CHAPTER 5

Phosphate mineralization and solubilization abilities of *E. asburiae* PSI3 containing *Aspergillus fumigatus* phytase (*phyA*) gene and *C. braakii* Phytase (*appA*)

5.1 Introduction

Organic phosphorus generally present in soil varies from 29-65% depending upon the nature of soil. In soils, predominant form of organic phosphate is *myo*-inositol hexakisphosphate (phytic acid) or its salts (Turner et al., 2002). Presence of six phosphate groups provide high charge density to IP6 therefore it undergoes strong interaction with the soil or being adsorbed to clays (Anderson and Arlidge, 1962; Celi et al., 1999) or precipitated as insoluble salts such as sesquioxides of Fe and Al in acidic soil and insoluble calcium salts in alkaline soils (Jackman and Black, 1951). Due to this strong reaction, interaction of phytate with hydrolyzing enzyme prevented which results in accumulation of inositol phosphate as major organic P in most of the soil (Harrison., 1987). Conversion of hexakisphophate to monophosphate decrease numbers of P groups associated with inositol decrease adsorption in soil due to decrease charge densities of the molecule (Anderson and Arlidge, 1962; Anderson and Malcom, 1974). pH of the soil play major role in stabilization of inositol phosphate (Hawkes et al.,1984). Complexes of inositol phosphates with Fe, Al, Ca and clays show pH dependent variations in solubility, being most stable at pH 5 and 7.5 (Jackman and Black,1951; Emsley and Niazi, 1981), which is further regulated by the type of cations (Celi et al., 2001).

Adsorbed form of phytate can not be taken up by plants due to the absence of phytase secretion from plant roots (Hayes et al., 1999; Richardson et al., 2000). Non specific enzymes like phosphatases secreted by soil microorganism and roots are unable to break phosphomonoester bond of phytates (Turner et al., 2002; Mudge et al., 2003). However, lower form of phytate hydrolysed by phytase can be acted upon non specific phosphatase like acid or alkaline (Wodzinski and Ullah, 1996). In soils, phytase positive microorganisms account for 30-50% total microflora (Greaves and Webley, 1965;

Richardson and Hadobas, 1997) and phytase positive rhizobacteria are in the range of 3-19 % in the rhizosphere of different plants (Patel et al., 2010). But phytate remains most recalcitrant pool of organic phosphate in soils (Ognalaga et al., 1994) due to inability of the phytase to act upon adsorbed and insoluble form. These complexes are insusceptible to enzymatic hydrolysis by all the three major types of phytases (Tang et al., 2006). Another reason for the inability of phytase producing rhizobacteria to increase availability of phytate P to plants is lack of suitable condition that favour optimum enzyme production and activity in soil.

Phytase enzymes secreted by several rhizosphere isolates belong to the genera *Pantoea, Citrobacter, Enterobacter, Klebsiella, Burkholderia, Pseudomonas, Bacillus* etc. Enzyme can be secreted in cell bound (periplasmic) or secreted form (Unno et al., 2005; Rodriguez et al., 2006; Lim et al., 2007; Patel et al., 2010). Expression of phytase gene in bacteria is inducible and is controlled by complex regulation (Konietzny and Greiner, 2004). In case of *Raoultella terrigena* (*Klebsiella terrigena*), phytase activity increased markedly at the stationary phase in the presence of glucose (0.25 %) in minimal medium compared to glucose supplied in nutrient rich LB medium (Zamudio et al., 2002). Phytase activity was subject to catabolite repression where cyclic AMP (cAMP) showed a positive effect in expression during exponential growth and a negative effect during stationary phase. *appA* gene encodes phytase in *E. coli* and is induced under nutrient rich, P deficient, stationary phase and anaerobic conditions (Greiner et al., 1993, Konietzny and Greiner, 2004). Level of inorganic phosphate generally controls regulated inhibition of phytase in all microbial phytase producers, including moulds, yeast and bacteria except *R. terrigena* and in the rumen bacteria (Yanke et al., 1998; Konietzny and Greiner, 2004; Greiner, 2007). In *Mitsuokella jalaludinii* and *Pseudomonas* sp., phytase was found to be

induced by substrate or by myo-inositol as the sole carbon source, respectively (Konietzny and Greiner, 2004). This condition does not appear suitable for successful application of phytase secreting bacteria for mobilizing P from soils phytates.

In present study the phytase genes of *A. fumigatus* and *C. braakii* were incorporated in P solubilizing rhizobacterium *E. asburiae* PSI3 to enhance organic phosphate mineralization.

5.2 Material and Methods

5.2.1 Bacterial strains, plasmids and growth condition

Table 5.1 Bacterial strains and plasmid 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., 1998b
Plasmids		
pCBappA	pBBR1MCS-2 containing *Citrobactor braakii* phytase (*app*A) Kan^r	Patel et al., 2010
pUCphyA	pUC18 containing *Aspergillus fumigatus* phytase gene (*phy*A) Amp^r	Pasamontes et al., 1997
pTTQKan^r	Amp^r, Kan^r, *tac* promoter	This study
pUC18T-mini-Tn*7*T-Gm-*eyfp*	Amp^r, Gm^r, *eyfp*	Choi et al., 2006

CNK9	Amp^r, Kan^r , pTTQKan^r containing *Aspergillus fumigatus* phytase gene (*phy*A)	This study
pCNK10	Amp^r, Kan^r , pTTQKan^r *C. braakii* phytase (*app*A)	This study
pCNK13	Amp^r, Gm^r, pUC18T-mini-Tn*7*T-Gm-*eyfp*, constitutive *tac, C. braakii* phytase (*app*A)	This study

5.2.2 Incorporation of *Aspergilus fumigatus* phytase (*phy*A) gene under *tac* promoter vector pTTQKan^r

The pUC18 based vector pTTQ18 was used to clone *phy*A of *A. fumigatus* and *app*A of *C. braakii.* Since *E. asburiae* PSI3 has ampicillin resistance, it was necessary to incorporate another antibiotic marker gene addition with phytase gene. Hence following was strategy employ to clone both genes.

