Genetic manipulation of *Enterobacter asburiae* PSI3 for enhanced Phosphorus nutrition

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STATEMENT UNDER O.Ph.D 8/ (iii) OF
THE M.S. UNIVERSITY OF BARODA, VADODARA

This is to certify that the work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of **Prof. G. Naresh Kumar.**

Vadodara Chanchal Kumar
Date:

This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph.D. degree of The M.S. University of Baroda by Mr. Chanchal Kumar is his original work. The entire research work and the thesis have been built up under my supervision.

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DEDICATED TO

MY BELOVED PARENTS

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List of Abbreviations:

X-Gal	5-Bromo-4-chloro-3-indolyl-β-D galactopyranoside
IPTG	Isopropyl β-D thio galactopyranoside
LB	Luria broth
SDS	Sodium Dodecyl Sulphate
kb	kilobase (s)
bp	base pair (s)
kDa	kilodalton
rpm	Revolutions per minute
OD	Optical Density
mM	millimolar
µl/ml	Microlitres / microlitres
Ori	Origin of replication
GDH	Glucose dehydrogenase
GAD	Gluconate dehydrogenase
EYFP	Enhanced fluorescent protein
PTS	Phosphoenolpyruvate:sugar phosphotransferase system
PQQ	Pyrolloquinolino quinone
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
cAMP	Cyclic adenosine monophosphate
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
EMP	Embden–Meyerhof–Parnas pathway
TCA	Tricarboxylic acid
PPP	Pentose phosphate pathway
ETC	Electron transport chain
ED	Entner-Duodoroff
PSM	Phosphate solubilizing microorganisms
P/Pi	Phosphate / inorganic phosphate
HPLC	High performance liquid chromatography
BLAST	Basic local alignment search tool
PCR	Polymerase Chain Reaction
PAGE	Polyacrylamid Gel Electrophoresis
EDTA	Ethylene diamine tetra acetic acid
Tris	Tris (hydroxymethyl) aminomethane buffer
CTAB	Cetyl trimethylammonium bromide
PGPR	Plant Growth Promoting Rhizobacteria
RE	Restriction Endonuclease

Note: The full forms of several other minor abbreviations have been described within the text.

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CHAPTER 1

Review of Literature and Introduction

1.1 Introduction:

Green revolution especially in developing countries like India had led extensive use of chemical fertilizers and other chemicals which have resulted in reduction in soil fertility and to environmental degradation (Gyaneshwar et al., 2002). Use of chemical fertilizers reach at theoretical maximum level beyond that no further improvement in grain production is possible (Ahmad, 1995). Current studies shows that by the year 2050, population touched the 9 billion mark to feed this population agriculture demand between now and 2050 will have to grow more than it has been from last several decades. To feed this population food production required to double. Phosphorus is chief macronutrient after nitrogen required for plant growth and grain yield (Bieleski, 1973; Vance et al., 2000). Nucleic acids, phospholipids, ATP and several other biologically active compound required phosphorus for their synthesis. It participates directly in generating the biochemical energy necessary to drive virtually every anabolic process within the cell and is a prerequisite in every phase of cellular metabolism (Goldstein, 1995).

Crop production needs to be improved to meet the global population demand which requires efficient utilization of phosphate reserves of earth. Since middle of twenty century phosphorus has been overflow four time from the environment which is essential for all form of life. Green revolution created one way overflow of phosphorus from rock to forms to lakes and oceans which dramatically impairing costal and marine ecosystem. In current scenario, supply of phosphorus for fertilizers is obtained from the rock phosphates.

1.1 **Global phosphorus status**

Recent IFDC survey produces an assessment of global phosphorus reserves. New survey increased the estimated phosphorus reserve of Moracco and Western Sahara territory from 15 billion ton to 65 billion ton (van Kauwenbergh, 2010). Overall, three countries have more than 85% of known phosphorus reserves.

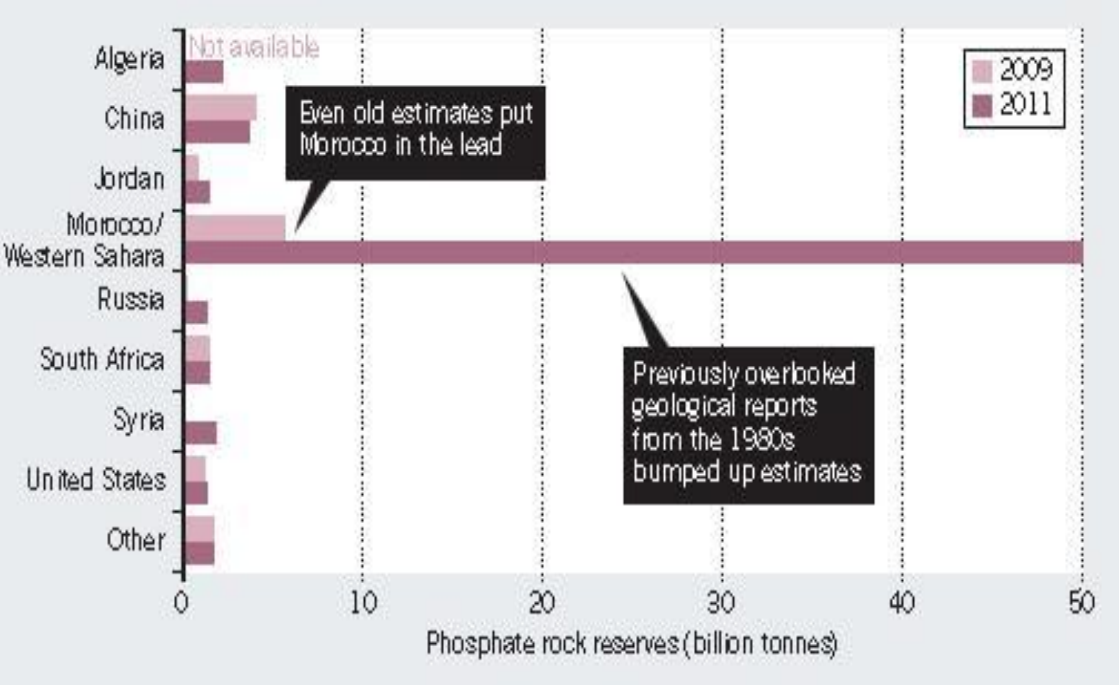

Fig. 1.1 Global distribution of phosphorus reserve (Elser and Bennett, 2011).

1.2 **Status of phosphorus in soil**

Amount of P in soil varies from 0.02 to 0.5% (400-1200 mg per kg of soil) depending upon the weathering of rock and other environmental factors (Kucey et al., 1989). P exists in soil in inorganic and organic forms. Inorganic P is present in the form of calcium salt in alkaline vertisol soil while Al and Fe salts of P are

predominantly present in acidic alfisols (Gyaneshwar et al., 2002). Most of the inorganic phosphate in the soil is present in poorly soluble mineral phosphate precipitate.

Organic phosphates make up largest fraction up to 40% of total soil phosphate bound with organic matter. Phytate, a hexaphosphate salt of inositol, is the major form of P in organic matter contributing about 50 and 80% of the total organic P (Molla and Chowdary, 1984). Different forms of organic P are present in soils and their classification depends upon the type of bond formation (Fuentes et al., 2008).

Orthophosphate monoesters are mainly present in agriculture soils supplemented with organic manure, compost and sewage sludge. Class includes *myo*-inositol hexakisphosphate, glucose-6-phosphate, p-nitrophenyl phosphate and nucleotides.

Orthophosphate diesters include nucleic acid derived from phosphoprotein and phospholipid.

Phosphonates include anion of phosphonic acid and similar to phosphates except that they have carbon-phosphorus bond (C-P) instead of carbon-oxygen- phosphorus (C-O-P) linkage. It includes highly water soluble and poorly soluble organic solvents and are non volatile. In most soil organic P is dominated by mixture of mono and diesters phosphate with small amount of phosphonates and organic polyphosphates (Turner, 2008). IP comprise maximum amount of organic P in all types of soil (Richardson 2001b; Turner, 2007; Fuentes et al, 2008), frequently over 60% (Turner et al., 2002).

1.3 **Status of Microorganisms in Phosphate Mobilization**

PSM population varies from soil to soil. In soils, phosphate solubilizing bacteria (PSB) dominate over P-solubilizing fungi by about 5-150 fold (Banik and

Dey, 1982; Kucey, 1983; 1989). PSB accounts for approximately 1-50% total population of soil microorganisms whereas PS fungi contribute for about 0.1-0.5%. These PSM are effective in field when supplement with rock phosphate (Kucey, 1983). PSB isolated from rhizospheric soil are metabolically more active compared to PSB isolated from bulk soil (Katznelson and Bose, 1959; Baya et al., 1981). PS fungi exhibit better P-solubilization ability than PSB in both liquid and solid medium due to secretion of high amount of citric and oxalic acids (Kucey, 1983; Gyaneshwar et al., 2002; Khan et al., 2006; Zaidi et al., 2009).

1.3.1 **Phosphate Solubilizing Microorganisms**

PSMs are integral component of the soil which controls the biofertility of the soil throughout biogeochemical cycle. Numerous microorganisms reported to have MPS ability (Gyaneshwar et al., 1998; Hameeda et al., 2008; Henri et al., 2008; Zaidi et al., 2010). Important genera reported to PSMs include *Bacillus*, *Enterobactor* and *Pseudomonas* (Illmer and Schinner, 1992; Wani et al., 2007a; Archana et al., 2012) while *Penicilium, Aspergillus* and *Rhizopus* are the important fungal genera (Souchie et al., 2006; Pandey et al., 2008). The reported bacilli includes, *B. brevis*, *B. cereus*, *B. circulans*, *B. firmus*, *B. licheniformis*, *B. megaterium*, *B. mesentricus*, *B. mycoides*, *B. polymyxa*, *B. pumilis*, *B. pulvifaciens* and *B. subtilis* were isolated from rhizosphere of legumes, cereals (rice and maize), arecanut palm, oat, jute and chilli. *Pseudomonas striata*, *P. cissicola*, *P. fluorescens*, *P. pinophillum*, *P. putida*, *P. syringae*, *P. aeruginosa*, *P. putrefaciens* and *P. stutzeri* have been isolated from rhizosphere of *Brassica*, chickpea, maize, soybean and other crops, desert soils and Antarctica lake.

In addition, *Escherichia freundii, Serratia phosphaticum* and species of *Achromobacter, Brevibacterium, Corynebacterium, Erwinia, Micrococus,* and *Sarcina* are known to actively play role in solubilizing insoluble phosphates. Cyanobacteria, *viz*. *Anabaena* sp., *Calothrix brauni, Nostoc* sp., *Scytonema* sp. and *Tolypothrix ceylonica* can also solubilze phosphate (Tilak et al., 2005). Many other genera also show MPS ability which include *Rhodococcus, Arthrobacter, Chryseobacterium, Gordonia, Phyllobacterium, Arthrobacter, Delftia* sp. (Chen et al., 2006), *Azotobacter* (Kumar et al., 2001), *Xanthomonas* (de Freitas et al., 1997), *Pantoea,* and *Klebsiella* (Chung et al., 2005). Some symbiotic nitrogen fixers also showed MPS ability. *Rhizobium leguminosarum* bv. *trifolii* (Abril et al., 2007), *R. leguminosarum* bv. *viciae* (Alikhani et al., 2007) and *Rhizobium* species nodulating *Crotalaria* sp. (Sridevi et al., 2007) showed to improved plant P-nutrition by mobilizing inorganic and organic P from the soil.

1.3.2 **Factors influencing the efficacy of PSMs in field conditions**

Many factors influence the efficacy of PSMs in field conditions. Many successful field studies reported with consortium of microorganism which improved plant growth due to P availability or other plant growth promoting activities like nitrogen fixation, efficient nutrient uptake, alleviation of metal toxicity and PGPR traits including siderophores production, antibiotics production, phytoharmones etc. (Archana et al., 2012).

1.3.2.1 Root colonization ability

Competitiveness of the PSMs depends upon the nutrient availability, survival and continuous population increase in the soil (Van Veen et al., 1997). Effectiveness

of the inoculated microorganism depends upon the ecological condition. Introduced microorganism involved in the process gives the selective advantage in the soils; certain minimal number of cells are required to be successful in the field condition. Reports in understanding the factors influencing the survival and efficacy of PSMs in field conditions are scanty. In case of *Rhizobia,* 300 cells per seed are sufficient for optimal nodulation (Giddens et al., 1982). Efficiency of *Rhizobia* can also be improved by favoring ecological competence by amending soil with specific substrate.

Generally microbial population size decreases rapidly after inoculation in soil (Ho and Ko, 1985). Survival depends upon the abiotic, biotic factors, soil composition (Bashan et al., 1995; Heijnen et al., 1993; 1995;), temperature, moisture, carbon status (Richardson and Simpson, 2011) and presence of recombinant plasmid (Van Veen et al., 1997). Biotic factors also play an important role in the survival of the inoculated strains as decline was observed in non sterile soil which is minimal in sterile soils (Heijnen et al., 1988; Heijnen and Van Veen, 1991). Increased population of inoculated microbes was observed in sterile soil (Postma et al., 1988).

Root colonization is a major factor for the successfulness of inoculants. Majority of the microbe population found in the soil are associated with the plant roots where their population can reach up to 10^9 to 10^{12} per gram of soil (Whipps, 1990), leading to biomass equivalent to 500kg ha^{-1} (Metting, 1992). Abundance of the microbes in rhizosphere is due to secretion of high amount of root exudates (Brimecombe, 2001). Root associated bacterial diversity; growth and activity vary in response to the biotic and abiotic rhizospheric environment of the particular host plant

(Berg and Smalla, 2009). Rhizobacteria interaction with plant root is mediated by secreted compounds (signals and nutrients) that vary in abundance and diversity depending upon the characteristics of the particular host (Bais et al., 2006; Lugtenberg and Kamilova, 2009).

1.3.2.2 **Soil Properties**

Soil properties vary in term of texture, particle and pore size of soil. Pore size distribution determines the efficacy of the microorganism and different behavioral pattern in bacteria when released in different texture soil can be correlated by protective pore spaces present in these soil (van Veen et al., 1997). *P. fluorescens* inoculation study in loamy sand and silt loam soils observed for three years and its showed that better survival was in finer-texture soil i.e. silt loam soil than in the sandy soil (van Elsas et al., 1986). P solubilization varies with composition of the medium and type of mineral phosphate provided. These phenomenon leads to the buffering effect of these components (Cunningham and Kuiack, 1992). Nature of the secreted organic acid changes in PSB depending on nature of rock phosphate and ultimately affects the P solubilization ability (Vyas and Gulati, 2009). Efficacy of PSMs drastically reduced in soil due to high buffering capacity of soil like alkaline vertisol (Gyaneshwar et al., 1998b).

1.3.2.3 Nutrient Availability

Substrate availability often limits the performance of the inoculants. In natural environment nutrient are generally present in very limited amount (Paul and Clark, 1988; Gottschal et al., 1992; Koch et al., 2001). Physiological status of the microorganism also determines the efficacy in field conditions. In rhizosphere, the

abundance and activity of microorganisms significantly is correlated with increased exudation of photosynthetic carbon from root. 5-20% photosynthetic carbon generally secreted into the rhizosphere as high molecular weight mucilage, simple hexose sugar and organic anions along with complex carbon derived from root turn over (Jones et al., 2009). These available carbon sources are utilized by microorganism which result in significant increase in microbial biomass (C and P) within the rhizosphere was monitored in perennial ryegrass and radiate pine by using the rhizobox system (Chen et al., 2002).

Scarcity of C in bulk soil compared to rhizospheric soil was shown by reporter gene technology using carbon limitation dependent gene expression system fused with *lacZ* and introduce in *P. fluorescens* (Koch et al., 2001). Different root portions like sub apical zone, root hair zone and emerging site of secondary ramification release specific exudates (Curl and Truelove, 1986; Bais et al., 2006). These exudates are directly accessible to microorganism without the help of exoenzymes (Bremer and van Kessel, 1990; Bremer and Kuikman, 1994). These exudates used as convenient source of carbon (may be nitrogen), energy and likely to favor fast growth of metabolically versatile microorganism. Rhizobacteria having broad substrate utilization for organic acid production appear to be better PSB (Sharma et al., 2005; Patel et al., 2008).

Amount of carbon used in the laboratory studies (several mg per gram soil) are very high compared to carbon present in the rhizosphere (Kozdroj and van Elsas, 2001; Schutter and Dick, 2001; Griffiths et al., 2004). Daily carbon input estimated around 50-100mg per gram rhizospheric soil (Trofymow et al., 1987; Iijima et al.,

2000). Plant roots secrete complex mixture of organic compounds *viz*. organic acid, amino acid and sugars. Sugars secreted are glucose, fructose, maltose, ribose, sucrose, arabinose, mannose, galactose and glucuronic acid (Jaeger III et al., 1999; Lugtenberg et al., 1999; 2001; Kupier et al., 2002; Bacilio- Jimenez et al., 2003; Bais et al., 2006; Kamilova et al., 2006). Organic acids secreted in rhizosphere are malate, citrate, oxalate, succinate, formate, etc. (Jones, 1998) but not in sufficient amount for releasing P from soils. Amongst amino acids, histidine, proline, valine, alanine and glycine are present (Bacilio-Jimenez et al., 2003; Phillips et al., 2004).

