

further confirmed PCR amplification. Functionality of the gene was confirmed by *E. coli* transformant grown on sucrose M9 minimal medium and enzyme activity. Invertase enzyme activity was 0.3mU. Phytase and acid phasphatase activity was similar to *E. asburiae* PSI3 (pCNK21).

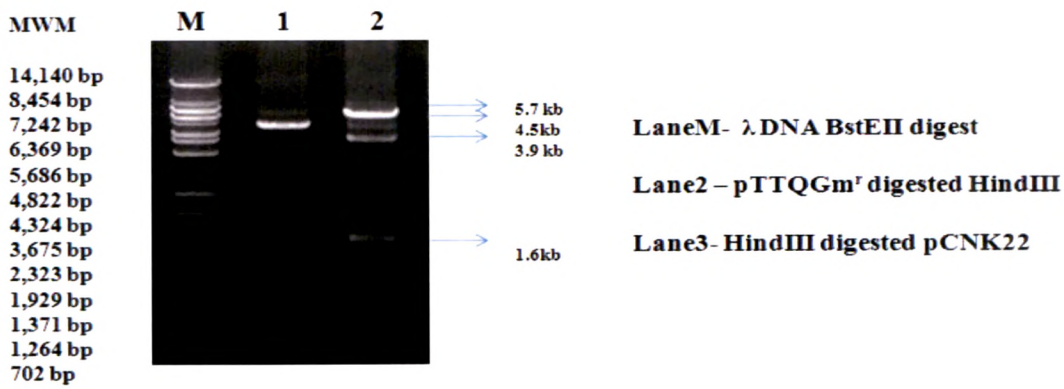


Fig.6.5 Restriction digestion pattern of pCNK22

6.3.4 Growth and MPS ability of *E. asburiae* PSI3 (pCNK15) in buffered TRP medium

E. asburiae PSI3 (pCNK15) showed acidification and MPS ability on TRP plates containing 60mM glucose and 100mM Tris buffer whereas *E. asburiae* PSI3 (pTTQGm^r) did not show MPS ability below 75mM glucose and 100mM Tris buffer. In liquid medium, *E. asburiae* PSI3 (pCNK15) grew up to 0.35 O.D. 600_{600} where as vector control showed 0.26 O.D. 600_{600}. *E. asburiae* PSI3 (pCNK15) acidified the medium up to pH 4.5 where as control failed to drop pH below 7.0 in 94 h. Pi release in medium was 0.53 mM whereas vector failed to release P. In TRP medium containing 100mM Tris and 60 mM glucose, enzyme activity of the *E. asburiae* PSI3 (pCNK15) was enhanced three fold compare to vector control (**Fig.6.6**).

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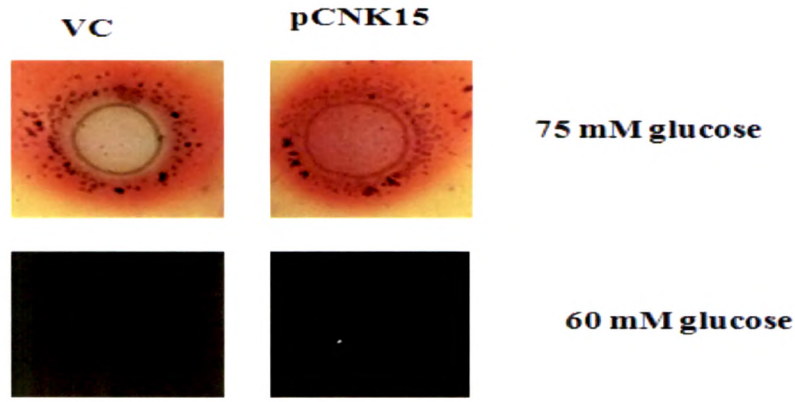

Fig. 6.6 MPS ability of *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK15) in TRP agar medium with glucose as carbon source.