Step1- Incorporation of kanamycine resistance gene to pTTQ18

The Kan^r gene fragment was obtained from pYanni3 plasmid by digesting with *Bam*HI and *Hind*III. The ~1.6 kb fragment containing Kan^r was ligated to the purified plasmid pTTQ18 to obtain pTTQKan^r. Ligation mixture was transformed into *E. coli* DH10B and transformants were selected on LA plates containing ampiciline and kanamycine. The plasmid isolated from the transformants was confirmed for the presence of kan^r by restriction digestion with *Bam*HI/*Hind*III and *Pst*I.

Step 2:- Incorporation of *phy*A gene of *A. fumigatus* in expression vector pTTQKan^r

Plasmid pUCphyA was digested with *Eco*RI and *Bam*HI to obtain the 1.3kb *phy*A gene. 1.3 kb fragment was gel eluted and ligated to purified plasmid pTTQKan^r digested with *Eco*RI and *Bam*HI to obtained pCNK9. Positive clone was confirmed by restriction digestions.

5.2.3 Incorporation of *Citrobactor braakii* phytase (*app*A) gene under *tac* promoter in vector pTTQKan^r

Plasmid pCBAppA was digested with *Kpn*I and *Bam*HI to obtain the 1.3 kb *app*A fragment. *app*A fragment was gel purified and ligated to gel purified plasmid pTTQKan^r digested with *Kpn*I and *Bam*HI to obtained pCNK10. Positive clone was confirmed by restriction digestions and PCR amplification with gene specific primers.

5.2.4 Incorporation of *Citrobactor braakii* phytase (*app*A) gene under constitutive *tac* promoter in pUC18T-mini-Tn*7*T-Gm-*eyfp*

*app*A gene was PCR amplified using primers

Ctac FP2 as forword primer

TCGTATAATGGATCGAATTGTGAGC GGAATCGATTTTCACACAGGAAA

And *appA* RP as reverse primer

TCTTTGCAAAAGATCAAACTTTATTCCGTAACTGCACACT .

To make *app*A under control of constitutive *tac* promoter second PCR was done using

Ctac FP1 CGCGCAACGAGCTCACTAGTGGATCTTGACAATTAATCATCGGC*TCG TATAATGGATCGAATTGTGAGC*

as a forward primer and appA RP as reverse primer. Final PCR product of *app*A with constitutive *tac* was cloned in *Stu*I digested pUC18T-mini-TnT-Gm-*eyfp* to obtained pCNK16. Positive clone were confirmed by RE digestion and PCR amplification.

5.2.5 Functional confirmation of phytase gene expressed from pCNK9 and pCNK10

pCNK9 and pCNK10 was transformed in *E. coli* DH10B. Functionally the transformants were screened by qualitative estimation of their phytase activities. Transformants were inoculated into 3 ml LB broth containing 50µg/ml kanamycin and incubated on orbital shaker for 16 h at 37°C. The overnight grown culture suspension was taken into 1.5 ml centrifuge tube and tubes were centrifuge for 1 min at 13,000 × g. The supernatant was removed and cell pellet was suspended into1 ml autoclaved normal saline, centrifuged for 1 min at 13,000 × g. Further the pellet was resuspended in to 1 ml autoclaved deionized H_2O and 5µl of lysozyme (30 mg/ml) solution was added. The content was mixed by vortexing and incubated for 1 h at 37 ° C. After incubation, 1 ml of 0.1 M Na-acetate (pH 4.5) buffer containing 250 nmol of Na-phytate was added in to tube and it was incubated for 1 h at 37° C.

5.2.6 Phytase and phosphatase activity

E. asburiae PSI3 transformants were grown in 30 ml LB media at 37°C till they reached stationary phase. Cell-associated acid phytase and phosphatase activity of transformatns were checked as described in Material and Method section. In case of pCNK9 phytase enzyme activity was done at pH-4.0 and 8.0.

5.2.6 Phytate mineralization ability of *E. asburiae* PSI3 (pCNK9, pCNK10 and pCNK13) transformants

Ca-phytate hydrolysis by transformants was studied in tris buffered minimal medium containing 100mM tris, 75mM glucose, NH_4Cl, 10mM; KCl, 10mM; $MgSO_4$, 2mM; $CaCl_2$, 0.1mM; micronutrient cocktail and 100 µM calcium chloride ($CaCl_2$). Ca-phytate (0.3%) was used as the sole source of P and added separately after autoclaving.

For plate assay 1.5% agar and methyl red was added. After inoculating the bacterial cultures, the pH of medium, O.D. and P released in solution was measured in aliquots sampled at regular time intervals. In case of plate assay appearance of red zone was monitored.