1.3.3: Phosphate solubilization

Phosphate mineralization in rhizosphere is achieved by different mechanisms – acidification, chelation and proton secretion,

1.3.3.1 Acidification

MPS ability of PSM has been attributed to the reducing the pH of the surrounding by release of organic acids (Gyaneshwar et al., 1999; Whitelaw, 2000; Sharma et al; 2005; Patel et al., 2008). Protons of the organic acid are the major reason for the release of inorganic P from the bound P (Lin et al., 2006). Secreted organic acid can dissolve mineral phosphate by anion exchange of PO_4^{-2} by acid anion (acid hydrolysis) or chelation of Ca, Fe and Al ions associated with phosphate or competes with phosphate for adsorption in soil site (Gyaneshwar et al., 2002; Khan et al., 2006; Zaidi et al., 2009). PSM generally secretes gluconic, 2-ketogluconic, citric, malic, acetic acid etc. when grown on simple carbohydrates. In case of *E. asburiae* PSI3, gluconic acid secretion is induced by phosphate starvation (Gyaneshwar et al., 1999). PSM ability of *Rhizobium/Bradyrhizobium* was demonstrated due to the presence of

2-ketogluconic acid (Halder and Chakrabarty, 1993). Biochemical and molecular mechanism PSM ability of these nodulating bacteria is not clear.

1.3.3.2 Chelation

Acidification is not sufficient to release P from medium (Illmer and Schinner, 1995). Chelation ability has been shown to contribute for the MPS ability along with acidification. P release was observed when 0.05M EDTA added to the medium as *Penicillium bilaii* inoculation (Kucy, 1988). Complex formation of cations (Al^{+3}, Fe^{+3} and Ca^{+2}) depends upon the number and kind of functional groups present on chelator. Organic acid having more number of carboxylic acid functional group are more effective P solubilizer (Kpomblekou and Tabatabai 1994; Xu et al. 2004).Citric form complex with Ca^{+2} more easily than dicarboxylic acids like malic and tartaric (Whitelaw, 2000). Dicarboxylic acids like oxalic and tartaric acids are more efficiently release P into the medium as compared to citric acid even though these two acids poorly solubilize precipitate of Ca^{+2} due to effectively lowering the solution saturation point (Sagoe et al., 1998). In acidic alfisol condition, oxalic acid is much better in releasing P from RP than that of citric acid (Srivastava et al., 2007).

1.3.3.3 H^+ excretion

Many microorganisms excrete H^+ in response to the assimilation reactions of cations which accounts for P solubilization. This phenomenon was well reported in fungi which excrete H^+ in exchange for NH_4^+ (Beever and Burns, 1980; Banik and Dey, 1982; Asea et al., 1988). This exchange reaction is more potent in presence of NH_4^+ as source of N source rather than NO_3^- due to higher acidity (Whitelaw et al., 1999). Ammonium sulphate and other N sources are also reported to facilitate RP

solubilization in many gram positive bacteria *viz Bacillus circulans*, *Bacillus brevis* and *Bacillus coagulans* (Vora and Shelat, 1998). In many cases, release of H^+ from assimilation of NH_4^+ seems to be the sole mechanism of RP solubilization. *Penicillium fuscum* could decrease pH and solubilize RP when media contains NH_4^+ where as *Pennicilium billai* maintain ability of lowering pH and RP dissolution in absence of NH_4^+ in the medium (Asea et al., 1988).

MPS ability of PSMs depends upon the secretion of organic acids but amount of P release in the medium does not always correlate with the amount of organic acid (Thomas, 1985; Asea et al., 1988). Therefore, nature (chelation ability) and strength of organic acid seems to effective in P solubilization. Oxalic acid is more effective as compared to citric acid in acidic alfisols supplemented with RP but situation is reverse in case of alkaline vertisol (Gyaneshwar et al., 1998b; Srivastava et al., 2006).

1.3.4 Phytate mineralization

Microbial phytase has been used for many purposes including animal feed supplement and human food stuff to improve P bioavailability. Phytase addition reduces antinutritive phytate along with this it also prevent eutrophication and supplementation of inorganic phosphate in animal feed (Vohra and Satyanarayana, 2003; Haefner et al., 2005; Vats et al., 2005; Greiner and Konietzny, 2006; Jorquera et al., 2008b). Phytase mediated hydrolysis of phytate produces lower phosphoryl intermediates which play a major role transmembrane signaling and Ca^{+2} ion mobilization from intracellular store in animal as well as plant tissue (Vohra and Satyanarayana, 2003). Many phytase producing bacteria have been explored for plant growth promotion by making P available to plants. Soil microorganisms that mobilize

P are important in providing this nutrient to plants and are crucial for the development of sustainable agriculture practices (Gyaneshwar et al., 2002; Rodriguez et al., 2006). Most of the plant roots can not hydrolyze phytate (Hayes et al., 1999; Richardson et al., 2000). Hence, rhizospheric microorganisms have been considered to be important for P mobilization in soil conditions where phytate is major source of bound organic P.

Mineralization of phytic acid involves release inorganic P and formation of less phosphorylated myo-inosital. Due to phosphate ester linkage phytic acid is very stable in basic condition. In acidic medium, hydrolysis is slow and maximum hydrolysis occurs at pH-4.5. Phytases are capable of initiating the step wise release of P from phytate (Greiner, 2007). During hydrolysis, phytases follow different pattern of sequential attack on P at different carbon position and generate varied final end product (**Table 1.1**). Acid phytases produced from *S. cerevisiae*, *Pseudomonas*, *E. coli*, rice, rye, barley P1, barley P2, oat, wheat PHY1, wheat PHY2, wheat F2, lupine L11, lupine L12, lupine L2 and mung bean cleave five of the six phosphate groups of phytate, and the final degradation product was identified as Ins(2)P (Konietzny and Greiner, 2002). This pattern of hydrolysis suggests that these phytases have strong preferences for equatorial phosphate group cleavage instead of axial phosphate group. Alkaline phytases (pH range 6.5-8.0) from lily pollen and *Bacillus subtilis* do not accept three or less phosphate residue as a substrate and form myo-inositol triphosphate as end product.

Table 1.1: myo-inositol phosphate intermediate generated during enzymatic phytate degradation **(**Greiner, 2007**)**

Enzyme	IP5-isomer	IP4-isomer	IP3-isomer	IP2-isomer	IP-isomer
Barley P1; P2, spelt D21, Wheat PHY1; PHY2, Rye, Oat, Rice, Lupine L2	D-Ins(1,2,3,5,6)P5	D-Ins(1,2,5,6)P4	D-Ins(1,2,6)P3	D-Ins(1,2)P2	Ins(2)P
Wheat F2	D-Ins(1,2,3,5,6)P5	D-Ins(1,2,3,6)P4	Ins(1,2,3)P3	D-Ins(1,2)P2	Ins(2)P
Mung bean	D-Ins(1,2,3,5,6)P5	D-Ins(1,2,3,6)P4	D-Ins(1,2,6)P3/ Ins(1,2,3)P3	D-Ins(2,6)P2/ D-Ins(1,2)P2	Ins(2)P
S. cerevisiae, *Pseudomonas*, Lupine L11, Lupine L12	D-Ins(1,2,4,5,6)P5	D-Ins(1,2,5,6)P4	D-Ins(1,2,6)P3	D-Ins(1,2)P2	Ins(2)P
E. coli	D-Ins(1,2,3,4,5)P5	D-Ins(2,3,4,5)P4	Ins(2,4,5)P3	Ins(2,5)P2	Ins(2)P
Paramecium	D-Ins(1,2,3,4,5)P5	D-Ins(1,2,3,4)P4	Ins(1,2,3)P3	D-Ins(2,3)P2	Ins(2)P
Lily	D-Ins(1,2,3,4,6)P5	D-Ins(1,2,3,4)P4/ D-Ins(1,2,3,6)P4	Ins(1,2,3)P3		
B. subtilis	D/L-Ins(1,2,3,4,5)P5/ D/L-Ins(1,2,4,5,6)P5	Ins(1,2,3,5)P4/	Ins(1,3,5)P3/ Ins(2,4,6)P3		
B. subtilis, *B. amyloliquefaciens*	D-Ins(1,2,4,5,6)P5/ D/L-Ins(1,2,3,4,5)P5	Ins(2,4,5,6)P4/ Ins(2,4,5,6)P4	Ins(2,4,6)P3/ D-Ins(1,2,6)P3		
Pantoea agglomerans	D-Ins(1,2,4,5,6)P5	D-Ins(1,2,5,6)P4			

Acid phytases produced from *S. cerevisiae, Pseudomonas, E. coli*, rice, rye, barley P1, barley P2, oat, wheat PHY1, wheat PHY2, wheat F2, lupine L11, lupine L12, lupine L2 and mung bean cleave five of the six phosphate groups of phytate, and the final degradation product was identified as Ins(2)P (Konietzny and Greiner, 2002). This pattern of hydrolysis suggests that these phytases have strong preferences for equatorial phosphate group cleavage instead of axial phosphate group.

Alkaline phytases (pH range 6.5-8.0) from lily pollen and *Bacillus subtilis* do not accept three or less phosphate residue as a substrate and form myo-inositol triphosphate as end product (Konietzny and Greiner, 2002).

Many reports showed intrinsic role of phytase producing microorganism in plant growth promotion with respect to increasing shoot P, shoot to root length, and dry weight of plant in gnobiotics condition (**Table 1.2**). Inoculation of phytase producing *Pseudomonas* to wheat plants improves plant dry weight and shoots P content compare to uninoculated control (Richardson, 2001a). Culture filtrate of phytase producing *Bacillus amyloliquefaciens* was used in artificial sterile system, provided phytate as major P source showed improved maize seedling growth (Idriss et al., 2002). Similarly, Unno et al. (2005) have shown *Burkholderia sp.* increased height, dry weight and P content of *Lupinus albus.*

Table 1.2: Phytase producing microorganisms used for plant growth promotion.

Phytase producing microorganism	Plants	References
Bacteria		
Pseudomonas sp.	Pasture grass (*Danthonia richardsonii* and *Phalaris aquatica*)	Richardson et al., 2001b
Bacillus amyloliquefaciens FZB45	Maize seedlings	Idriss et al., 2002
Burkholderia spp †	*Lupinus albus*	Unno et al., 2005
Pseudomonas sp. strain CCAR59	*Triticum aestivum L.*	Richardson et al., 2000
Bacillus mucilaginosus (native strain)*	Tobacco	Li et al., 2007
Bacillus mucilaginosus (Transgenic expressing phytase)*	Tobacco	Li et al., 2007
Citrobactor DHRSS, *Enterobactor asburiae* PSI3, *Pentoea* sp. PP1, *Pseudomonas putida* KT2440,	Mung Bean (*Vigna radiata*)	Patel et al., 2011

Ensifer meliloti (ATCC9930), *Rhizobium* sp. ST1, *Rhizobium* sp. IC-3109 (Transgenic expression)		
Fungus		
*Emericella rugulosa**	Pearl millet	Yadav and Tarafdar, 2007
*Chaetomium globosum**	Wheat and pearl millet	Tarafdar and Gharu, 2006
Sporotrichum thermophile †	*Triticum aestivum L.*	Singh and Satyanarayana, 2009
Phytase from *Aspergillus niger*	*Trifolium subterraneum* L.	Hayes et al., 2000

1.3.4.1: Phytase expressing transgenic plants to improved P nutrition

Transgenic plants expressing phytase were developed for different purposes, expression of phytase in seed has been done to eliminate P supplementation in monogastric animal feeds, and also it was express to improve plant growth promotion by improved P nutrition. *Aspergillus niger phyA2* gene was express in maize under control of embry specific globulin-1 promoter resulted 50 fold increase in phytase activity in transgenic maize seed compare to control (Chen et al., 2008). *Aspergillus niger* phytase expression in *Arabidopsis thaliana* was successful when expression was done extracellular, using signal peptide of carrot extension gene. Transgenic line showed 20 fold phytase expression and 4.3 fold shoot P concentration compare to control plants (Richardson et al., 2001a). Phytase producing *B. amyloliquefaciens* culture supernatant along with phytate as a sole P source apply in artificial sterile condition resulted maize seedling growth promotion where as phytase mutant bacteria culture supernatant fail to improve growth (Idriss et al., 2002). *Lupinus albus* height,

dry weight and P content were improved by application of *Burkholderia sp.* (Unno et al., 2005).

1.3.4.2: Cloning and overexpression of different phytases

For various commercial application of phytase, it was cloned and expresses in various expression vectors suitable to express in host microorganism.

Table 1.3: Phytase overexpressing transgenic plants

Organisms phytase gene	Plant used for experimental study	References
Bacteria		
168*phy*A of *Bacillus subtilis* †	Tobacco and *Arabidopsis*	Lung et al., 2005
168 *phy*A of *Bacillus subtilis †*	Tobacco	Yip et al., 2003
Fungus		
*phy*A gene of *Aspergillus niger* †*	Tobacco	George et al., 2005b
*phy*A gene of *Aspergillus niger* Ψ	white clover	Shengfang et al., 2007
*phy*A gene of *Aspergillus niger* †	*Trifolium subterraneum L.*	George et al., 2004
*phy*A gene of *Aspergillus niger* †	*Arabidopsis thaliana*	Mudge et al.,2003
*phy*A gene of *Aspergillus niger* †	*Arabidopsis thaliana*	Richardson et al., 2001a
Plants		
*MtPHY*1 of *Medicago truncatula* Ψ	*Trifolium repens L.*	Ma et al., 2009

*Sphy*1 of Soybean †	*Tobacco*	Guo et al., 2009
*MtPHY*1 of *Medicago truncatula* †	*Arabidopsis*	Xiao et al., 2005
Synthetic gene		
Synthetic phytase gene (*PHY*) ±	Potato	Zimmermann et al.,2003

† – Synthetic medium; * - Soil amended with Na-phytate; Ψ - Sand culture

1.3.5 Role of organic acids in P mobilization organic phosphates

Organic acids secreted by microorganisms play a major role in P solubilization. P solubilizing bacteria (**Table 1.4**) and fungi (**Table 1.5**) secret high amount of organic acid, chelate mineral ions or drop the pH to brings inorganic P in the medium. Gluconic acid is generally produced in the periplasmic space via direct oxidation pathway and secreted to outside of cell which leads to pH drop of the surrounding.

Table 1.4: Major organic acid secreted by PSBs

Microorganism	**Organic acid produced**	**Reference**
Burkholderia cepacia DA23	Gluconic acid	Song et al., 2008
Pseudomonas corrugata (NRRL B-30409)	Gluconic, 2-ketogluconic acid	Trivedi and Sa., 2008
Citrobacter sp. DHRSS	Acetic and gluconic acid	Patel et al., 2008
Burkholderia, Serratia, Ralstonia and *Pantoea*	Gluconic acid	Elizabeth et al., 2007

Bacillus, Rhodococcus, Arthrobacter, Serratia, Delftia,Chryseobacterium, Gordonia, Phyllobacterium, Arthrobacter ureafaciens, Phyllobacterium myrsinacearum, Rhodococcus erythropolis and *Delftia* sp.	Citric, gluconic, lactic, succinic and propionic acid	Chen et al., 2006
Enterobacter intermedium	2-ketogluconic	Hwangbo et al., 2003
Enterobacter asburiae PSI3	Gluconic, acetic acid	Gyaneshwar et al., 1998a
*Bacillus amyloliquefaciens, B. licheniformis, B. atrophaeus, Penibacillus macerans, Vibrio proteolyticus, Xanthobacter agilis, Enterobacter aerogenes, E. taylorae, E. asburiae*PSI3, *Kluyvera cryocrescens, Pseudomonas aerogenes, Chryseomonas luteola*	Lactic, itaconic, isovaleric, isobutyric, acetic	Vazquez et al., 2000
Pseudomonas cepacia	Gluconic, 2-ketogluconic	Bar-Yosef et al., 1999

Bacillus polymyxa, B. licheniformis, Bacillus spp.	Oxalic, citric	Gupta et al., 1994

Table 1.5: Major organic acid produced by PS fungi

Fungi	Organic acid produced	References
Aspergillus niger	Gluconic, oxalic	Chuang et al., 2007
Penicillium oxalicum	Malic, gluconic,oxalic	Shin et al., 2006
Aspergillus flavus, A. niger, Penicillium canescens	Oxalic, citric, gluconic succinic	Maliha et al., 2004
Penicillium rugulosum	Citric, gluconic	Reyes et al., 2001
A. niger	Succinic	Vazquez et al., 2000
Penicillium variabile	Gluconic acid	Fenice et al., 2000
Penicillium rugulosum	Gluconic	Reyes et al., 1999
Penicillium radicum	Gluconic	Whitelaw et al., 1999
P. variabile	Gluconic	Vassilev et al., 1996
A. niger	Citric, oxalic, gluconic	Illmer et al., 1995
A. awamori, A. foetidus, A. terricola, A. amstelodemi, A.tamari	Oxalic, citric	Gupta et al., 1994
A. japonicus, A. foetidus	Oxalic, citric gluconic succinic, tartaric acid	Singal et al.,1994

1.4 **Genetic modifications for developing P solubilizing ability**

In gram negative bacteria, direct oxidation of glucose is major pathway in which glucose gets converted into gluconic and 2-ketogluconic acid (Goldstein, 1995). These acids play a major role in MPS ability and their secretion has been incorporated to achieve and improve the MPS ability of non- or weak-PSMs. Gene responsible for MPS ability was cloned from *Erwinia herbicola* in *E. coli* HB101 which enables it to solubilize hydroxyapetite (Goldstein and Liu, 1987).