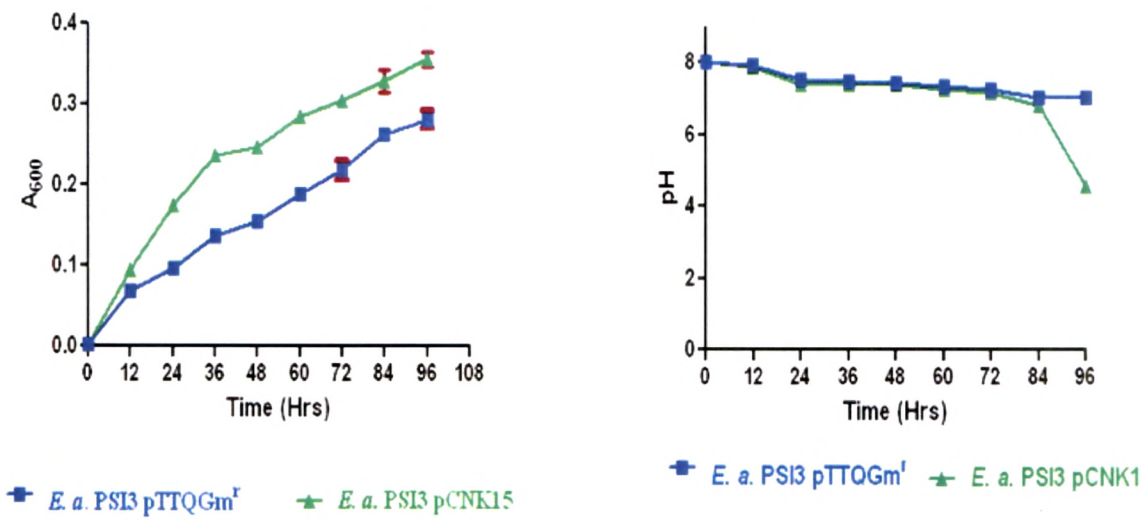

Fig. 6.7 Growth and pH profile of *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK15).

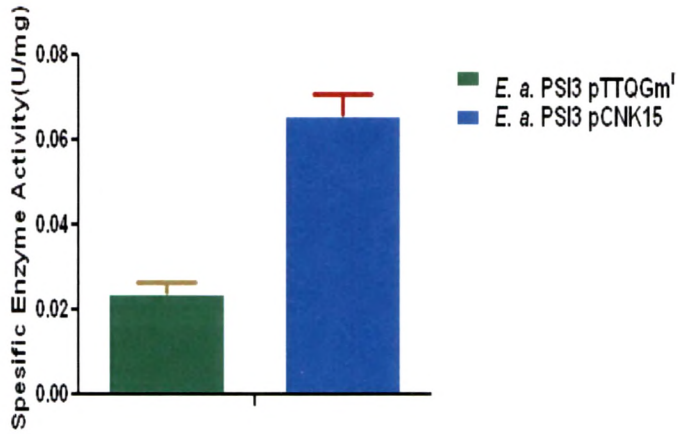

Fig. 6.8 GDH activity of *E. asburiae* PSI3 pTTQGm^r and *E. asburiae* PSI3 pCNK15.

Table 6.2- pH drop and Pi release in TRP medium after 96 h.

Recombinant strains	**pH**	**Gluconic acid (mM)**	**Pi (mM)**
E. asburiae PSI3 (pTTQGm^r)	6.97 +0.092	ND	UD
E. asburiae PSI3 (pCNK15)	4.49+0.117	38.36 ±1.65	0.53+0.016