5.2.7 Root colonization study

Mung bean (*Vigna radiata*) seeds were surface sterilized with 0.1% $HgCl_2$ followed by 70% ethanol wash thoroughly. Finally, washed thrice with sterile distilled water and the seeds were germinated in distilled water. The sterile grown seeds were transferred to standardized hydroponic plant assay system (half strength MS liquid medium) in sterile test tubes containing paper wick moistened with 15 ml medium. Germinated seeds were incubated with saline washed suspension of *E. asburiae* PSI3 transformants containing at least 10^8 exponential phase grown cells for 30 min. Three grown seeds were placed on paper wick. Similar sets of seeds were also inoculated in non sterile soil. Root was protected from light by wrapping aluminum foil to lower portion of test tube. Test tubes kept at 30^0C for 18 h with light and 8h in dark. After 5 day incubation plants root were washed with sterile saline. For Confocal microscopy 1-2cm root pieces were cut and mounted with phosphate buffer saline containing glycerol on glass slide. Root colonization study was done using Laser Scanning Confocal Microscope (LSM 410, Zeiss Welwyn Garden City, UK). Root colonization ability of *E. a.* PSI3 in non sterile soil was monitored on 5^{th} and 10^{th} day.

5.3 Results

5.3.1 Construction of plasmid pCNK9 containing *phyA* gene of *A. fumigatus* under *tac* promoter

The schematic representation of the cloning strategy is depicted in **Fig. 5.1**. 1.3 kb *phy*A was cloned into pTTQKan^r under *Eco*RI and *Bam*HI RE sites. Clone was confirmed by digestion with *Eco*RI and *Bam*HI which gives release of 1.3 kb *phy*A fragment and 6.1 kb vector backbone. RE digestion with *Eco*RI/*Hind*III gives release of 1.6 kb kanamycine, 1.1kb from *phy*A gene due to presence of two *Hind*III sites in the gene and vector backbone of 4.7. Functionality of the cloned gene was confirmed by enzyme assay.