Cloned gene showed similarity to gene III of *pqq* gene cluster from *Acinetobacter calcoaceticus*, and to *pqq*E of *Klebsiella pneumoniae* (Liu et al., 1992). Since *E. coli* does not possess cofactor *pqq* synthesis genes, it is unable to produce gluconic acid. Some *E. coli* strains suggested to possess cryptic *pqq* biosynthesis genes, as single open reading frame (ORF) of *E. herbicola* PQQ synthase gene could complement the cofactor requirement and showed the MPS phenotype. 7.0kb genomic DNA fragment of *Rahnella aquatilis* incorporation in *E. coli* showed hydroxyapatite solubilization. Sequence analysis showed two complete ORF and one partial ORF showing similarity to *pqqE* of *E. herbicola*, *K. pneumoniae*, *A. calcoaceticus* and *pqqC* of *K. pneumoniae* respectively (Kim et al., 1998a). These genes proposed to complement cryptic PQQ in *E. coli* and other non PSMs.

Many individual or *pqq* gene clusters from P solubilizing bacteria were cloned and express in *E. coli* enables them to produce GA which leads to MPS phenotype (**Table 1.6**). Expression of *gabY*(396bp) gene in *E. coli* JM109 induces MPS ability and GA production. Sequence analysis does

not show any similarity with direct oxidation pathways genes. Sequence was similar to membrane bound histidine permease component. GA was seen when PQQ was added externally in medium. Gene might be playing role as PQQ transporter (Babu-Khan et al., 1995). Mutation in *gdh* gene resulted loss of MPS phenotype. *Serratia marcenses* genomic DNA fragment induces GA production in *E. coli*. Gene does not show any homology with either *gdh* or *pqq* genes. When DNA fragment was replaced with other *pqq* producing strain, GA was not produced in *E. coil* (Krishnaraj and Goldstein, 2001). This gene product could be an inducer of GA production since *gdh* mutant did not show any phenotype. Similar reports showed genes that are not directly involved in *gdh* or *pqq* biosynthesis induce MPS ability. Genomic DNA fragment of *Enterobacter agglomerans* showed MPS ability in *E. coli* JM109 without any significant difference change in pH (Kim et al., 1997). MPS genes from *R. aquatilis* showed higher GA production and hydroxyapatite dissolution in *E. coli* compared to native strain (Kim et al., 1998).

Table 1.6: Genetic modifications for enhanced MPS by bacteria

Gene/Function	Source	Host	Mineral P solubilized	Organic acid	References
PQQ biosynthesis	*Serratia marcescens*	*E. coli*	TCP	GA	Krishnaraj and Goldstein, 2001
pqqE	*Erwinia herbicola*	*Azospirillum sp.*	TCP	GA ?	Vikram et al., 2007
pqqED genes	*Rahnella aquatilis*	*E. coli*	HAP	GA	Kim et al., 1998

Unknown	*Enterobacter agglomerans*	*E. coli*		GA ?	Kim et al., 1997
pqqABCDEF genes	*Enterobacter intermedium*	*E. coli* DH5α	HAP	GA	Kim et al., 2003
Ppts-gcd, P gnlA-gcd	*E. coli*	*Azotobacter vinelandii*	TCP	GA	Sashidhara and Podille, 2009
gabY Putative PQQ transporter	*Pseudomonas cepacia*	*E. coli* HB101		GA	Babu-Khan et al., 1995
Unknown	*Erwinia herbicola*	*E. coli* HB101	TCP	GA	Goldstein and Liu, 1987
gltA/ citrate synthase	*E. coli* K12	*Pseudomonas fluorescens* ATCC 13525	DCP	Citric acid	Buch et al., 2009
Unknown	*Synechocystis* PCC 6803	*E. coli* DH5α	RP	Unknown	Gyaneshwar et al., 1998a
gad / Gluconate dehydrogenase	*P. putida* KT2440	*E. asburiae* PSI3	RP	GA and 2KG	This Study

GA- Gluconic acid, 2KG- 2 ketogluconic acid, DCP- Dicalcium phosphate, TCP- Tricalcium phosphate, RP- Senegal rock phosphate, HAP- Hydroxyapatite

1.5 **Ovreexpression of Phytase genes for improved mineralization**

Many phytase producing transgenic plants have been developed for improve P nutrition and growth promotion (Details are given in earlier sections). Few reports are available using phytase overexpressing rhizobacteria to improve P mobilization for plant growth promotion. *Citrobactor brakii* phytase encoding gene *appA* was expressed through

broad host range vector pBBR1MCS-2 (Patel et al., 2010). Expression of *appA* gene in plant growth promoting rhizobacteria resulted improved P nutrition and growth in Mung bean (*Vigna radiata*) plant under semisolid agar medium containing Ca or Na-phytate as sole P source.

1.6 Importance of *Vitreoscilla* hemoglobin for enhancing the efficacy of bioremediation and biofertilizers.

Oxygen is very important for life as it required primarily in the energy metabolism as an electron acceptor molecule. Additionally, oxygen is required for regulation of various cellular functions and byproduct accumulated by stress response (Frey and Kallio, 2003). Oxygen and cognate oxygen radicals can affect bacterial cell either by altering the function or biosynthesis of specific proteins mediated by global transcription regulation.

Globins are present in all five kingdoms of life and divided into vertebrate and non-vertebrate groups including plants, fungi and protozoans. Common characteristics of all hemoglobins (Hb) bind reversibly to oxygen. Obligate anaerobe bacteria *Vitreoscilla* synthesize homodimeric hemoglobin (VHb) which is the best characterized member of bacterial Hb family (Webster and Hacket, 1966). *vhb* gene encodes 15.7 kDa protein and is expressed by oxygen dependent promoter (Pvhb) which is induces under oxygen limiting condition in *Vitreoscilla*. Pvhb has been characterized in *E. coli* and shown to be functional in many heterologous hosts *viz Pseudomonas*, *Azatobactor*, *Rhizobium etli*, *Streptomyces* sp., *Burkhulderia* sp., *Enterobactor aerogens*, yeast etc.

Pvhb expression occurs when dissolved oxygen level less than 2% air saturation in both *Vitreoscilla* and *E. coli* (Khosla and Bailey, 1988). Pvhb activity in *E. coli* positively is modulated by CRP and FNR. In absence of CRP or cAMP, expression of Pvhb decreased substantially. Pvhb maximal induction increased recombinant protein production to about 10% of total cellular protein (Khosala et al., 1990). Pvhb regulatory mechanism and high expression level rendered interesting for biotechnological process applications. Kallio at al., (1994) had proposed that VHb acting as a additional oxygen source, increases intracellular dissolved oxygen level under microaerobic condition which in turn raises the activities of cytochrome o and cytochrome d. Increase in terminal oxidase activity facilitates proton pumping which generates ATP when proton reenters the cytoplasm via ATPase. Increased proton pumping results increased amount of ATP production subsequently (**Fig. 1.2**). This model was further supported by study on energy metabolism in *E. coli* and *Saccharomyces cerevisiae* (Chen et al., 1994; Kallio et al., 1994; Tsai et al., 2002).

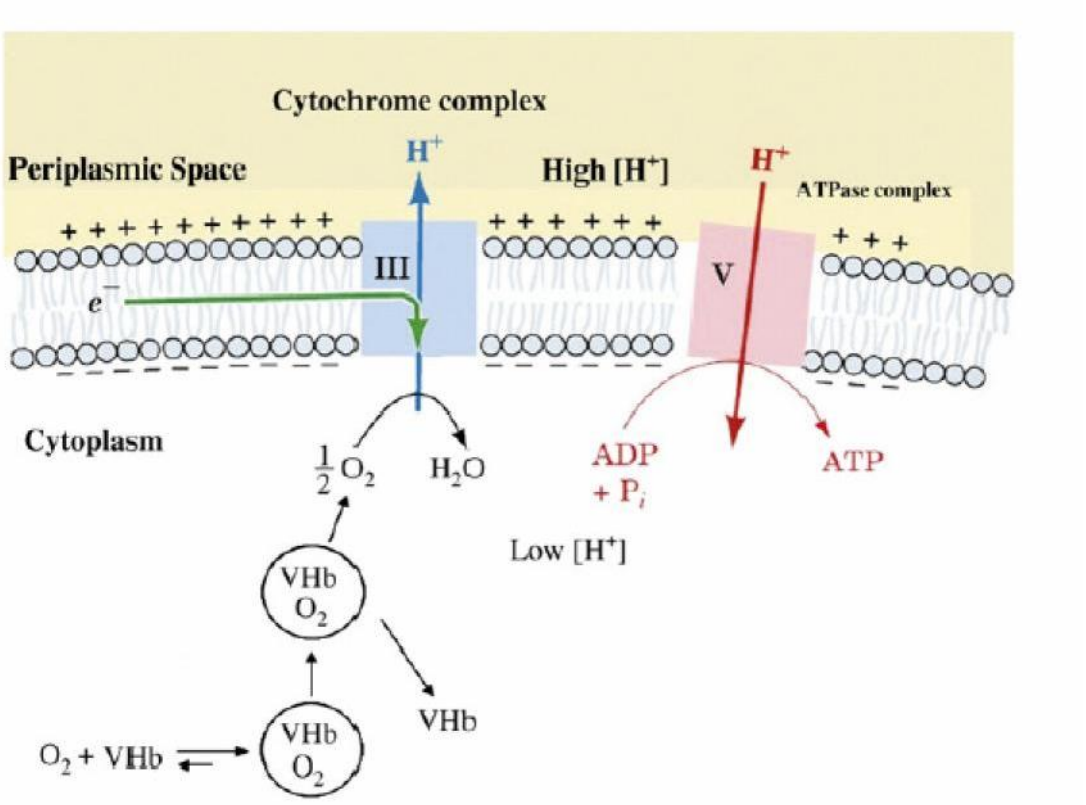

Fig. 1.2: Proposed Energy generation model in presence of VHb (Zhang et al., 2007).

Investigation of tRNA and ribosome content revealed cell expressing VHb showed higher level of tRNA and ribosome compared to control cells which help in enhancing translational component by VHb (Roos et al., 2002). VHb provides oxygen to respiration apparatus to host organism under limited oxygen condition (Webster, 1988; Chi et al., 2009). This hypothesis supported by its localization and concentration near periphery of cytosolic face of cell membrane in *Vitreoscilla* and *E. coli* (Ramandeep et al., 2001), improved the uptake of oxygen (Erenler et al., 2004), and specifically interacts with lipids of cell membrane (Rinaldi et al., 2006) and cytochrome O (Ramandeep et al., 2001; Park et al., 2002). VHb binding with lipid decreases oxygen binding by several folds and simultaneously releases oxygen

compared to lipid free VHb, this might allow VHb to transfer oxygen to respiratory apparatus for efficient utilization (Rinaldi et al., 2006). VHb stimulates oxygenase activity, acts as terminal oxidase (Dikshit et al., 1992), modulates redox status of cells (Tsai et al., 1995) and detoxifies nitric oxide (Frey et al., 2002; Kaur et al., 2002) and give protection against oxidative stress (Geckil et al., 2003; Kvist et al., 2007). VHb presence in cells altered antioxidant enzymes status to protect cells from oxidative damage. VHb expressing *Enterobactor aeruginosa* showed elevated level of catalase and become more tolerant to oxidative stress (Geckil et al., 2003). Similarly, VHb expressing *Streptomyces lividens* exhibits significant upregulation of *katA* (catalase peroxidase A) and *sodF* (superoxide dismutase F), two antioxidant enzymes (Kim et al., 2007). In oxidative stress condition, despite low content of VHb expression, cells display significantly minimizes H_2O_2 effect; enhance growth rate and survival (Anand et al., 2010). Protective effects of VHb are reported in many heterologous hosts where *vhb* not expressed from native promoter could get induced from an alternate promoter by OxyR transcriptional activator (Abrams et al., 1990; Anand et al., 2010) (**Fig. 1.3**).

VHb has been extensively used for metabolic engineering and improved many fermentation processes. Industrial production of metabolite requires modification in complex metabolic pathway to obtained final active metabolites. Limited aeration condition during the cultivation of various microbial strains can decrease the activity of cytochrome P-450 monooxygenase which is required for the processing of pathway intermediates into final form leading to the overflow of intermediate metabolic products instead of final product. To minimize this problem, *vhb* was expressed in *E. coli* which enhanced the growth and protein production under microaerobic condition

(Khosla and Bailey, 1988a,b; Khosla et al., 1990). Since then VHb was expressed in many organisms for enhanced growth and metabolite production (**Table 1.7**).

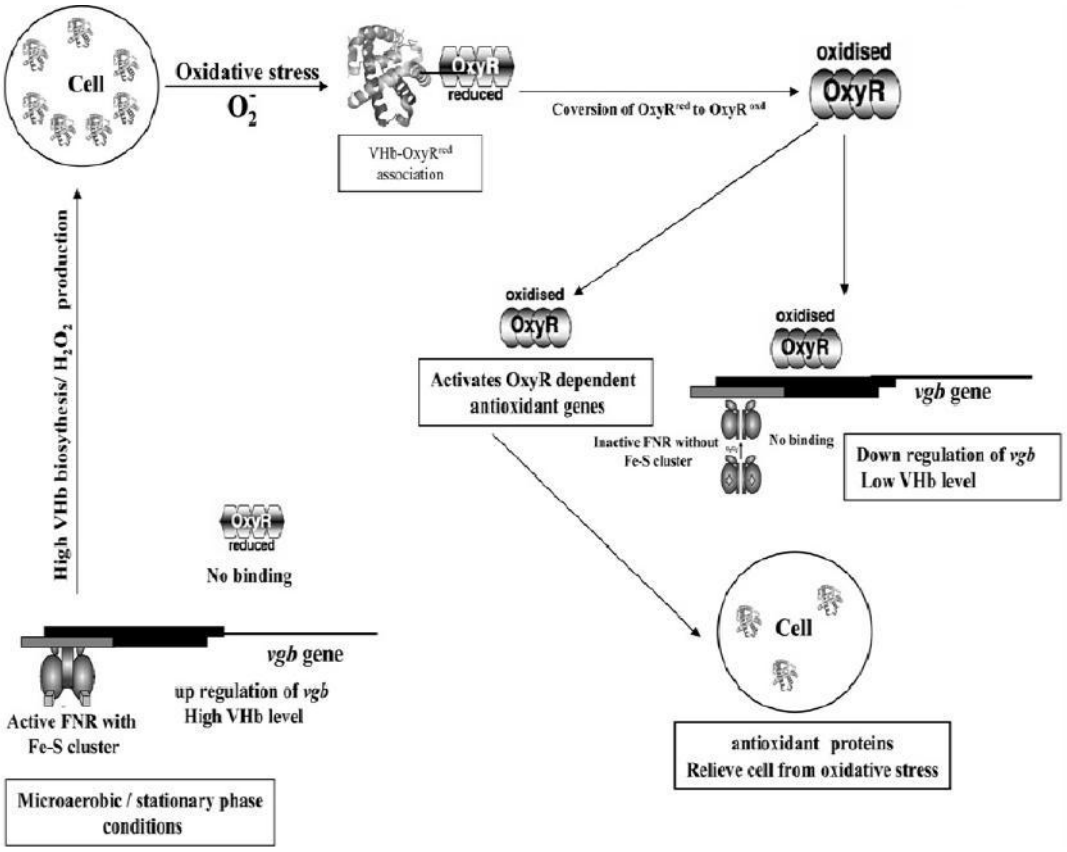

Fig. 1.4: Proposed role of *vgb* during oxidative stress (Anand et al., 2009)