6.3.5 Growth and MPS ability of pCNK21 unbuffered and Tris buffered phytate minimal medium.

Heterologous overexpression of *app*A gene showed ~1350 mU phytase activity where as vector control shows negligible activity. Acid phasphatase activity was also increased six fold compare to vector control (**Fig. 6.9**). *E. asburiae* PSI3 (pCNK21) and *E. asburiae* PSI3 (pTTQGm^r) showed similar growth pattern and pH drop when grew in unbuffered minimal medium containing 60 mM glucose and phytate as sole P source. Both transformants of

recombinant plasmids grew up to 0.25 O.D. 600 $_{600}$ and droped pH below 4.0 in 18 h. Thus, there was no difference in growth pattern was observed in unbuffered phytate minimal medium (**Fig. 6.11**). P released into the medium was 10 fold higher in *E. asburiae* PSI3 (pCNK21) as compared to vector control (**Table 6.3**). *E. asburiae* PSI3 (pCNK21) showed MPS ability on 100mM Tris phytate minimal medium containing 60mM glucose agar plates while vector control did not show any MPS ability in similar conditions (**Fig. 10**). In liquid medium, *E. asburiae* PSI3 (pCNK21) grew better than vector control due to the presence of *vgb*. *E. asburiae* PSI3 (pCNK21) grew up to 0.32 O.D. 600 whereas control grew up to 0.28 O.D. 600 Growth pattern of *E. asburiae* PSI3 pCNK21 indicated faster log phase as compared to vector control. *E. asburiae* PSI3 pCNK21 drops pH below 4.0 in 60 h but in vector control showed pH drop after 96 h. *E. asburiae* PSI3 (pCNK21) released 3.4 mM P in the medium where as vector control showed 0.17 mM of P release. Vector control releases these P in medium due to inherent alkaline phytase activity of *E. asburiae* PSI3 (Patel et al., 2010).

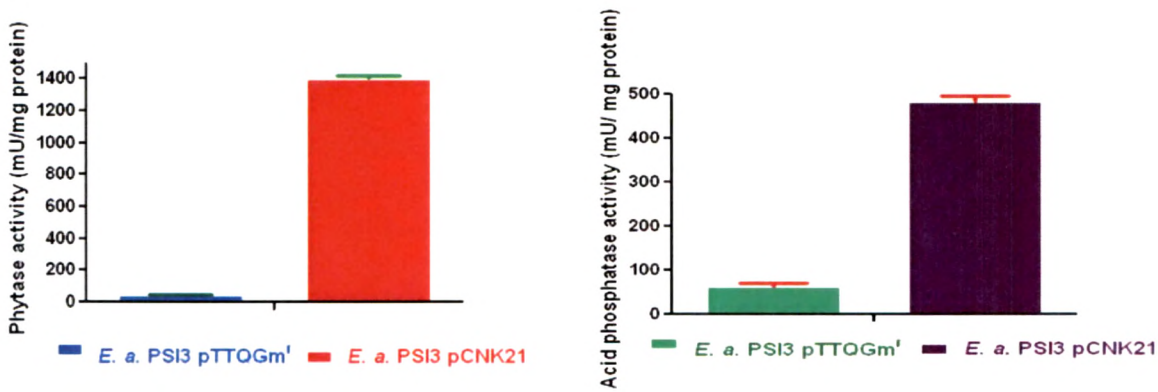

Fig. 6.9 Phytase and acid phosphatase activity of *E. asburiae.* PSI3 (pCNK21) and *E. asburiae* PSI3 (pTTQGm^r).

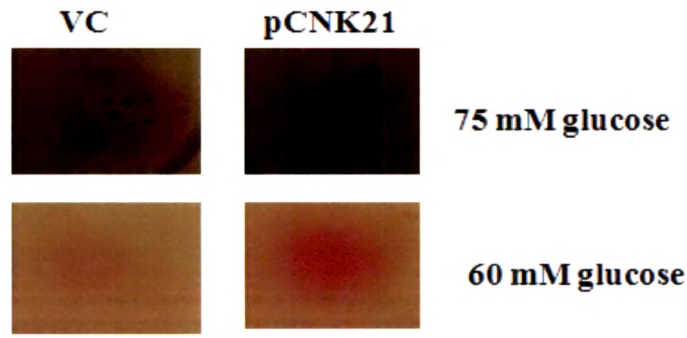

Fig. 6.10 MPS ability of *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK21) in Tris phytate agar medium with glucose as carbon source.