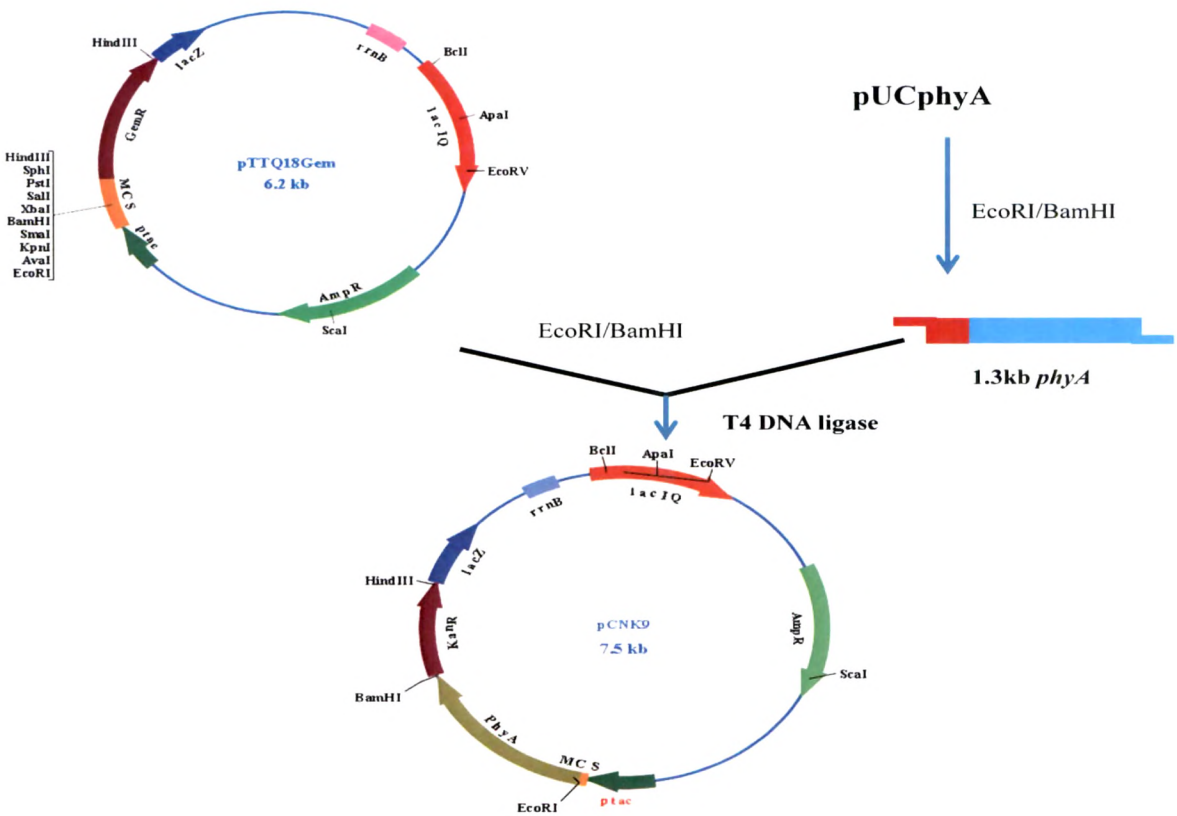


Fig. 5.1 Schematic representation of pCNK9 cloning strategy

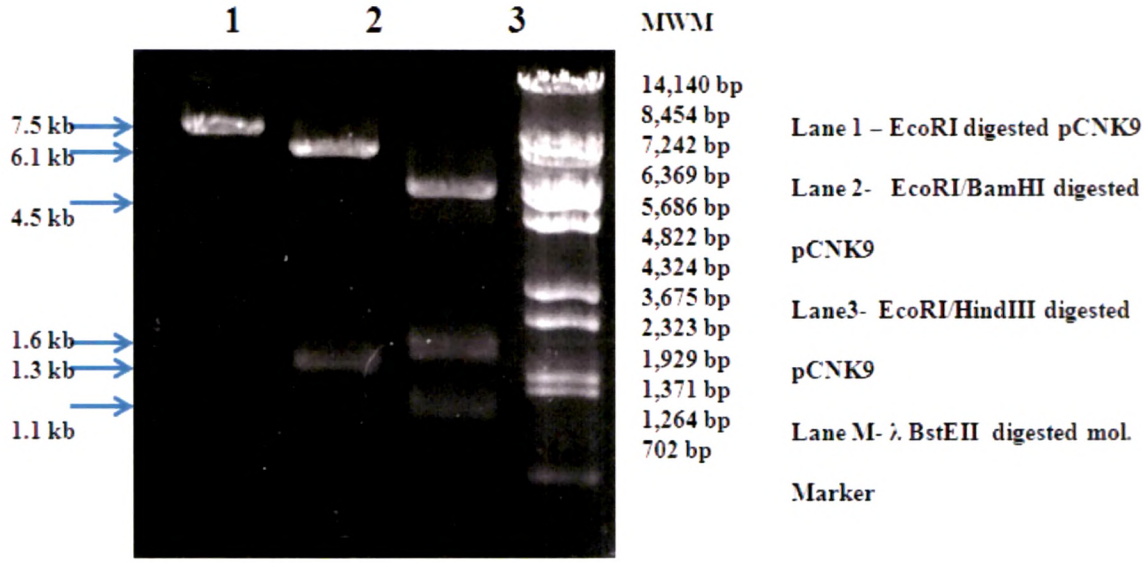


Fig. 5.2 Restriction digestion pattern of pCNK9

5.3.2 Enzyme activity

Enzyme activity of *E. a.* PSI3 (pCNK9) and *E. a.* PSI3 (pTTQGm^r) was monitored on pH- 8.0 to mimic the soil buffering condition. At pH-8.0 enzyme activity was 2.5 fold more as compared to vector control.

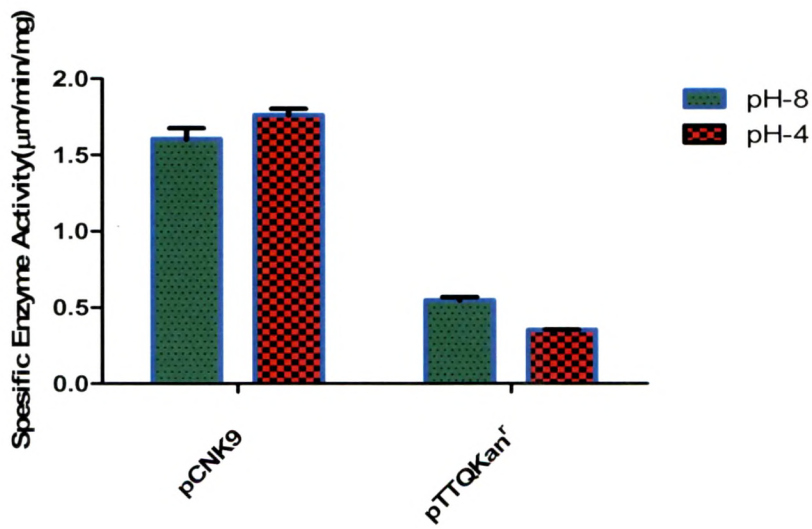


Fig.5.3 Enzyme activity of pCNK9 at pH-8.0 and 4.0

5.3.3 Construction of plasmid pCNK10 containing *appA* gene of *Citrobactar braakii* in pTTQKan under *tac* promoter

pCBappA was digested with *Kpn*I and *Bam*HI which gives ~1.3 kb release of *app*A. 1.3kb fragment was cloned under *Kpn*I and *Bam*HI RE site of pTTQKan^r. Positive clone was confirmed by digestion with *Kpn*I and *Bam*HI which give release of 1.3kb *app*A and 6.1kb vector backbone. *Eco*RI/*Hind*III digestion will give 2.9 kb appA with 1.6kb kanamycine and 4.5kb vector backbone. *Eco*RI/*Bam*HI/*Hind*III digestion gives release of 1.3kb *app*A, 1.6kb kanamycine and 4.5kb vector backbone. Functionality of the clone was confirmed by enhanced phytase activity of clones was observed by release of more P from Na-phytate compared to vector control strain using inorganic P estimation method (Ames 1979). Phytase enzyme activity was found 1.4 U in *E. asburiae* PSI3 where as in vector control it was 0.1U due to inherent alkaline phytase activity. *app*A possess acidic phasphatase activity along with phyatse activity was found 0.5U where as vector control showed 0.04U.

5.3.4 Construction of plasmid pCNK13 containing *appA* gene of *Citrobactar braakii* in pUC18T-mini-Tn*7*T-Gm-*eyfp* under constitutive *tac* promoter

1.5 kb PCR product of *appA* with constitutive *tac* promoter was obtained. This PCR product was cloned in pUC18T-mini-Tn7T-Gm-*eyfp*. Positive clone was confirmed by *Eco*RI and *Sac*I digestion which released gives release while vector control get linearize (**Fig. 5.6**).