Table 1.7: Application of VHb expression in metabolic engineering

Categories	Organisims	Comments	References
Improved protein production	*Gluconobacter oxydans*	Dihydroxyacetone	Li et al., 2010
	Bacillus subtilis	Poly c-glutamic acid	Su et al., 2010
	Escherichia coli	Ethanol	Sanny et al., 2010
	Citrobacter freundii, Erwinia herbicola	L-dopa, dopamine	Kurt et al., 2009

	Escherichia coli	Mussel adhesive protein	Kim et al., 2008
	Escherichia coli	D-amino acid oxidase	Yu et al., 2008
	Corynebacterium glutamicum	L-glutamate, L-glutamine	Liu et al., 2008
	Bacillus thuringiensis	Insecticidal crystal proteins	Feng et al., 2007
	Bacillus subtilis	Hyaluronic acid	Chien and Lee, 2007
	Pichia pastoris	S-adenosylmethionine	Chen et al., 2007
	Schwanniomyces occidentalis	Increased alpha-amylase production and total protein secretion	Suthar and Chattoo, 2006
	Pichia pastoris	Enhanced growth and heterologous protein production	Chien and Lee, 2005
	Escherichia coli	Improved growth and alpha-amylase production	Aydin et al., 2000
	Bacillus subtilis	Enhanced total protein secretion and alpha-amylase production	Kallio and Bailey, 1996
	E. coli	Increased total protein content	Khosla et al., 1990
Elevated chemical production	*Gordonia amarae*	Improved growth and increased biosurfactant production	Dogan et al., 2006
	Enterobacter aerogenes	Increased acetoin and butanediol production	Geckil et al., 2004a,b
	E. coli	Improved growth and poly-beta-hydroxybutyrate (PHB) accumulation	Yu et al., 2001
	Saccharomyces sp.	Improved yield and D-arabitol productivity	He et al., 2001
	Sphingomonas elodea	Improved growth and gellan gum production	Wu et al., 2012
Fortified Antibiotic Production	*Acremonium chrysogenum*	Increased cephalosporin C production	DeModena et al., 1993
	Saccharopolyspora erythraea	Increased erythromycin production	Brunker et al., 1998; Minas et al., 1998
	Streptomyces cinnamonensis	Enhanced growth and monensin production	Wen et al., 2001
	Streptomyces aureofaciens	Increase chlortetracycline (CTC) yield	Meng et al., 2002

Enhanced Bioremediation	*Pseudomonas delafieldii*	Sulfur (from diesel oil)	Li et al., 2009
	Rhodococcus erythropolis LSSE8-1	Biodesulfurization of dibenzothiophene (DBT)	Xiong et al., 2007
	Pseudomonas putida	Benzene, toluene, chlorobenzene	Ouyang et al., 2007
	Pseudomonas aeruginosa	Improved growth and degradation of 2,4-dinitrotoluene (2,4-DNT)	So et al., 2004; Kim et al., 2005
	Xanthomonas mathophillia	Enhances degradation of benzoic acid	Liu et al.,1996
	Burkholderia sp.	Improved growth and degradation of benzoic acid	Kim et al., 2005
	Burkholderia sp.	Improved growth and degradation of 2,4-dinitrotoluene (2,4-DNT)	Lin et al., 2003
	Burkholderia cepacia	Enhanced degradation of 2-chlorobenzoate (2-CBA)	Urgun-Demirtas et al., 2003; 2004 Zhu et al., 2006
Physiological improvement	*Tremella fuciformis*	Enhanced growth	Khleifat, 2006
	E. coli	Increased copper uptake	Geckil et al. 2004a,b
	E. aerogenes	Increased sensitivity to mercury and cadmium	Erenler et al., 2004
	E. aerogenes	Improved growth and oxygen uptake rates	
	P. pastoris	Increased β-galactosidase activity	Wu et al., 2003
	E. coli	Enhanced resistance against the NO releaser sodium nitroprusside (SNP)	Frey et al., 2002

Table adopted from **Stark et al., 2011** with some modifications

Oxygen is required in several step of degradation pathway of hazardous contaminants of soil and groundwater but due to low dissolved oxygen (DO) seems to be limiting factor for the bioremediation mediated by microorganisms (Zhang et al.,

2007). To overcome this problem, several attempts had been made to develop organism which showed better growth and bioremediation property 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).

VHb expressing *Burkholderia* sp. enhanced 2,4-dinitrotoluen (DNT) degradation in continuous flow sand column bioreactor. With effluent DNT concentration of 214mg/l using wild type, the reactor reaches 20mg/L in 40 day under oxygen poor condition (3.1mg/L DO), whereas it was decreased to 1.7mg/L in 25 day under bioreactor engineered with VHb (So et al., 2004). Similar strategy was employed for the degradation of benzoic acid by *Xanthomonas mathophillia* (Liu et al., 1996) and 2-chlorobenzoic acid by *Burkholderia cepacia* (Urgun-Demirtas et al., 2003; 2004). VHb incorporation in *Rhodococcus erythropolis* LSSE8-1 improved growth and biodesulfurization in lower oxygen condition (Xiong et al., 2007). Biodesulfurization ratio of recombinant and wild type showed 37.5% and 20.5% at 70 rpm shaking condition. Similarly desulfurization of diesel oil showed reduction of sulphur from 261.3 mg/L to 70.1 mg/L by recombinant.

Under oxygen limited condition, *R. etli* expressing VHb increased respiratory activity, chemical energy content and expression of *nifHC* gene which in turn increased the efficiency of ATP production, nitrogenase activity and total nitrogen content in bean plants inoculated with recombinant *R. etli*. (Ramirez et al., 2009) Similarly, VHb could significantly contribute to the effectiveness in field conditions. On the basis of above information's genetic manipulation *E. asburiae* PSI3 had carried out to improve P mobilization in soil (**Fig. 1.4**)

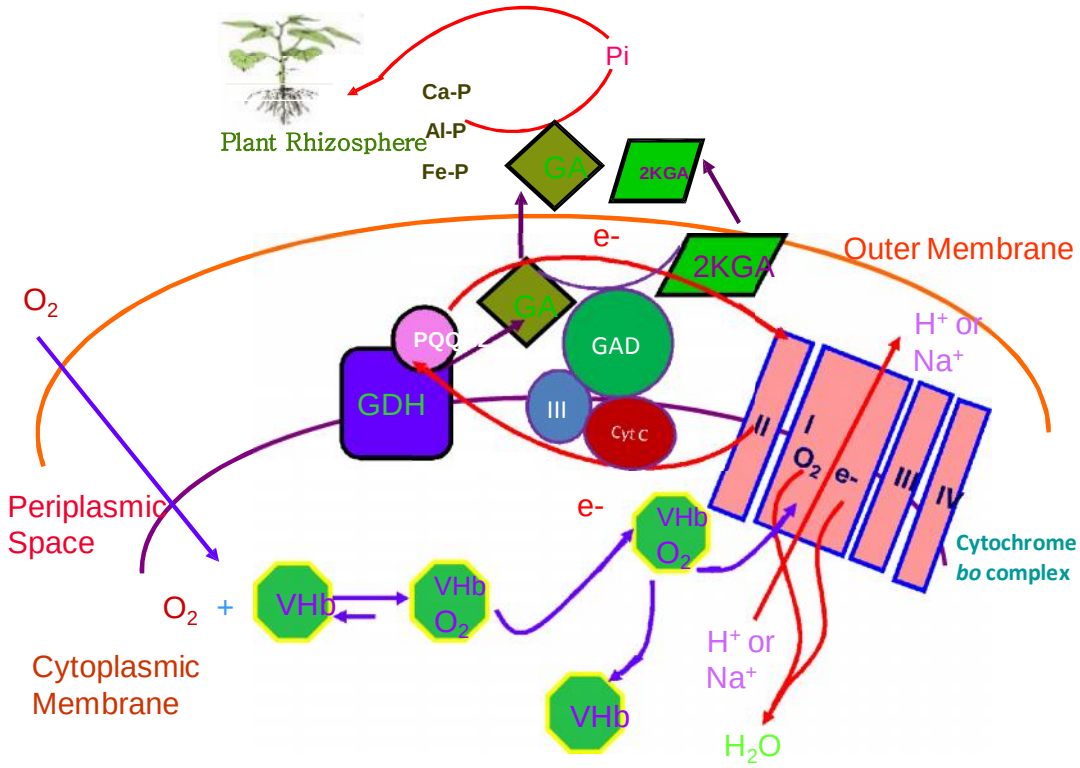

Fig. 1.4 Proposed model for genetic manipulation in *E. asburiae* PSI3

1.7 The objectives of the present study includes

(i) Characterization of MPS ability of *E. asburiae* PSI3 containing *Pseudomonas putida* KT2440 gluconate dehydrogenase (*gad*) operon.

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

(iii) Phosphate mineralization and solubilization abilities of *E. asburiae* PSI3 containing *Aspergillus fumigatus* phytase (*phyA*) gene and *Citrobactor braakii* phytase (*app*A).

(iv) Phosphate mobilization ability of *E. asburiae* PSI3 containing periplasmic *Uromycetes fabae* invertase, *C. braakii* phytase (*appA*) and *Vitreoscilla* haemoglobin (*vgb*) genes.

CHAPTER 2

Materials and Methods

2.1 Media and Culture conditions

The *E. coli* strains and *E. asburiae* PSI3 were cultured and maintained on Luria Agar (LA) (Hi-Media Laboratories, India). *E. coli* cultures were grown at 37ºC. For growth in liquid medium, shaking was provided at the speed of 200rpm. The plasmid transformants of both *E. coli* and *E. asburiae* PSI3 were maintained using respective antibiotics at the final concentrations as mentioned in **Table 2.1** as and when applicable. Both *E. coli* and *E. asburiae* PSI3 wild type strains and plasmid transformants grown in 3ml Luria broth (LB) containing appropriate antibiotics were used to prepare glycerol stocks which were stored at -20ºC.

Table2.1: Recommended dozes of antibiotics used in this study (Sambrook and Russell, 2001).

Antibiotics	Rich medium	Minimal medium
Kanamycin	50µg/ml	12.5µg/ml
Streptomycin	10µg/ml	2.5µg/ml
Trimethoprim	60µg/ml	15µg/ml
Ampicillin	50µg/ml	12.5µg/ml
Erythromycin	100µg/ml	25µg/ml
Gentamycin	20µg/ml	5µg/ml

The antibiotic dozes were maintained same for both *E. coli* and *E. asburiae* PSI3. All the antibiotics were prepared in sterile distilled water or recommended solvent at the stock concentrations of 1000x. The compositions of different minimal media used in this study

are as described below. Antibiotic concentrations in all the following minimal media were reduced to 1/4th of that used in the above mentioned rich media (**Table 2.1**).

2.1.1: Koser's Citrate medium

Composition of the Koser's Citrate medium included magnesium sulphate, 0.2g/L; monopotassium phosphate, 1.0g/L; sodium ammonium phosphate, 1.5g/L; sodium citrate, 3.0g/L and agar, 15g/L. The readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions.

2.1.2: M9 minimal medium

Composition of M9 minimal broth was according to Sambrook and Russell (2001) including Na_2HPO_4 $7H_2O$, 34g/L; KH_2PO_4, 15g/L; NH_4Cl, 5g/L; NaCl, 2.5g/L; 2mM $MgSO_4$; 0.1mM $CaCl_2$ and micronutrient cocktail. The micronutrient cocktail was constituted of $FeSO_4.7H_2O$, 3.5 mg/L; $ZnSO_4.7H_2O$, 0.16 mg/L; $CuSO_4.5H_2O$, 0.08 mg/L; H_3BO_3, 0.5 mg/L; $CaCl_2.2H_2O$, 0.03 mg/L and $MnSO_4.4H_2O$, 0.4 mg/L. Carbon sources used were glucose, xylose, arabinose, maltose, cellobios, mannose and sucrose as and when required. For solid media, 15g/L agar was added in addition to above constituents. 5X M9 salts, micronutrients (prepared at 1000X stock concentration) and carbon source (2M, 1M and 0.5M stock) were autoclaved separately. Fixed volumes of these were added aseptically into pre-autoclaved flasks containing distilled water to constitute the complete media with the desired final concentrations. Volume of water to be autoclaved per flask was calculated by subtracting the required volumes of each ingredient from the total volume of the media to be used.

2.1.3: Tris rock phosphate buffered medium

The media composition included Tris-Cl (pH=8.0), 100mM; NH_4Cl, 10mM; KCl, 10mM; $MgSO_4$, 2mM; $CaCl_2$, 0.1mM; micronutrient cocktail; Glucose, 50-100mM and phosphate (P) sources (Sharma et al., 2005). 1mg/ml Senegal Rock phosphate (RP) or KH_2PO_4 were used as insoluble and soluble P sources respectively. Each ingredient was separately autoclaved at a particular stock concentration and a fixed volume of each was added to pre-autoclaved flasks containing sterile distilled water (prepared as in Section 1.3) to constitute complete media.

2.1.4: Tris phytate buffered medium

Similar composition used in this medium as mention in Tris rock phosphate buffered medium. Rock phosphate replace with 0.3% Ca-phytate or Na- phyate.

2.1.5: Murashige-Skoog's Medium

Murashige-Skoog's (MS) medium was composed of macro elements and micro elements. The macro elements included $CaCl_2.2H_20$, 0.440g/L; KH_2PO_4, 0.17g/L; KNO_3, 1.9g/L; $MgSO_4.7H_2O$, 0.37g/L; NH_4NO_3, 1.65g/L and 10g/L agar. The micro elements included the essential trace elements. Micronutrients and desired carbon source (autoclaved separately), were added aseptically to reconstitute the complete media. The readymade micronutrient was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions while the macronutrients were reconstituted as and when required.

2.2: Molecular biology tools and techniques

2.2.1: Isolation of plasmid and genomic DNA

2.2.1.1: Plasmid DNA isolation from *E. coli* and *E. asburiae* PSI3

The plasmid DNA from *E. coli* and *E. asburiae* PSI3 was isolate using standard alkali lysis method (Sambrook and Russell, 2001).

2.2.1.2: Genomic DNA isolation from *Pseudomonas putida* KT2440

Fresh *Pseudomonas* culture obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30ºC was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed twice with sterile normal saline. Following this, the cells were used for genomic DNA isolation performed using standard genomic DNA extraction protocol for bacterial cell (Ausubel et al., 2006). The DNA was finally re-suspended in 40µl of sterile double distilled water.

2.2.2: Transformation of plasmid DNA

2.2.2.1: Transformation of plasmid DNA in *E. coli*

The transformation of plasmids in *E. coli* using $MgCl_2$-$CaCl_2$ method and blue-white selection of the transformants using IPTG and X-Gal (as and when applicable) was carried out according to Sambrook and Russell (2001).

2.2.2.2: Transformation of plasmid DNA in *E. asburiae* PSI3

Plasmid transformation in *E. asburiae* PSI3 was done using the NaCl-$CaCl_2$ method (Cohen et al., 1972) with slight modifications which are as follows. *E. asburiae* PSI3 was grown at 37ºC in LB broth to an O.D. 600 of 0.4-0.6. At this point, the cells were chilled for about 10minutes, centrifuged at 5000rpm for 5 minutes and washed once in 0.5 volume 10mM NaCl (chilled). After centrifugation (5000rpm for 5 minutes), bacteria were re-suspended in half the original volume of chilled 0.1M $CaC1_2$, incubated on ice-bath for 1hr, centrifuged (5000 rpm for 5 minutes) and then resuspended in 1/10th of the original culture volume of chilled 0.1M $CaC1_2$. 0.2ml of competent cells treated with $CaCl_2$ was used per vial (microcentrifuge tube) to add DNA samples (minimum 0.8-1.0µg is required) and were further incubated on ice-bath for 1hr. Competent cells were

then subjected to a heat shock at 42°C for 2 min to enable DNA uptake, immediately chilled for 5 minutes and then were supplemented with 0.8ml of sterile LB broth followed by incubation for 1hour at 37°C under shake conditions. These cells were then centrifuged and plated on Luria Agar or Koser citrate agar plates containing appropriate antibiotics. The colonies obtained after overnight incubation of the plates at 37°C were then subjected to plasmid DNA isolation.

2.2.3: Transfer of plasmid DNA by conjugation

The plasmids were transformed in *E. coli* S17.1, for mediating the conjugal transfer and the resultant transformant strain was used as the donor strain. *E. coli* S17.1 harboring the plasmid and the recipient *E. asburiae* PSI3 were separately grown in 3ml LB broth with respective antibiotics at 37°C under shake conditions for approximately 16h. The freshly grown cultures of recipient and the donor strains were aseptically mixed in 1:1 ratio (v/v) in a sterile centrifuge tube and the cells were centrifuged at 5000rpm for 5 minutes. The media supernatant was discarded to remove the antibiotics and the pellet was re-suspended in 0.2ml of fresh sterile LB and the bacteria were allowed to mate at 37°C. After 16h, the bacterial culture mix was centrifuged at 5000rpm for 5 minutes and the resultant pellet was re-suspended in 0.05ml of sterile normal saline, this cell suspension were plated on Koser Citrate agar containing the appropriate antibiotics for selection (antibiotic dose was as described in **Table 1**) to obtain the transconjugants.