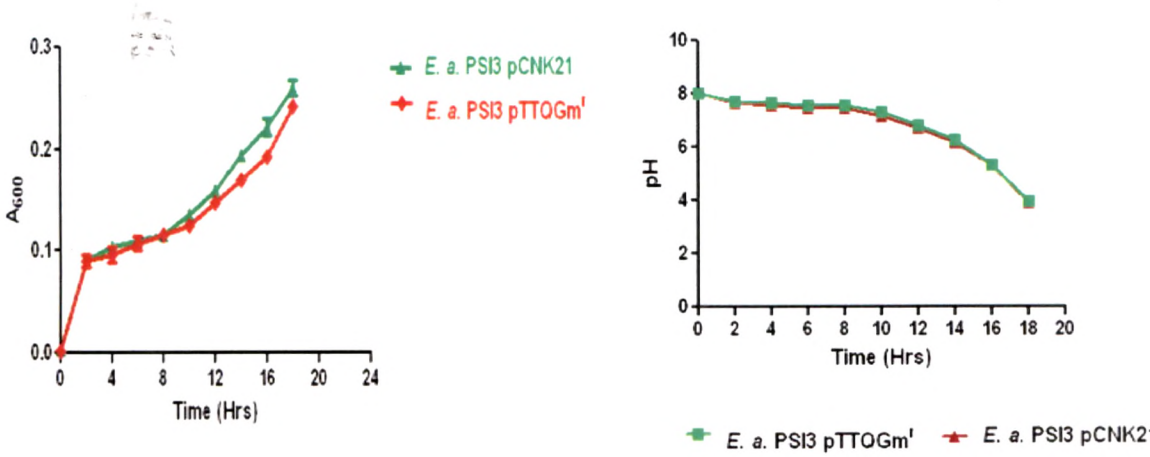

Fig. 6.11 Growth and pH profile of *E. asburiae* PSI3 (pCNK21) and vector control in unbuffered condition.

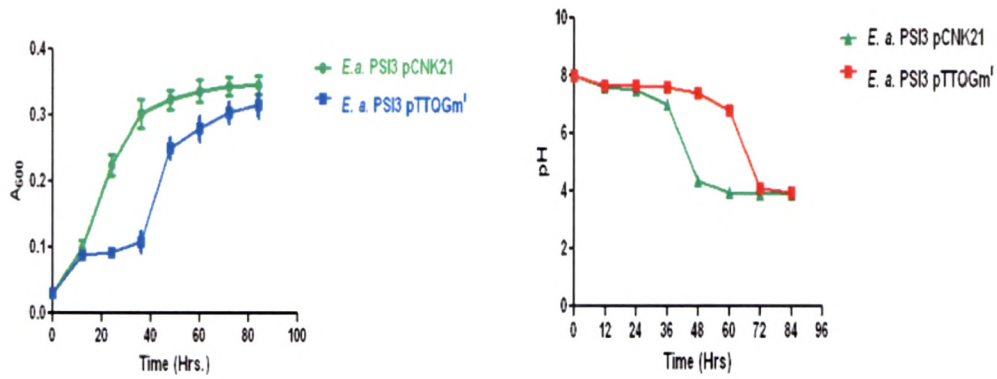

Fig. 6.12 Growth and pH profile of E. asburiae PSI3 (pCNK21) and vector control in buffered condition.

Table 6.3 pH drop and Pi release in varying Tris buffered phytate minimal medium with varying glucose.