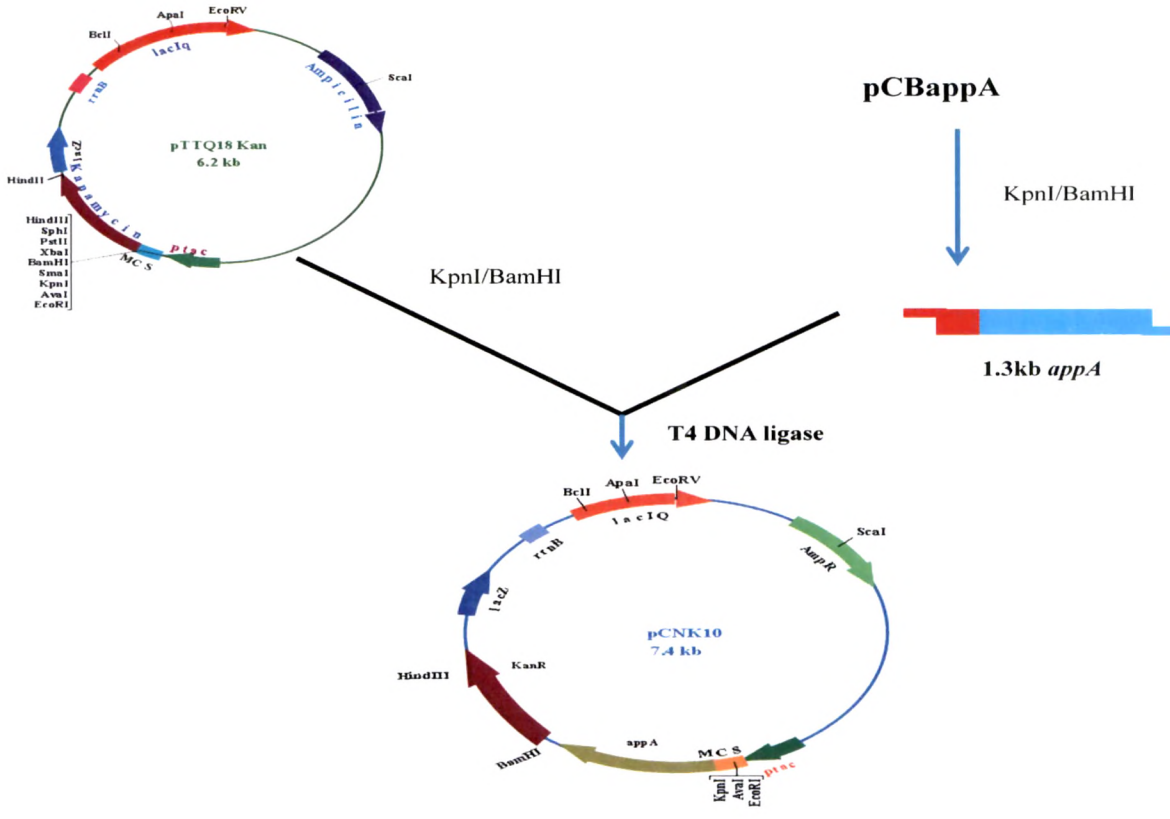


Fig. 5.4 Schematic representation of pCNK10 cloning strategy

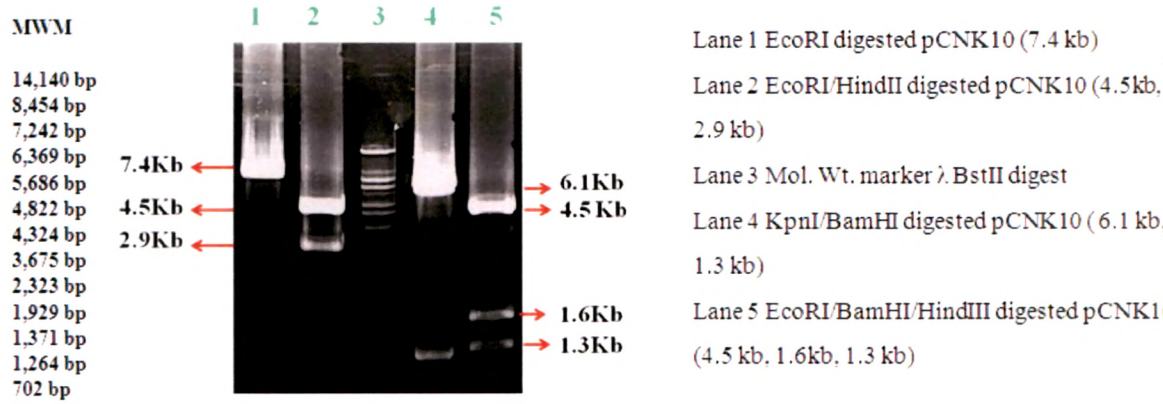
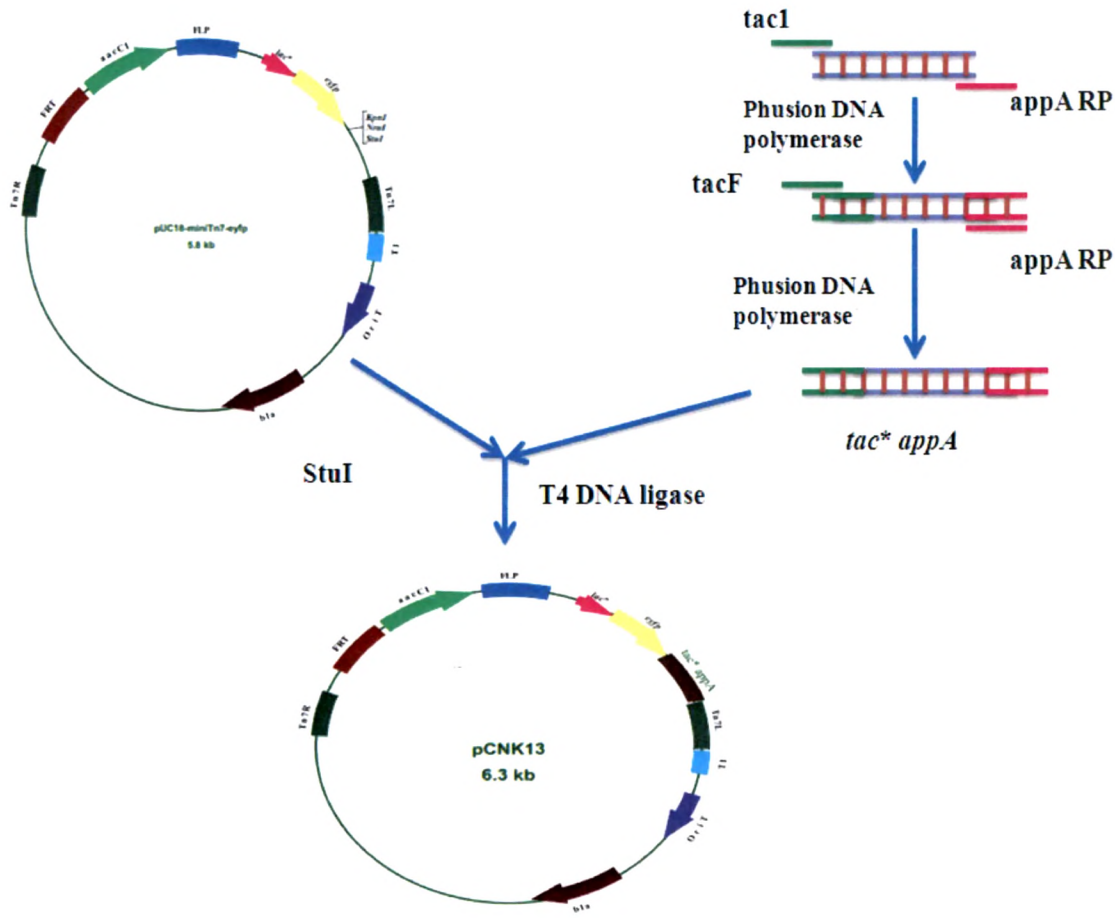


Fig. 5.5 Restriction digestion pattern of pCNK10.