2.2.4: Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% agarose (containing 1µg/ml ethidium bromide) gel in Tris-acetate-EDTA

(TAE) buffer at 5v/cm for 0.5-2h. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator.

2.2.5: Restriction enzyme digestion analysis

0.5-1.0µg DNA sample was used for each restriction enzyme digestion. 1-3U of the restriction endonuclease (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10µl. The reaction mixture was incubated overnight at 37°C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and were subsequently photographed. In case of double digestion, a compatible buffer for the two REs was essentially checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers. Restriction digestion was performed according to manufacturer instruction. Typical reaction follows

Components	**Quantity**
DNA (~300 ng/ µl)	3.0µl
Buffer (10X)	2.0µl
Restriction Enzyme (10U/µl)	0.3µl
Autoclaved D/W	14.7µl
Total	20 .0µl

Reaction mixtures were incubated for 4 hr at 37^0C. The samples ware analyze on 0.8% agarose

2.2.6: Polymerase Chain Reaction (PCR)

The PCR reaction set up was based on the guidelines given in Roche Laboratory Manual. The assay system and the temperature profile used are described as follows

PCR reaction mixture:

Forward primer (10pmol/ml)	**1.0 µl**
Reverse Primer (10pmol/ml)	**1.0 µl**
dNTPs (2.5mM)	**3.0 µl**
10X Pfu polymerase Buffer	**5.0 µl**
Pfu polymerase (3 units/µl)	**0.5 µl**
Template	**0.5 µl**
Nuclease free water	**39.0 µl**
Total system	**50.0 µl**

PCR amplifications were performed in DNA Engine thermal cycler (BioRad) or Variti thermocycler (Applied Biosystem). *Exact primer annealing temperature and primer extension time varied with primers (designed with respect to different templates) and has been specified in the text as and when applicable. Taq DNA polymerase, XT-20 polymerase, Pfu polymerase and Phusion polymerase used as when required mention in text. Polymerase, buffer, dNTPs and primers were obtained respectively from Bangalore Genei Pvt. Ltd., India, Sigma Chemicals Pvt. Ltd., Ocimum biosolutions, India, Integrated DNA Technology USA. The theoretical validation of the primers with respect to absence of intermolecular and intramolecular complementarities to avoid primer-primer annealing and hairpin structures and the appropriate %G-C was carried out with the help of online primer designing software Primer 3. The sequence, length and %G-C content of primers are subject to variation depending on the purpose of PCR and will be

given as and when applicable in the following chapters. The PCR products were analyzed on 0.8% agarose gel along with appropriate molecular weight markers.

PCR program

Lid temperature	**105°C**	
Step -1 (Initial denaturation)	**94^0 C - 1 min cycle 1**	
Step - 2 (Denaturation)	**94^0 C - 30 sec**	**30 cycle**
Step -3 (Annealing)	**52^0 C - 30 sec**	
Step – 4 (Extension)	**72^0 C - 1 min 30 sec**	
Step -5 (Final extension)	**72^0 C – 5 min cycle 1**	
Hold: - 4^0C - 20 min		

2.2.7: Gel elution and purification

The DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band. The agarose piece was weighed in a sterile microcentrifuge tube. Gel elution and purification was done by using PureLink Quick Gel Extractio Kit (Invitrogen) according to manufacturer protocol.

2.2.8: Ligation

The ligation reaction was usually done in 10µl volume containing the following constituents: Purified vector and insert DNA (volume varied depending on the respective concentrations); 10X T4 DNA Ligase buffer, 1µl; T4 DNA ligase (MBI Fermentas), 0.5-1.0U and sterile double distilled water to make up the volume 10 µl. The cohesive end ligation reaction was carried out at 22°C for 1h. The vector to insert molar ratio (molar concentrations calculated by the under mentioned formula) of 1:4 was maintained, with a total of 50-100ng of DNA in each ligation system.

$$\text{pmoles of DNA} = \frac{\text{Amount of DNA (µg)} \times 1,515}{\text{Size of the DNA fragment (no. of base pairs)}}$$

2.2.9: SDS-PAGE

SDS-PAGE slab gel electrophoresis was carried out using 10-15% acrylamide gel by following the procedures described by Sambrook et al (2001).

Table 2.2: Composition of SDS-PAGE reagents (Sambrook and Russell, 2001)

(A) Monomer solution (30%) (Store at 4° C in dark)		**(B) Resolving gel buffer-1.5M Tris (pH 8.8)** Adjust pH with HCl		**(C) Stacking gel buffer 1.0M Tris (pH 6.8)** Adjust pH with HCl	
Acrylamide	14.6 gm	Tris base	9.1 gm	Tris	3.02 gm
Bisacrylamide	0.4 gm	SDS	0.2 gm	SDS	0.20 gm
D. H_20	Till 50 ml	D. H_20	Till 50 ml	D. H_20	Till 50ml
(D) Tank Buffer (pH 8.3)		**(E) Sample Loading buffer (2X)**		**(F) Other reagents**	
Tris base	6.0 gm	SDS	4%	APS	10% (fresh)
Glycine	28.8 gm	Glycerol	20%	TEMED	2-3 µl
SDS	2.0 gm	Tris-Cl (pH6.8)	0.125M	Water saturated n-butanol	
D. H_20	Till 2 L	Bromophenolblue	0.05%w/v	Sigma protein molecular weight marker SDS6H2-(30,000-200,000).Used 14µg total protein/well	
Adjust the pH with HCl		β-mercaptoethanol	10mM		
		D. H_20	Till 10ml		
(G) Separating Gel (8%, 10ml)		**(H) Stacking Gel (3.9%, 5ml)**		**(I) Staining Solution**	
30% Monomer	2.7 ml	30% Monomer	0.65 ml	0.025% Commassie Blue R-250 in 40% Methanol and 7% Acetic acid	
Separating gel buffer (pH 8.8)	2.5 ml	Stacking gel buffer (pH 6.8)	1.25 ml		
D. H_20	2.3 ml	D. H_20	3.05 ml	**(J) De-staining solution**	
10% APS	50 µl	10% APS	25 µl	(10% methanol and 10% Acetic acid)	
TEMED	2 µl	TEMED	3 µl		

After electrophoresis the gel was stained using the staining solution (**Table 2.2**) for approximately 1h and then de-stained with de-staining solution (**Table 2.2**) by incubating overnight under mild shaking conditions. Result was recorded by direct scanning of gel.

2.3: P-solubilization phenotype

P-solubilizing ability of the native as well as the transformant *E. asburiae* PSI3 was tested on Tris buffered RP-Methyl red (TRP) agar plates which represents a much more stringent condition of screening for PSMs (Gyaneshwar et al, 1998b). Liquid medium for RP solubilization is described in Section 2.1.3 with an addition of methyl Red as pH indicator dye and 1.5% agar for all the plate experiments. *E. asburiae* PSI3 cell suspension for these experiments was prepared as described in section 2.4.1 and 3µl of it was aseptically spotted on the above mentioned agar plates and was allowed to dry completely followed by incubation at 37°C for 5-7 days. P solubilization was determined by monitoring red zone on the TRP agar plates. Media acidification from pH=8.0 to pH<5 on TRP broth was used as an indicator for P-solubilization and organic acid secretion.

2.4: Physiological experiments

The physiological experiments were carried out using various WT and transformant *E. asburiae* PSI3 which included growth, pH profile and enzyme assays.

2.4.1: Inoculum preparation

The inoculum for M9 and Tris minimal media containing free Pi was prepared by growing the *E. asburiae* PSI3 cultures overnight at 37°C in 3ml LB broth. Cells were harvested aseptically, washed twice by normal saline and finally re-suspended in 1ml

normal saline under sterile conditions. Freshly prepared inoculum was used for all the experiments.

2.5.2: Growth characteristics and pH profile

Growth parameters and pH profile of the native as well as transformant *E. asburiae* PSI3 were determined using three different media conditions including TRP medium with RP as P source (Gyaneshwar et al., 1998b). The media composition in this case was same as mentioned in Section 45- 100mM glucose or 50-100mM sucrose was used as the carbon source for all the experiments unless and until stated categorically. In 150ml conical flasks, 30ml of relevant minimal broth without free Pi was inoculated with cell suspensions to have 0.01-0.03 O.D600nm initially (0 hour O.D.).The batch culture studies were performed under aerobic conditions in Orbitek rotary shaker maintained at 37°C with agitation speed kept constant at 200rpm. 1ml samples were aseptically harvested at regular intervals (varying with every set of batch culture depending on media conditions) and were subjected to various analytical techniques.

2.4.3: Analytical techniques

The cell density determinations were done at 600nm as monitored spectrophotometrically. Change in absorbance was considered as the measure of growth and drop in pH of the media was taken as the measure of acid production. In all the cases, the observations were continued till the media pH reduced to less than 5. 1ml aliquots withdrawn aseptically at regular time intervals were immediately frozen at -20°C until further used for biochemical estimations. The stored samples were centrifuged at 9, 200x *g* for 1 min at 4°C and the culture supernatants derived were used to estimate residual glucose and organic acid analysis using HPLC. For HPLC analysis, the culture

supernatant was passed through 0.2µm nylon membranes (MDI advanced microdevices, India) and the secreted metabolites were quantified using RP-18 column or Luna column (Phenomenex). The column was operated at room temperature using mobile phase of 0.01M 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 membranes and were subjected to chromatography for determining the individual retention time. Measurements of area under peak with an external standard were used for quantification.

2.5: Enzyme assays

2.5.1: Preparation of cells and cell free extracts

Glucose grown cells under above mentioned in TRP medium conditions till pH goes below 6.0. Cells were collected by centrifugation 9,200x *g* for 2 minutes at 4°C. The whole cell preparation for GDH assay was done by washing the harvested cells (mid-late log phase cultures) thrice with normal saline to remove the residual glucose of the medium and resuspending in 0.01M phosphate buffer (pH 6.0) with 5mM $MgCl_2$.

2.5.2: Enzyme Assay Protocols

2.5.2.1: Invertase assay

The bacterial isolate was grown overnight in M9 minimal medium with sucrose or glucose as the carbon source. Cells were harvested by centrifugation at 5000g for 10 min, then washed with and resuspended in sterile saline. An aliquot of the cells was used for toluenization to permeabilize the cells. Whole cells as well as toluenized cells were used for enzyme assay. The assay system (2 ml) consisted of 0.01 M sucrose, 0.1 M buffer (acetate buffer, pH 5.0, phosphate buffer, pH 7.0, or Tris Cl buffer, pH 8.0), with an

appropriate amount of cells as the source of enzyme. The reaction system was incubated at 37°C for 30 min and the reducing sugar produced was measured by the method of Miller (1959). One unit of activity is defined as the amount of enzyme that produced reducing sugar equivalent to 1 µmol of glucose per h. Specific activity is defined as units per milligram of protein.

2.5.2.2: GDH assay

GDH (D-glucose phenazine methosulphate oxidoreductase, (EC 1.1.5.2) was determined spectrophotometrically by following the coupled reduction of 2,6-dichlorophenolindophenol (DCIP) at 600nm (Quay et al., 1972). Molar absorbance of DCIP was taken as 15.1 mM-1 cm-1 at pH 8.75. The reaction mixture included: Tris-Cl buffer (pH 8.75), 16.66mM; D-glucose, 66mM; DCIP, sodium salt, 0.05mM; phenazine methosulfate, 0.66mM; sodium azide, 4mM; whole cells, and distilled water to 3.0ml.

2.5.2.3: GAD assay

Wild type and recombinant PSI3 were grown on RP containing minimal medium containing 75mM glucose and 100 mM Tris Cl (pH- 8.0) as carbon source. After pH drop below 5.5, cells were harvested (10000 g for 5 min) and washed with sterile saline and resuspend in 0.1M potassium phosphate buffer pH 6.0. Hole cells were used as GAD enzyme source and assay was performed according to Matsushita and Ameyama (1982). Units of activity of GAD that catalyze the oxidation of 1 µmole of D- gluconate or reduction of 1 µmol of DCIP per minute at 25 C. Specific activity is defined as units per milligram of protein.

2.5.2.4: Phytase assay

Phytase and phosphatase activities were determined at acid (pH 4.5) and

alkaline (pH 8.0) pH. Transformants were grown till stationary phase in 25 ml LB media or Minimal medium containing 50mM glucose as carbon source. Culture supernatants obtained by centrifuging culture suspensions at 4ºC for 10 min at 12,000 × g were used for determining extracellular enzyme activities. For assaying cell associated enzyme activities, the cell pellets so obtained were washed two times with sterile 0.85 % NaCl solution, resuspended in 3 ml chilled sterile 0.85 % NaCl and subjected to sonication for 3.33 min at 9.9 sec on/off pulsed at 28 % amplitude by using Branson Sonifire ultrasonicator. Sonicated suspensions were centrifuged as above for 15 min to remove cell debris and supernatant was used for assaying cell associated enzyme activity.

Phytase activity was measured using 0.7 ml assay buffer consisting of 0.1 M Na-acetate pH 4.5 (for acid phytase) or 0.1 M of Tris-Cl pH 8.0 (for alkaline phytase), both containing 0.7 mM of Na-phytate and the total system was made up to 1.0 ml with enzyme aliquot and/or distilled water and incubated at 37°C for 30 min. Release of P from Na-phytate was estimated by Ames method (Ames, 1964).

Unit activity of phytase is defined as the amount of enzyme liberating 1µmol P/min.

2.5.2.5: Phosphatase assays

Phosphatase activity of the transformants was determined by using *p*-nitro phenyl phosphate (pNPP) as a substrate. For acid phosphatase, 0.5M Na-acetate buffer pH 4.5 and for alkaline phosphatase, 0.5M Tris-Cl (pH 8.0) were used. Different aliquots of the sample were incubated with 0.1 ml of 0.5 M pNPP along with 0.1 ml of 0.1M magnesium chloride ($MgCl_2$) and 0.1 ml of appropriate buffer. Total system was made up to 2 ml by using distilled water and incubated in dark at 30° C for 30 min, after which the reaction was stopped by adding 2 ml of 2N NaOH. The reaction mixture was vortexed and the

amount of p-nitro-phenolate (pNP) released was estimated by measuring absorbance at 405 nm. One unit of phosphatase is defined as the amount that releases 1 µmol of pNP/min.

2.6: Inoculation of mung beans (*Vigna radiata*)

Mung bean seeds were surface sterilized by treating with 0.1% (w/v) mercuric chloride for ~3-5 minutes followed by 5 rounds of thorough washing with 100ml freshly sterilized distilled water (Ramakrishna et al., 1991). These seeds were placed in autoclaved petri-dishes containing moist filter paper and were allowed to germinate in dark for 24h at room temperature. *E. asburiae* PSI3 was inoculated in 25 ml sterile LB broth and was allowed to grow at 37°C with overnight shaking. Fresh culture was harvested by centrifuging at 9, 200x *g* for 2 minutes; the pellet was aseptically washed twice with sterile normal saline and finally was re-suspended in 4 ml of normal saline (Ramos et al., 2000). Healthy germinated seeds were incubated with this cell suspension in a sterile petri-dish under aseptic conditions for 10-15 minutes. Root tips of hence obtained *E. asburiae* PSI3 coated mung bean seeds were implanted with the help of sterile forceps in autoclaved alkaline vertisol soil. Plants were allowed to grow at room temperature with sufficient light conditions for 15 days after which effect of *E. asburiae* PSI3 inoculation was monitored in terms of overall plant growth.

2.7: Root colonization study

Colonization of *E. asburiae* PSI3 on mung bean root was monitored by tagging with fluorescent plasmids. Plants were grown in hydroponics or in non sterile soil inoculated with tagged bacteria. In case of hydroponics system root colonization was monitored at 5^{th} day of plant growth. In case of soil it was monitored on 5^{th} and 10^{th} day

of plant growth. 10^6 to 10^8cfu/ml bacterial cells were used to soak the sterile condition germinated seed for 30 minute. Before doing confocal microscopy roots were washed twice with sterile saline and fixed on glass slide.

CHAPTER 3

Characterization of MPS abilities of *E. asburiae* PSI3 containing Gluconate dehydrogenase operon (*gad*) from *Pseudomonas putida* KT2440.