E. asburiae PSI3 **Transformants**	**Tris buffer (mM) and glucose (mM)**	**Gluconic acid (mM)**	**pH**	**Pi (mM)**
pTTQGm^r	0+ 75	41.2±1.7	3.98±0.11	0.313±0.017
pCNK21	0+ 75	48.5±2.3	3.91±0.046	3.20±0.065
pTTQGm^r	100 + 75	42.3±01.83	3.98±0.22	0.107±0.037
pCNK21	100 + 75	48.7±2.4	3.92±0.04	3.408±0.046
pTTQGm^r	100 + 60	ND	7.03±0.07	UD
pCNK21	100 + 60	39.7±1.9	4.35±0.11	3.1±0.053

6.3.6 Growth and phytate mineralization of pCNK22 in Tris buffered phytate minimal medium containing sucrose and mixture of sugar

MPS ability of *E. asburiae* PSI3 (pCNK22) was monitored on 75mM Tris buffer pH-8.0 phyate minimal medium containing 50mM sucrose. *E. asburiae* PSI3 (pCNK22) showed MPS ability while vector control fail to give MPS ability on sucrose. In liquid medium, *E. asburiae* PSI3 (pCNK22) grew up to 0.52 O.D. $_{600}$ and dropped the pH up to 4.3 in 96 h, released 2.8mM P in the medium and produced 21mM gluconic acid while vector control had no gluconic acid and P was not detected.

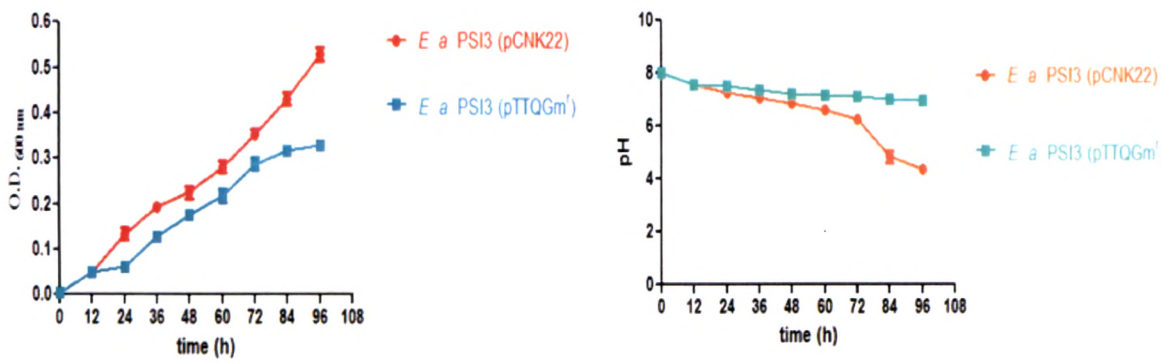

Fig. 6.13: Growth and pH profile of *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK22) in Tris phytate minimal medium containing 75mM Tris and 50mM sucrose.

When mixture of 8 sugars (D-glucose, L-Arabinose, D-Xylose, D-galactose, D-Maltose, D-Mannose, D-Cellobiose and Sucrose), each at 10 mM was used in 100 mM Tris buffered (pH-8.0) condition cultures grew up to 0.6 O.D. $_{600}$ and drop pH to 4.0 in 60h. *E. asburiae* PSI3 (pCNK22) releases 3.3 mM P in buffered medium. While vector control grew till 0.35 O.D. $_{600}$ but failed to drop pH below 7.0.