Schematic representation of pCNK13 cloning strategy

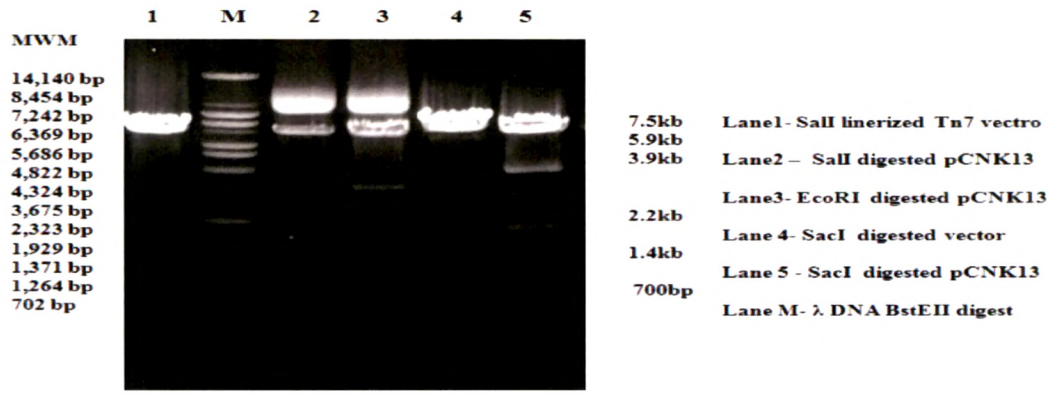
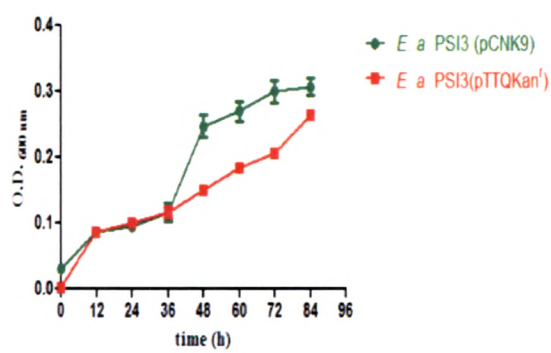
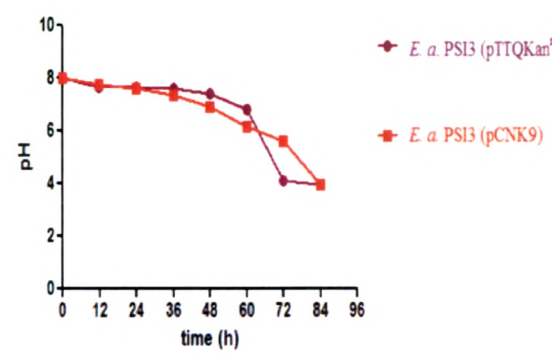


Fig. 5.6 Restriction digestion pattern of pCNK13.