3.1: INTRODUCTION

Phosphorus is a major macronutrient after nitrogen required for plant growth (Theodorou et al.,1991). P is abundant in the soil in the form of organic and inorganic metal complexes (Ca, Al, Fe) depending on nature of soil but not available to plants. Soluble P forms, $H_2PO_4^-$ and HPO_4^{--}, are used by plants (Ae et al., 1991). Application of chemical fertilizers in the field is not very efficient due to high P refixing capacity of the soil (Goldstein et al., 1995). Rhizobacteria promote plant growth by different mechanism and one of them is mineral phosphate solubilization (Kucey et al., 1989). A large number of bacteria belong to different genera and many fungi possess mineral phosphate solubilization ability which has been attributed to secretion of organic acid *viz*, D- gluconic acid (GA), citric acid, oxalic acid and 2-keto-D-gluconic acid (2-KG) etc. (Goldstein 1995; Khan et al., 2006; Archana et al., 2011).

Solubilization of mineral phosphate has been extensively studied in Gram negative bacteria using glucose as a carbon source (Goldstein 1995; Gyaneshwar et al., 1999; 2002). GA is prominent organic acid produced via direct oxidation pathway by membrane bound quinoprotein dependent glucose dehydrogenase (GDH). Pyrroloquinoline quinine (PQQ) is the cofactor for GDH enzyme and its biosynthesis requires many genes which vary in bacteria (Choi et al., 2008). Genes conferring mineral phosphate solubilization ability were cloned from phosphate solubilizing microorganisms (PSMs) like *Erwinia herbicola, Pseudomonas cepacia, Enterobacter intermedium, Serratia marcescens, Rahnella aquatilis* and non-PSMs *Synechocystis* PCC 6803 (Gyaneshwar et al., 1998a; Zaidi et al., 2009). Incorporation of *pqqE* gene from these PSMs has enabled to develop mineral phosphate solubilization ability in

Escherichia coli, *Burkholderia cepacia* IS-16, *Pseudomonas* sp. PSS and *Azospirillum* sp. Field studies of plant inoculations with PSMs had inconsistent effect on plant growth and crop yields. This has been attributed to variations in soil, crop and environmental factors influencing the survival and colonization of the rhizosphere. Buffering capacity of Alkaline vertisol, refixation of acidic alfisols, availability of carbon sources, nature of nitrogen source, nature of mineral phosphates and catabolite repression of organic acid secretion have been demonstrated to determine the efficacy of PSMs in field conditions (Kucey et al., 1989; Gyaneshwar et al., 1998b; Srivastava et al., 2007; Patel et al., 2011).

Enterobacter asburiae PSI3, an isolate of pigeon pea (*Cajanus cajan*) rhizosphere, solubilizes RP in buffered conditions by secretion of GA mediated by phosphate starvation inducible GDH (Gyaneshwar et al., 1999). In addition to glucose, *E. asburiae* PSI3 can utilize various mono- and di-saccharides for solubilizing RP due to the broad substrate specificity of GDH and it requires a mixture of seven aldosugars at 15mM concentrations for solubilizing RP in buffered medium (Sharma et al., 2005). 2-ketogluconic acid (2-KG) is much stronger than GA and also efficiently chelates calcium in soils (Moghimi et al., 1978; Moghimi and Tate 1978). Many bacteria are known to secrete 2-KG (Misenheimer et al., 1965; Webley and Duff 1965; Yum et al., 1997; Hwangbo et al., 2003; Saichana et al., 2009; Vyas and Gulati, 2009; Gulati et al., 2010; Park et al., 2010). GA gets converted to 2-KG in the periplasm by gluconate dehydrogenase (GAD) enzyme encoded within *gad* operon (Yum et al., 1997; Toyama et al., 2007; Saichana et al., 2009). GAD has been characterized and purified from *Pseudomonas*, *Kleibsiella pneumoniae*, *Serratia*

marcescens, acetic acid bacteria (*Gluconobacter* sp.) and *Erwinia cypripedii* ATCC 29267. *gad* operon encodes for three subunits namely, FAD dependent gluconate dehydrogenase, cytochrome c and smallest third subunit of unknown function (Matsushita et al., 1982; Yum et al., 1997; Toyama et al., 2007; Saichana et al., 2009). In present study, *Pseudomonas putida* KT 2440 *gad* operon was expressed in *E. asburiae* PSI3 which led to secretion of 2-KG and solubilized rock phosphate more efficiently in buffered condition.

3.2 Material and Methods

3.2.1 Bacterial strains, plasmids and media

The bacterial strains and plasmid used in this study are listed in **Table 3.1**. Routine DNA manipulation was done in *E. coli* DH10B (Invitrogen) using standard molecular biology protocols (Sambrook and Russel, 2001). pTTQ18 and pGM160 were generously gifted by Dr. Michael J.R. Stark (Leicester Biocentre, University of Leicester) and Dr. Günther Muth (University of Beilfeld, Germany). *E. coli* DH10B, *E. asburiae* PSI3 (*Cajanus cajan* rhizosphere isolate) and its plasmid derivatives were grown on Luria-Bertanni (LB) medium (Hi Media, India) containing 20 µg/ml streptomycin, 50 µg/ ml ampicillin, erythromycin (20µg/ml) and 10 µg/ml gentamycin as and when required.

Table 3.1 Bacterial strains and plasmids used in this study

Plasmid/Strains	Relevant characteristics	Source/Reference
Plasmids		
pTTQ18	Cloning vector-ColE1 origin, P_{tac}, Ap^r	Stark, 1987
pGM160	Cloning vector, Gm^r	Muth *et al*., 1989
$pTTQGm^r$	pTTQ18 with *acc*, Ap^r Gm^r	This study
pJET 2.1	Blunt end PCR cloning vector	Fermantas
pCNK12	pJET2.1 with *gad* operon	This study
pCNK14	$pTTQGm^r$ with *acc* gene, *gad* operon, Ap^r Gm^r	This study
Bacterial Strains		
E. coli DH10B	Host strain for routine DNA manipulation experiments and plasmid maintenance	Invitrogen
E. asburiae PSI3	*Cajanus cajan* rhizosphere isolate	Gyaneshwar *et al*., 1998b
E. a. PSI3(pCNK14)	*E. asburiae* PSI3 containing pCNK14	This study
E. a. PSI3($pTTQGm^r$)	*E. asburiae* PSI3 containing $pTTQGm^r$	This study

3.2.2: Cloning of gluconate dehydrogenase (*gad*) operon in blunt end cloning vector pJET2.1

Genomic DNA isolation from *Pseudomonas putida* KT2440 was done as described by Sambrook and Russel (2001). Sequence of *gad* operon was obtained from *P. putida* KT2440 genome database (Accession No. AE015451). Gene specific primer pair 5'CGGATCCCG*AGGAGG*AATGTCATGCCTGAGCATGCCCC3' and 'GCTCTAGAGCTCAGCGAAGCGACTTTACATC 3'were used for obtaining *gad* amplicon. Underline sequence showed *Bam*HI and *Xba*I restriction enzyme site used for cloning. Italic sequence indicates RBS. PCR amplification was done using XT-20 polymerase (Banglore Genei, India) from 10 ng template DNA of *Pseudomonas putida* KT2440. Amplified PCR product was ligated into blunt end PCR cloning vector pJET2.1 (MBI Fermantas, India) according to manufacturer instruction. Positive clones were confirmed by restriction digestion and designated as pCNK12.

3.2.3: Cloning of *aacC3* gene confer for gentamycin in pTTQ18

E. asburiae PSI3 is naturally resistant to ampicillin. Hence, aminoglycoside-(3)-N-acetyltransferase III (*aacC3*) gene from pGM160 (1.6kb *Hind*III fragment) was cloned in *Hind*III site of pTTQ18 to obtain pTTQGm^r. Recombinant plasmid was confirmed by growing them on gentamycin containing LA plates and restriction digestion. Plasmid was designated to pTTQGm^r.

3.2.4: Construction of pCNK14 containing gluconate dehydrogenase (*gad*) operon of *Pseudomonas putida* KT2440 under control of *tac* promoter

pCNK12 was digested with *Bam*HI and *Xba*I which gives 3.8 kb release of GAD operon. 3.8 kb fragment was gel eluted and subcloned in gel purified pTTQGm^r digested with *Bam*HI and *Xba*I to obtained pCNK14. Clone was confirmed by restriction digestion. Functionality of the operon was confirmed by GAD enzyme assay.

3.2.5: Physiological experiments

Bacterial cells were prepared by growing the cells overnight at 37 °C in LB. Batch culture studies were performed under aerobic conditions in Orbitek rotary shaker with temperature maintained at 37°C and agitation speed was kept constant at 200 rpm. The composition of minimal medium used for the physiological studies is as follows: TRP minimal medium- 100mM Tris-Cl (pH 8.0), (45mM, 50 mM and 60mM) glucose, 10mM NH_4Cl, 10mM KCl, 2mM $MgSO_4$, 0.1mM $CaCl_2$ and micronutrient cocktail (Gyaneshwar et al., 1998b) with 1mg per ml Senegal Rock phosphate (RP) as sole phosphorus source. Erythromycin and gentamycin was added to a final concentration of 7.5 µg ml^{-1} and 2.5µg ml^{-1}, respectively.

3.2.6: Monitoring of MPS ability of recombinant *E. asburiae* PSI3

MPS ability of recombinant *E. asburiae* PSI3 was determined on TRP minimal medium plates (100mM tris buffer pH-8.0, RP 1mg/ml, methyl red, 1.8% agar and varying concentration of glucose *viz* 60 mM, 50mM and 45 mM). Same combination was used for shake flask experiments without agar. Absorbance at 600

nm was used for cell growth and pH drop was used as acidification of medium. Supernatant of medium was used for Pi estimation using KH_2PO_4 as standard (Ames, 1964).

3.2.7: Measurement of GDH and GAD activities

Wild type and recombinant *E. asburiae* PSI3 were grown on TRP containing minimal medium containing 75mM glucose as carbon source. After pH dropped below 5.5, cells were harvested (10000 g for 5 min) and washed with sterile saline and resuspended in 50mM Tris buffer pH 8.75. Whole cells were used for GDH and GAD enzyme assay and the assay was performed according to Matsushita and Ameyama (1982) and Matsushita et al (1982). GDH (EC 1.1.5.2) was determined spectrophotometrically by following the coupled reduction of DCIP at 600nm. The molar absorbance of DCIP was taken as 15.1 $mM^{-1}cm^{-1}$ at pH 8.75. The reaction mixture included: Tris-Cl buffer (pH 8.75), 16mM; DCIP, sodium salt, 0.05mM; PMS, 0.66mM; sodium azide, 4mM; D-glucose, 66mM; whole cells and water to 3.0 ml. For GAD activity, the protocol was similar to GDH assay except the 0.01M potassium phosphate buffer pH 6.0 containing 66mM D-gluconate. The molar absorbance of DCIP was taken as 10.0 $mM^{-1}cm^{-1}$ at pH 6.0.

Total whole-cell protein was estimated using a modified Lowry's method (Peterson, 1979). Units of activity of GDH and GAD are defined as nmol of 2, 6-dichlorophenolindo-phenol (DPIP) reduced per minute using glucose or gluconate as substrates, respectively. Specific activity is defined as units per mg total protein.

3.2.8: Analytical methods

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 Luna C-18 column (Phenomenex, India). 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.

3.2.9: Plant inoculation study

For plant inoculation study was carried out in pots of sterile local alkaline vertisol. Mung bean (*Vigna radiata*) seeds were surface sterilized with 0.1% $HgCl_2$ solution, followed by 70% ethanol washed thoroughly and germinated in sterile distilled water. Overnight 50 ml LB grown wild type and genetic transformants of *E. asburiae* PSI3 were washed thrice with normal saline and dissolved in 5 ml saline.

Mung bean seeds were soaked in bacterial culture for 30 min. Uninoculated control seeds were treated with saline. 15 to 20 grown seeds were inoculated in a single pot. Three sets of 5-6 pots each of uninoculated control, *E. asburiae* PSI3 (pTTQGm) and *E. asburiae* PSI3 (pCNK14) were grown under natural light at ambient temperature for 15 days. Uprooted plant shoot and root were separated and dried at 65^{o}C for constant dry weight measurement. Dried plant shoots (n=6) were ashed in muffle furnace at 550^{0}C for 15 h and ashes were dissolved in 0.9M H_2SO_4 at 10 mg dry weight per ml acid (Richardson et al., 2001). Subsequently P contents of H_2SO_4 extract was estimated by molybdate- blue method (Murphy and Reilay, 1962).

3.2.10: Data analysis

Physiological experiments were done in three independent triplicates. Data are expressed in mean and standard deviation. In plant experiments, three independent duplicate studies were performed. Differences in mean values were determined using general analysis of varience (ANOVA) and linear regression analysis was done using GraphPad Prism5.0.

3.3 Results

3.3.1 Cloning and heterologous overexpression of *gad* operon of *P. putida* KT 2440 in *E. asburiae* PSI3.

Cloning strategy of gad operon schematically represented in Figure 2. *gad* operon was cloned from genomic DNA of *P. putida* KT 2440 in blunt end cloning vector pJET 2.1. Clone was confirmed by RE digestion with *Bam*HI/*Xba*I, *Bgl*lII and *Bam*HI/*Hind*III. *Bam*HI/*Xba*I digestion gives release of 3.8kb gad operon and 2.6kb vector. BglII present in both side of MCS so digestion will give 3.8 kb gad operon release and vector backbone. *Bam*HI/*Hind*III digestion gives 4.2kb due to presence of *Hind*III in vector, 400bp downstream of MCS. 3.8 kb *gad* operon was subcloned in *Bam*HI/*Xba*I RE sites of expression vector pTTQGm^r under the control of *tac* promoter to obtain pCNK14. pCNK14 was confirmed by RE digestion with *Bam*HI/*Hind*III. *Bam*HI /*Hind*III digestion give 3.8 kb *gad* operon, 1.6kb gentamycin and 4.5 kb vector backbone.

pTTQGm and pCNK14 were transformed into *E. asburiae* PSI3. *E. asburiae* PSI3 *gad* operon transformant showed similar GAD activity in the presence and absence of IPTG indicative of constitutive expression. In LB, GAD enzyme activity was found to be 985±44 which was enhanced by ~1.7 fold in presence of 2% D-gluconate. In TRP medium containing 75mM glucose, GAD and GDH specific activities of the *E. asburiae* PSI3 (pCNK14) were found to be 438.3± 0.113 U and 884.2±0.182 U, respectively. There was no significant difference in GDH activity was observed as compared to wild type and plasmid control (**Table 3.2**).

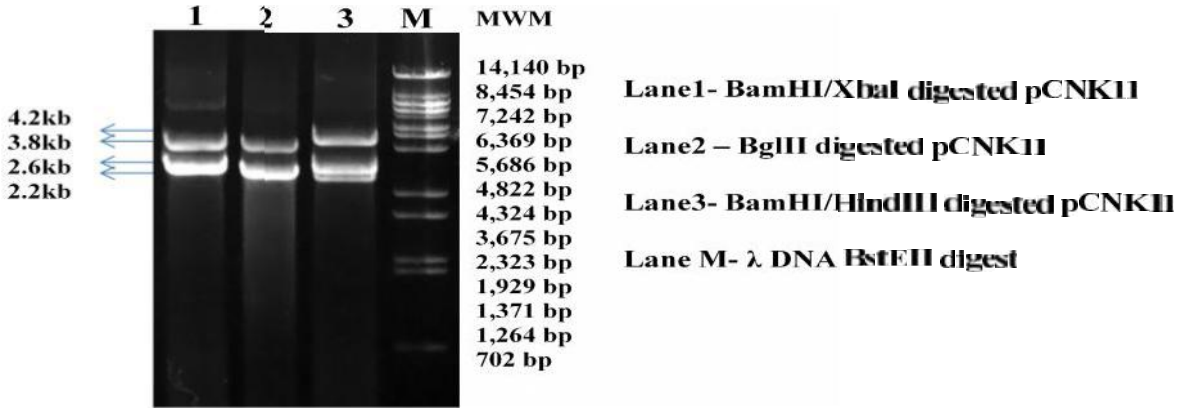

Fig.3.1 Restriction digestion pattern of pCNK11

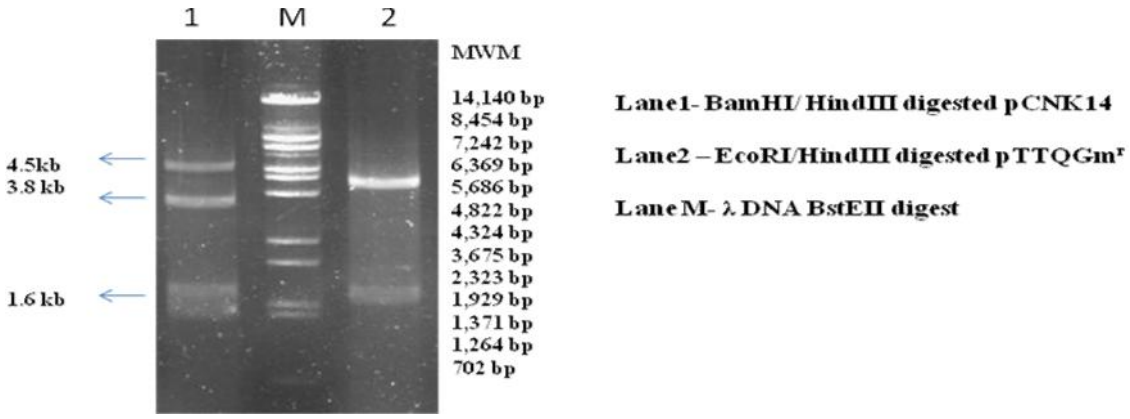

Fig.3.2 Restriction digestion pattern of pCNK14

3.3.2 Effect of *gad* overexpression on mineral phosphate solubilization ability of *E. asburiae* PSI3

E. asburiae PSI3 (pCNK14) showed acidification and mineral phosphate solubilization ability on TRP plates containing 45mM glucose while *E. asburiae* PSI3 and *E. asburiae* PSI3 (pTTQGm^r) did not show acidification and mineral phosphate solubilization ability when the glucose concentration was less than 75 mM (**Fig.3.3**).