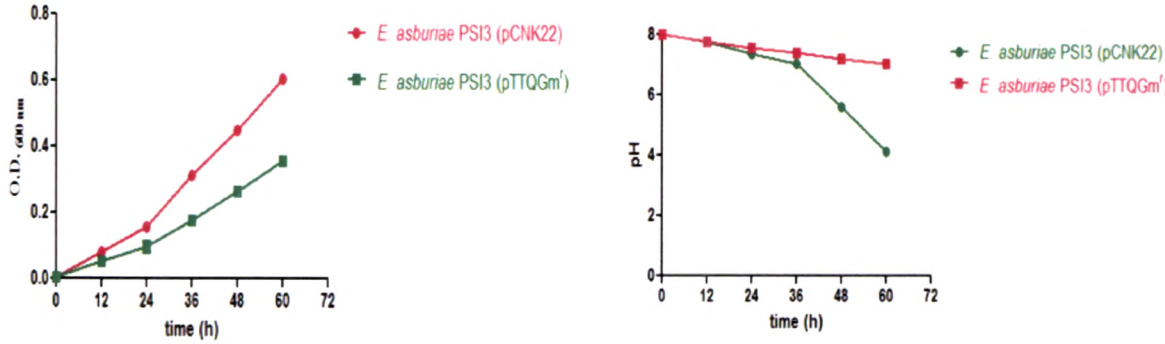

Fig. 6.14 Growth and pH profile of *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK22) in Tris phytate minimal medium containing 100 mM Tris and mixture of sugars (each at 10 mM).

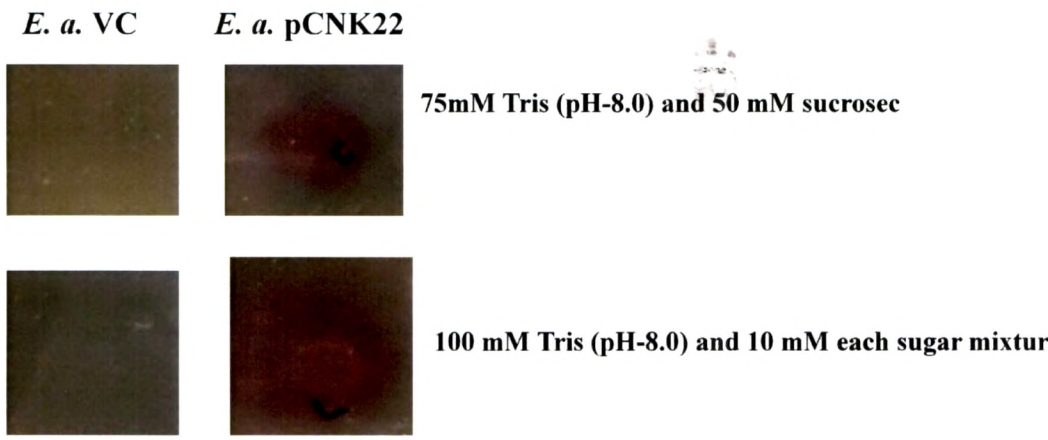

Fig. 6.15 Ca- phytate mineralization by *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK22) in buffered phytate minimal medium containing 50 mM glucose and mixture of sugars (each at 10 mM).

6.4 Discussion

vgb gene expression in *E. asburiae* PSI3 improved growth, enhanced three fold GDH activity and showed better MPS ability compare to vector control in buffered TRP medium. This result is agreement with 37.36% increase in the production of dihydroxyacetone (DHA) after incorporation of VHb in *Gluconobacter oxydans* (Li et al., 2010). Glycerol dehydrogenase is a membrane bound PQQ dependent which uses oxygen as final electron acceptor (Cleret et al., 1994). Thus similar mechanism could operate in PQQ dependent GDH conversion of glucose into gluconic acid.

Coexpression of *appA* and *vgb* in *E. asburiae* PSI3 improved growth, mineralization of Ca-phytate and P release in buffered medium at lower amount of sugar but it did not affect the AppA enzyme activity. VHb did not improved alkaloid production in *Hyoscyamus muticus* hairy root cultures (Wilhelmson et al., 2006), ectomycorrhizal fungus *Apsen* induced root formation (Zokipii et al., 2008) and submerge tolerance in white poplar (Zelasco et al., 2006). On the other hand, coexpression of *appA*, *Uinv* and *vgb* genes improved phytase mineralization in *E. asburiae* PSI3 on sucrose and 10 mM of each of a mixture of sugars while 15 mM each of sugar mixture required in case of wild type organism. These results indicate that phosphate mobilization by *E. asburiae* PSI3 can be further improved by incorporation of *gad* operon along *appA*, *Uinv* and *vgb* genes.