5.3.5 Effect of *A. fumigates* and *C. braakii appA* overexpression on organic phosphate mineralization ability of *E. asburiae* PSI3

E. asburiae PSI3 (pCNK8,9 and 10) showed acidification and phytate mineralization on 100mM Tris buffered pH-8.0 phytate minimal medium containing 75mM glucose while vector control acidify the medium but not able to mineralize the phytate. In Tris phyate minimal medium, organism grew till 0.4 O.D. and dropped pH below 4.0 in 84 h (**Fig. :**). ۵.۶





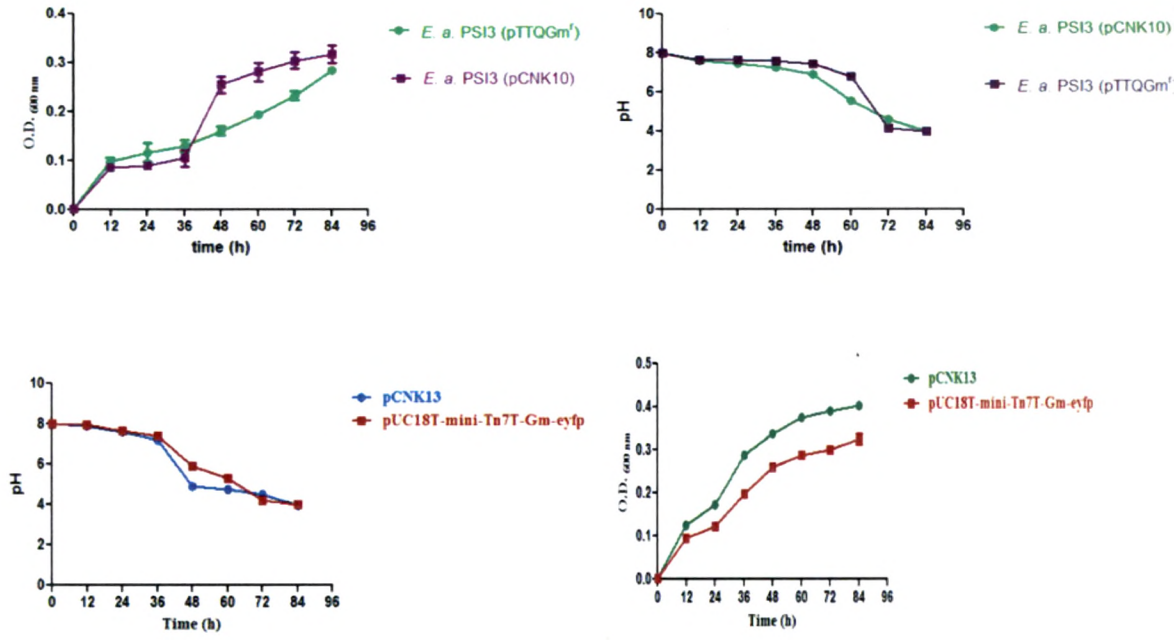


Fig.5.7: Growth and pH profile of *E. a.* PSI3 (pCNK9, pCNK10 and pCNK13) in 100 mM Tris buffered pH-8.0 phytate minimal medium containing 75 mM glucose.

pCNK9, 10 and 13 secreted ~43 to 47mM gluconic acid and release 3.93, 4.51 and 4.46 mM inorganic P, respectively in buffered Ca-phytate minimal medium (**Table 5.2**).

Table 5.2 Organic acid secretion and P release in Tris buffered (pH-8) phytate minimal medium by *E. asburiae* PSI 3 (pCNK9, 10 and 13)

E. asburiae PSI 3	Gluconic acid (mM)	Final pH	P release from Ca-phytate (mM)
pTTQKan^r	39.2±1.3	3.97±0.022	0.123 ±0.021
pCNK9*	43.3±1.45	3.96±0.046	3.93±0.154
pCNK10*	45.7±2.6	4.00±0.035	4.51±0.065
pUC18T-mini-Tn7T-Gm-eyfp	41±1.7	4.03±0.017	0.135 ±0.027
pCNK13	47.3±2.3	3.95±0.058	4.46±0.035

* In presence of 0.2mM IPTG.

5.3.6: Root colonization ability of *E. asburiae* PSI3 (pCNK13) in non sterile soil

Colonization study in non sterile soil showed heavy colonization on root tip of Mung bean after 5 day of inoculation (**Fig. 5.8**). Colonization in the soil decrease with the time. After 10 day colonization was reduce in the non sterile soil (**Fig. 5.9**).

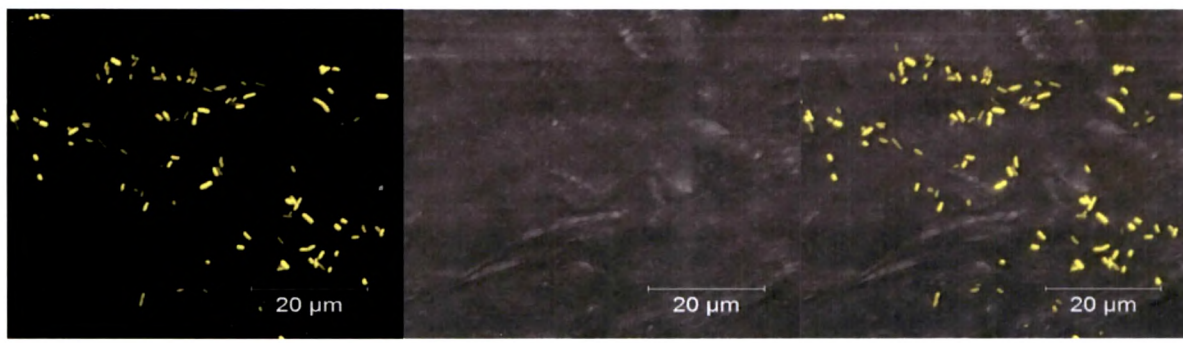


Fig. 5.8: Colonization of *E. asburiae* PSI3 (pCNK13) in non sterile soil after 5 day of inoculation