In liquid TRP medium, *E. asburiae* PSI3 (pCNK14) grew upto 0.3 O.D. and acidified the medium to pH 3.9 in 96 h (Fig.2). Pi release was found to be 0.84mM. *E. asburiae* PSI3 secreted ~ 50 mM GA in presence of 75 mM glucose in TRP medium whereas *gad* transformant secreted 11.5 mM 2-KGA and 21.6 mM GA when grown in TRP medium containing 45mM glucose as a carbon source (**Table 3.3**). Thus, the efficiency of GA conversion to 2KGA by *E. asburiae* PSI3 (pCNK14) is ~30%.

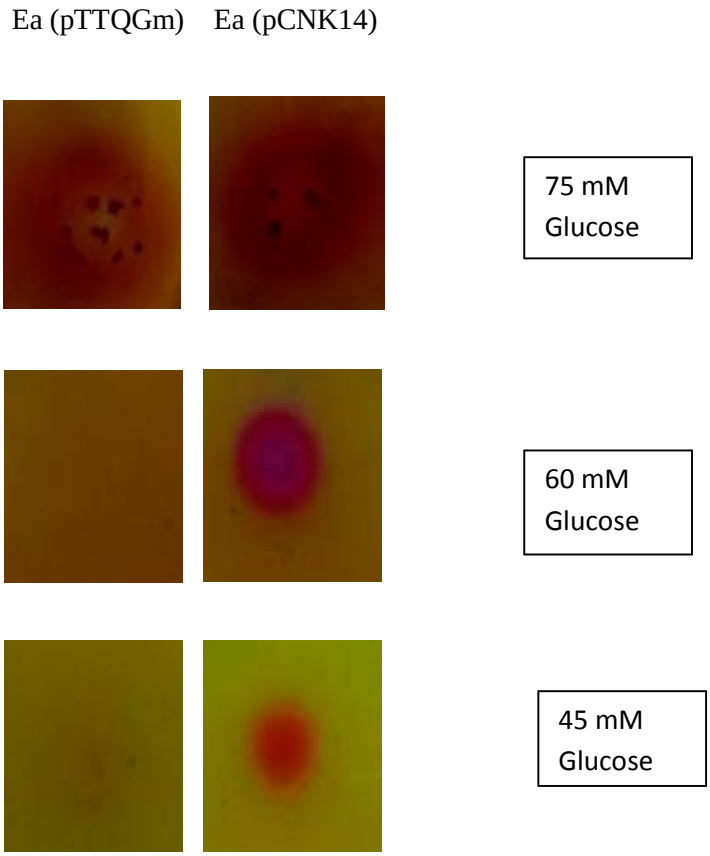

Fig.3.3 MPS ability of *E. asburiae* PSI3 (pTTQGm) and *E. asburiae* PSI3 (pCNK14) in TRP agar medium with glucose as carbon source.

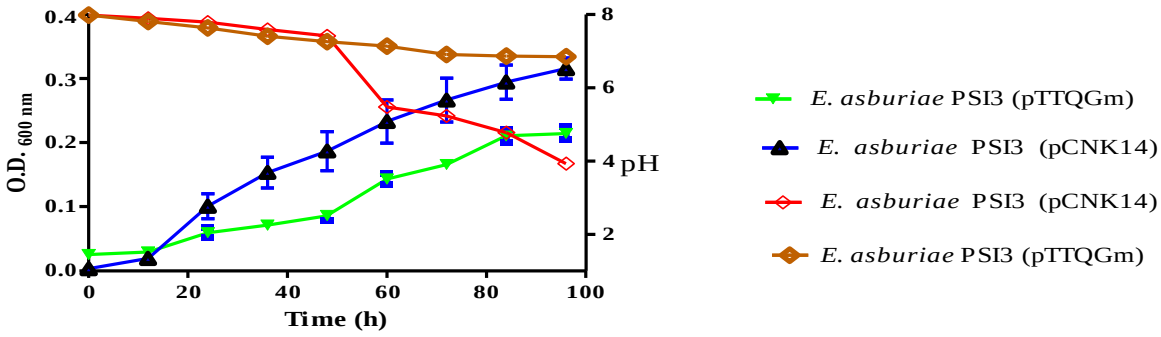

Fig. 3.4: Growth and pH profile of *E. asburiae* PSI3 (pTTQGm) and *E. asburiae* PSI3 (pCNK14) in TRP medium containing 45 mM glucose.

Table 3.2 Activities of GAD and GDH enzymes.

Strain	GAD activity (U)	GDH activity (U)
P. putida KT2440	328± 0.048	ND
E. asburiae PSI3 (pTTQGm)	UD	991±0.13
E. asburiae PSI3 (pCNK14)	438.3±0.032	884±0.052

E. asburiae PSI3 was grown in 75mM glucose and 100mM Tris bufferd medium. *P. putida* KT2440 was grown in 75mM glucose and 50mM Tris buffered condition. UD- Undetected, ND- Not Done

Table 3.3 Amount of Organic acid secretion and P release by *E. asburiae* PSI3 (pTTQGm) and *E. asburiae* PSI3 (pCNK14) in TRP medium containing 45 mM glucose.

Strain	Initial O.D.	Final O.D	Gluconic acid	2-ketogluconic acid	Pi (mM)
E. asburiae PSI3 (pTTQGm)	0.02±0.002	0.21±0.01	33.74 ±1.78	UD	UD
E. asburiae PSI3 (pCNK14)	0.03±0.01	0.31±0.03	21.65±0.94	11.63±0.93	0.84±0.04

UD- Undetected

3.3.3 Effect of *gad* overexpression *E. asburiae* PSI3 transformant on *Vigna radiata.*

Mung bean (*Vigna radiata*) growth was monitored in pot experiments in sterile alkaline vertisol soil. Dry shoot weight, dry shoot/root weight and shoot P content were significantly improved in *E. asburiae* PSI3 pCNK14 as compared to vector control as as well as uninoculated control while dry shoot weight and dry shoot/root weight were improved in vector control compared to uninoculated control but no significant P content was observed (**Table 3.4**).

Table 3.4 Effect of *E. asburiae* PSI3 (pCNK14) inoculation on the mung bean (*Vigna radiata)* dry shoot weight, dry shoot /root weight ratio and shoot P content after 15 days.

Growth Parameters						
Bacterial strain	Dry shoot weight (mg)		Dry shoot/root weight ratio of plant		Shoot P concentration of plants (mg/g dry weight)	
Uninoculated control	22.65		3.654		5.98	
E. asburiae PSI3	pTTQGm	pCNK14	pTTQGm	pCNK14	pTTQGm	pCNK14
	25.49*	29.39**†	5.02*	8.67**††	6.69ns	7.75**††

Plants were grown (12 plants, 5 replicates) for 15 days and data are means of individual experiments (for dry weight and shoot/root ratio of plants, n=12, and for shoot P content, n=6). n**s**: comparison with uninoculated control (Fisher LSD test, $p<0.05$); *****: significance in comparison with uninoculated control; **†**: significance in comparison with the corresponding vector control (single sign: $p<0.05$; double sign: $p<0.001$).

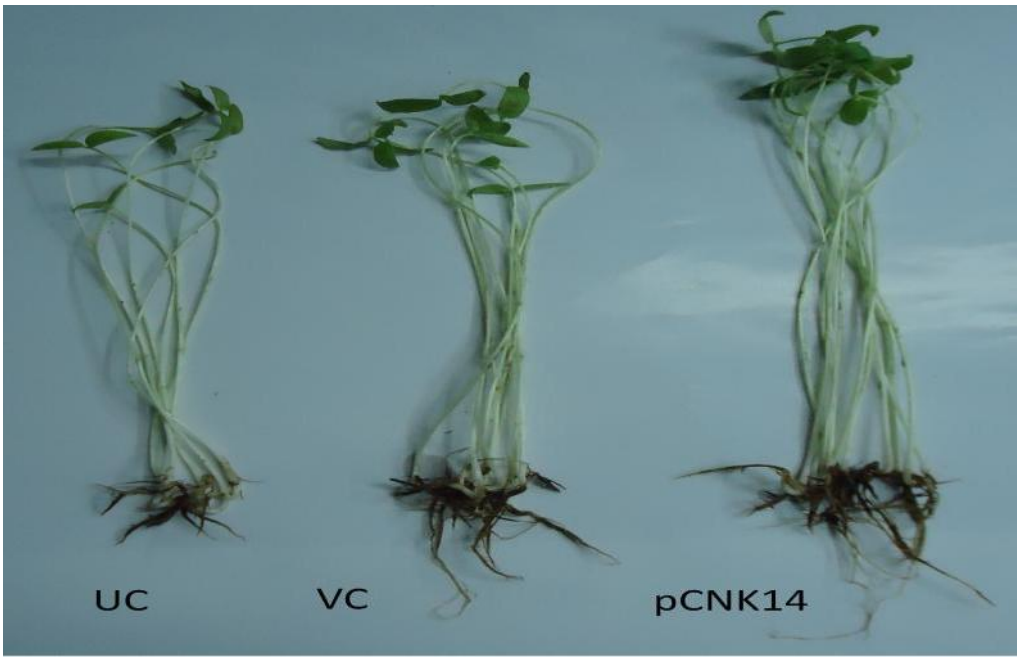

Fig. 3.5: Effect of *E. asburiae* PSI3 (pCNK14) on the growth of mung bean (*Vigna radiata*) after 15 days in alkaline vertisol soil. UC- Uninnoculated control, VC- *E. asburiae* PSI3 (pTTQGm),

3.4 Discussion

Nature and amount of organic acid determine the efficacy of phosphate solubilization by rhizobacteria. Most of the PSMs secrete GA but not 2KGA which is much stronger acid. Hence, incorporation of *gad* in GA producing PSM could significantly improve their plant growth ability. In the present study, incorporation of *P. putida* KT 2440 *gad* operon in *E. asburiae* PSI3 showed ~ 438 U and 985 U of enzyme activity in TRP and LB media respectively. Earlier reports of incorporation of the *gad* operon from *E. cypripedii* ATCC 29267 with ~ 1360 U enzyme activity in *E. coli* resulted in ~410 U GAD activity in LB medium which was increased to 2150 U in presence of gluconate (Yum et al., 1997). Similar increase in GAD activity (1738 U) was found in *E. asburiae* PSI3 *gad* transformant in presence of gluconate. *E. asburiae* PSI3 requires 75mM glucose to solubilize rock phosphate in TRP medium. However, *gad* transformant could solubilize rock phosphate in presence of 45 mM glucose due to secretion of ~21 mM GA and ~11mM 2KGA. In contrast, *E. coli* containing *E. cypripedii* ATCC 29267 *gad* operon could secrete ~ 13mM 2KGA in LB containing 100mM glucose (Yum et al., 1997).

Recent plant growth studies with PSMs have demonstrated improvement in P status upon supplementation with tricalcium phosphate (Vyas and Gulati, 2009; Gulati et al., 2010). *Acinetobacter rhizosphaerae* strain, which produced very high (upto ~ 87mM) GA, enhanced maize plant growth upon supplementation with tricalcium phosphate as compared to the plants supplemented with super phosphate (Gulati et al., 2010). On the other hand, phosphate solubilizing fluorescent pseudomonads strains improved maize plant growth as well as P content on

CHAPTER 4

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

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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,

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 and Russel, 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 and Russel, 2001
E. asburiae PSI3	*Cajanus cajan* rhizosphere isolate	Gyaneshwar et al., 1998b
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	Stratagene
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 1/4th 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 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. 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*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.

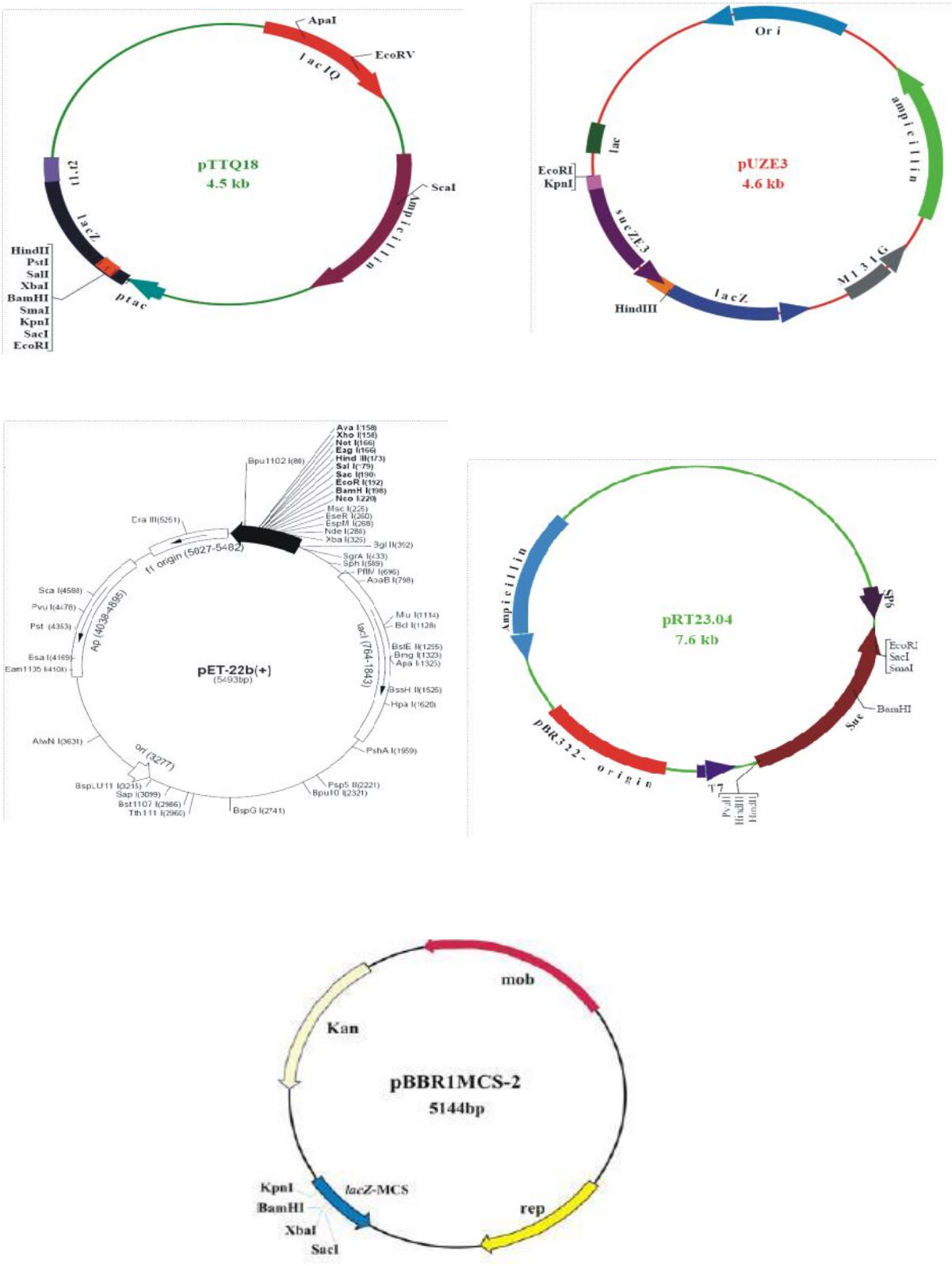


Fig. 4.1 Map of plasmids used in study

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'*GCGGAATCGATTTTCACACAGGAAACAGCG*ATG 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 94^0C for 15 s, 52^0C for 15 s, 72^0C for 30 s and followed by final extention at 72^0C 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 94^0C for 1 min followed 30 cycles each consisting 94^0C for 15 s, 55^0C for 15 s, 72^0C for 30 s and followed by final extention at 72^0C 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 94^0C for 1 min followed 30 cycles each consisting 94^0C for 15 s, 55^0C for 30 s, 72^0C for 90 s and followed by final extention at 72^0C 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 94^0C for 1 min followed 30 cycles each consisting 94^0C for 15 s, 55^0C for 30 s, 72^0C for 120 s and followed by final extention at 72^0C 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 (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 37^0C. 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., 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, 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 $FeSO_4 \cdot H_2O$ 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, 1964).