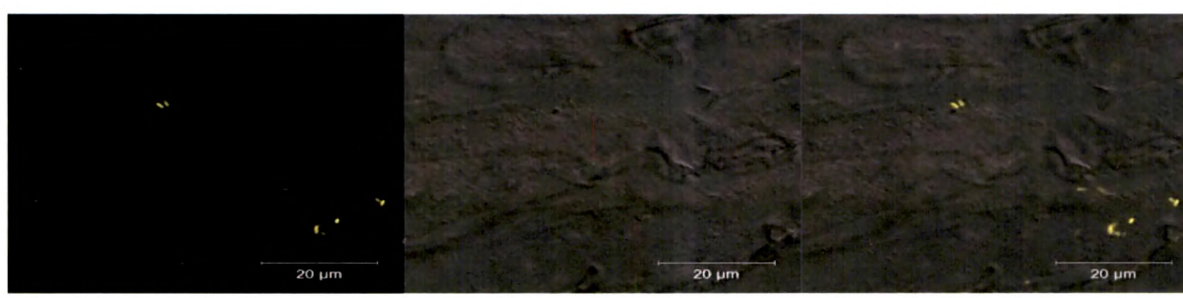


Fig. 5.9: Colonization of *E. asburiae* PSI3 (pCNK13) in non sterile soil after 10 day of inoculation.

5.4 Discussion

Phosphate solubilizer rhizobacteria *E. asburiae* PSI3 has been explored for phytate mineralization by heterologous overexpression of *C. braakii appA* and *A. fumigatus phyA* genes. *app*A gene of *C. braakii* encodes phytase which belongs to HAP family and is highly specific for phytate but it also possesses acid phosphatase activity (Kim et al., 2007). PhyA catalyzes phytic acid hydrolysis over range pH 2.5-8 (Luise et al., 1997). Overexpression of *phyA* in *E. asburiae* PSI3 under *tac* promoter showed 1.7 U activity while PhyA enzyme activity ranges from 23-28U in fungi. *A. fumigatus* phytase gene expression in *Bacillus mucilagenosus* increased phytase activity by 46 fold (Li et al., 2005). A large number of bacterial phytases have been expressed in *E. coli* using various expression systems (**Table 5.3**). *C. braakii appA* overexpression from *tac* promoter enhanced phytase level 12 fold in *E. asburiae* PSI3 compared to vector control while acid phosphatase activity was enhanced 6 fold. Level of expression from *lac* and *tac* promoters is similar in *E. asburiae* PSI3 (Patel et al., 2010). Generally, non-specific phosphohydrolases play a major role in the release of last stage P from phytate (Turner et al., 2002). Thus AppA possessing both phytase and acid phosphtase activities will be very efficient in complete hydrolysis of phytate and could be beneficial in the field condition.

Environmental conditions are very important for the efficacy of phytases in the soil. Phytase expressing transgenic plants unable to release P from phytate as phytate is present in bound form with cations (Lung and Lim, 2006). Hence, microorganisms possessing organic acid secretion and phytase enzyme could release P from soil phytates (Patel, 2009). *E. asburiae* PSI produces high amount of gluconic acid and AppA which is sufficient to solubilize bound phytate and mineralize phytate. HAP type phytases are most

suitable for the field condition due to its resistance to the metal ions binding agent and organic acid which affects the activity of enzyme (Tang et al., 2006). The overexpression of HAP type phytases (AppA) in rhizobacteria adds advantage to plant growth promotion by making available P for plant nutrition. However, *E. asburiae* PSI3 containing PhyA demonstrated similar extent of P release from Ca-phytate. *E. asburiae* PSI3 containing either of the phytases (AppA or PhyA) could be beneficial to the plants P nutrition regardless of nature of phytate available.

Table 5.3 Phytase overexpression using different expression system

Source of Phytase	Promoter	Host	Expression level	Reference
Pectobacterium wasabiae DSMZ 18074	T7	*E. coli* BL21 (DE3)		Shao et al., 2008
Pseudomonas syringae MOK1	T7	*E. coli* BL21 (DE3)		Cho et al., 2005
Pedobacter nyackensis MJ11 CGMCC 2503	T7	*E. coli* BL21 (DE3)		Huang et al., 2009a
Citrobacter braakii	T7	*E. coli* BL21 (DE3)		Kim et al., 2006
Enterobacter sp. 4	T7	*E. coli* BL21 (DE3)		Kang et al., 2006
Obesumbacterium proteus	T7	*E. coli* BL21 (DE3)	100 fold	Zinin et al., 2004
Bacillus subtilis VTT E-68013	*pst*	*B. subtilis* strain BD170	100 fold	Kerovuo et al., 2000
Bacillus licheniformis	Φ105	*B. subtilis* IA304MU331	100 fold	Kim et al., 1999
Citrobacter braakii	*lac*	Rhizobacteria	10-530 fold	Patel et al., 2010
Citrobacter braakii	*tac*	*E. asburiae* PSI3	12 fold	This study