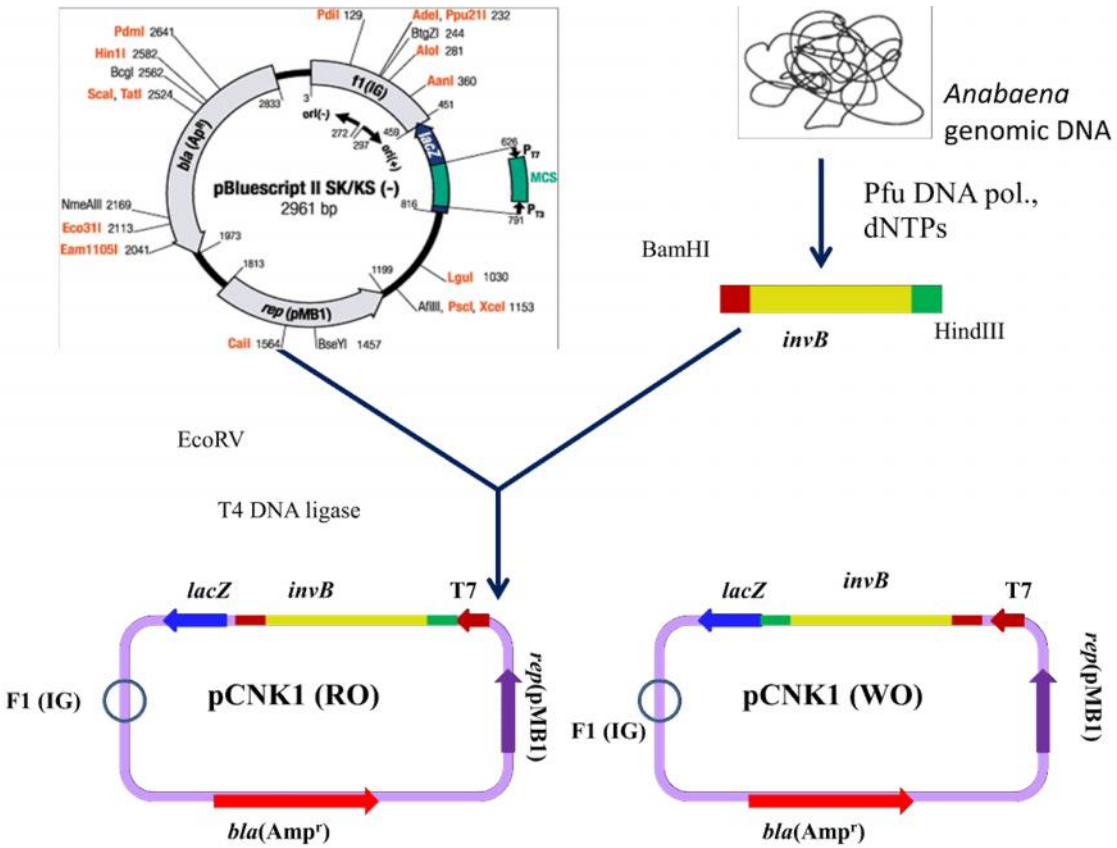
4.2.13 Analytical method

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.

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**).



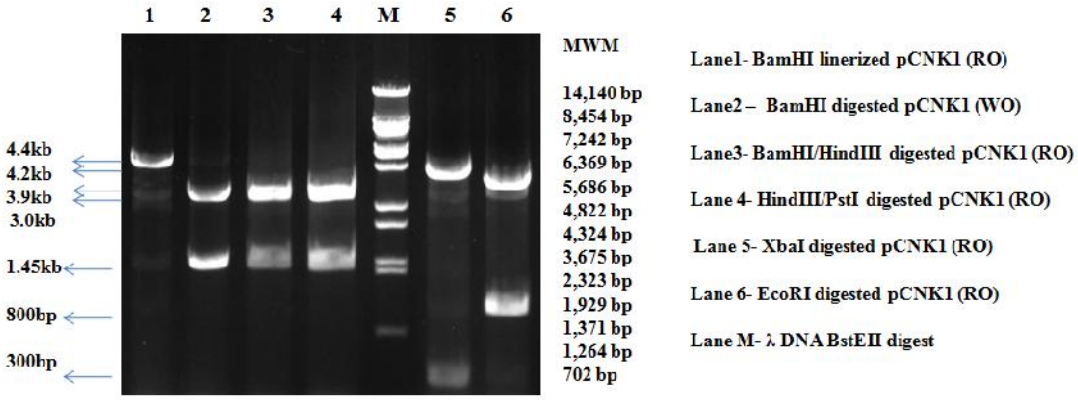


Fig. 4.2 Restriction digestion pattern of pCNK1(RO) and pCNK1 (WO)

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.

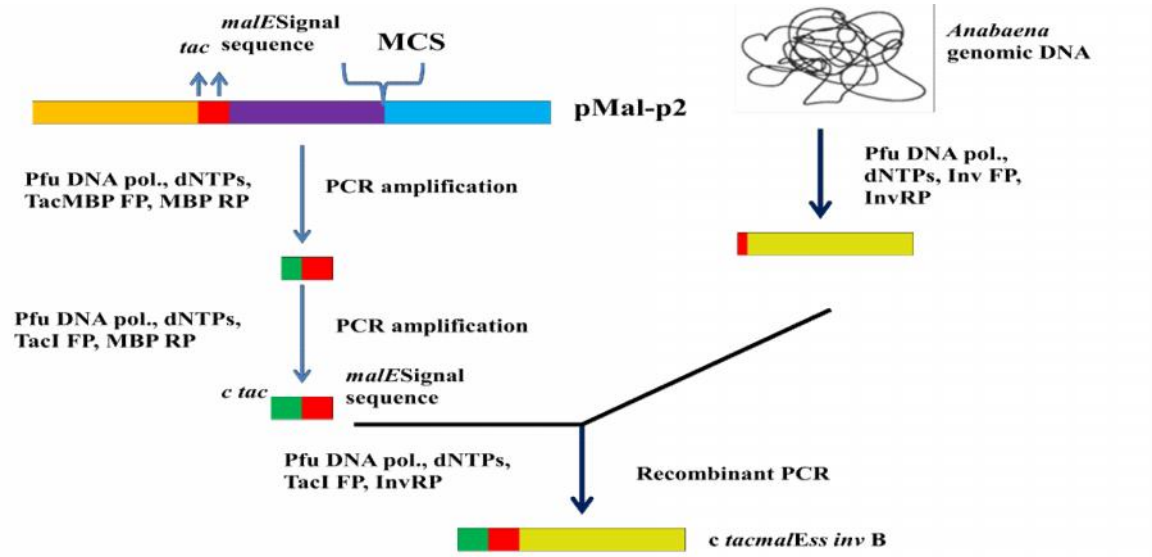


Fig. 4.4 Schematic representation of recombinant PCR strategy of *tac*SS- *invB.*

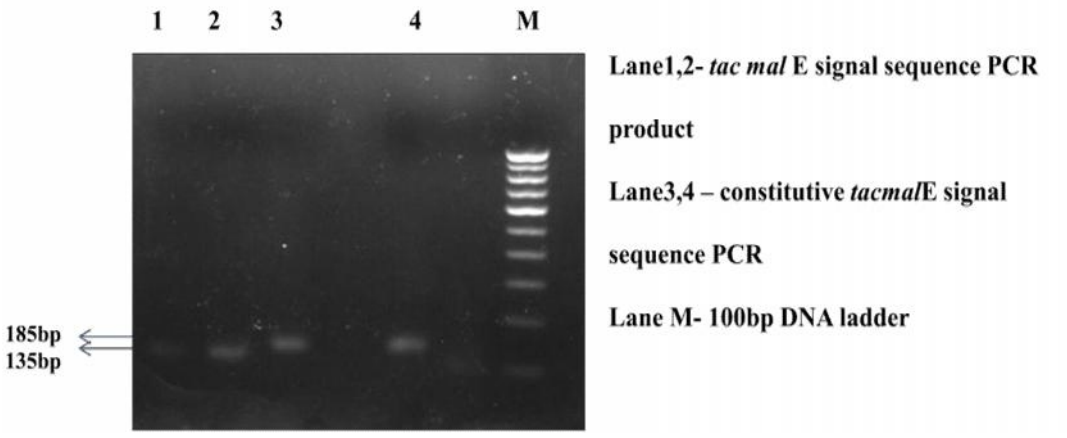


Fig. 4.5 Recombinant PCR of constitutive *tac* promoter with *malE* signal equence.

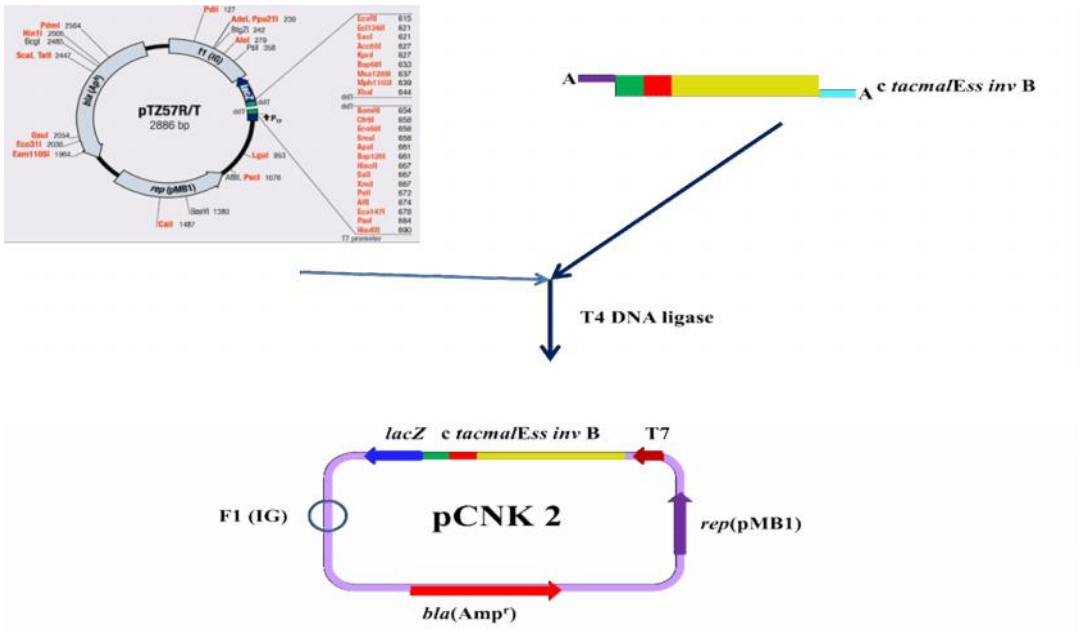


Fig. 4.6 Schematic representation of cloning strategy of pCNK2

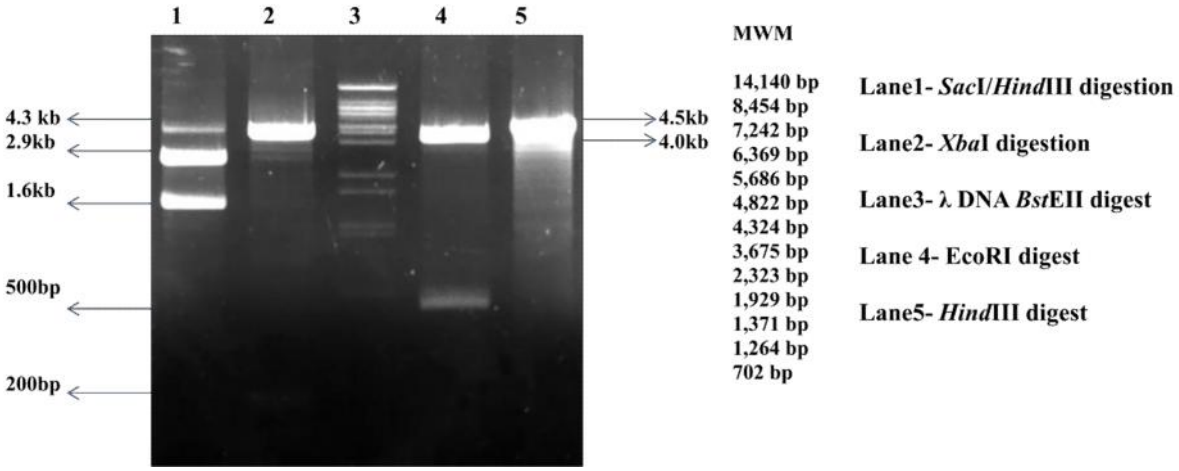


Fig. 4.7 Restriction digestion pattern of pCNK2.

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.

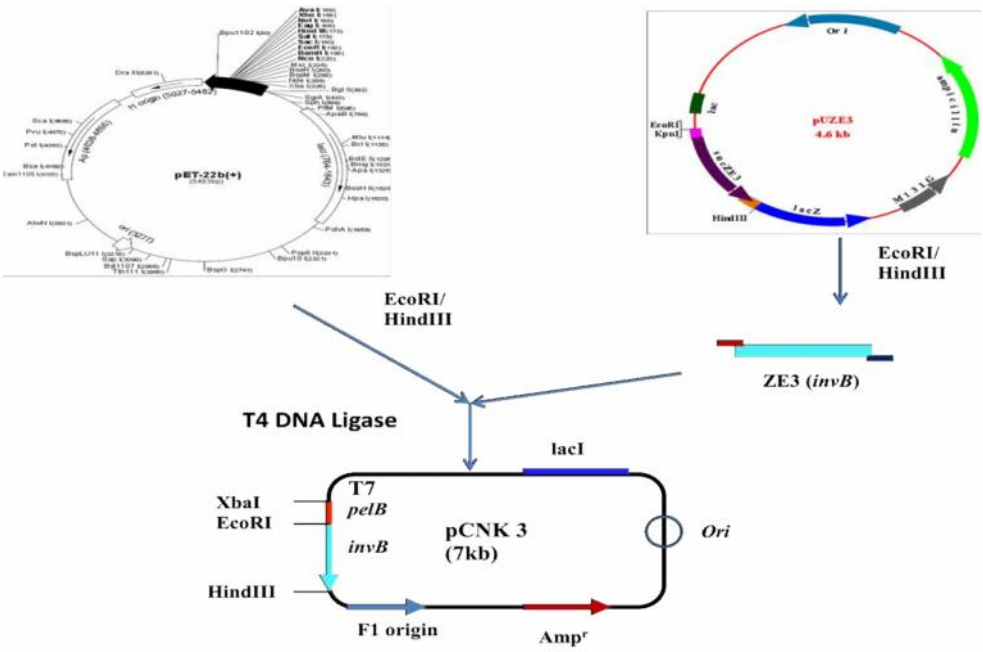


Fig. 4.8 Schematic representation of cloning strategy of pCNK3

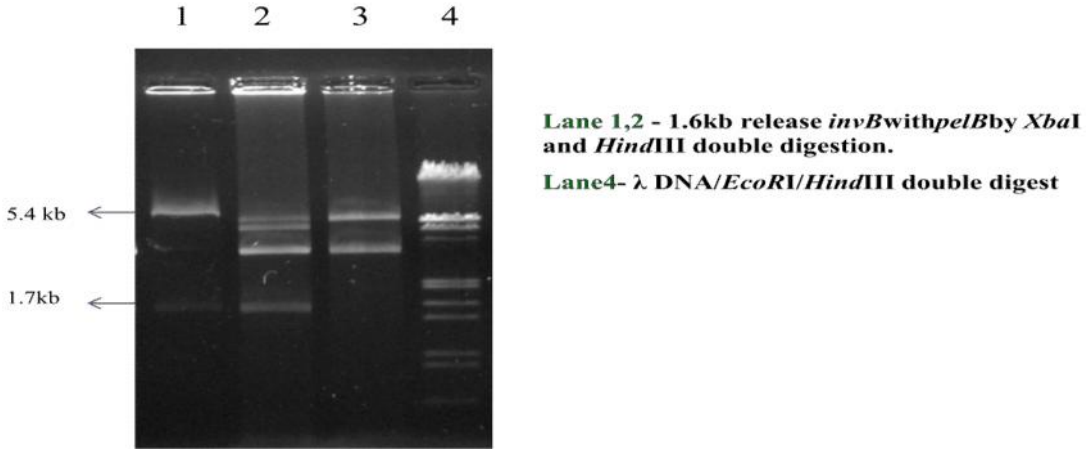


Fig. 4.9 Restriction digestion pattern of pCNK3.

*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 fra gment was blunt ended by Klenow fragment. Plasmid 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**).

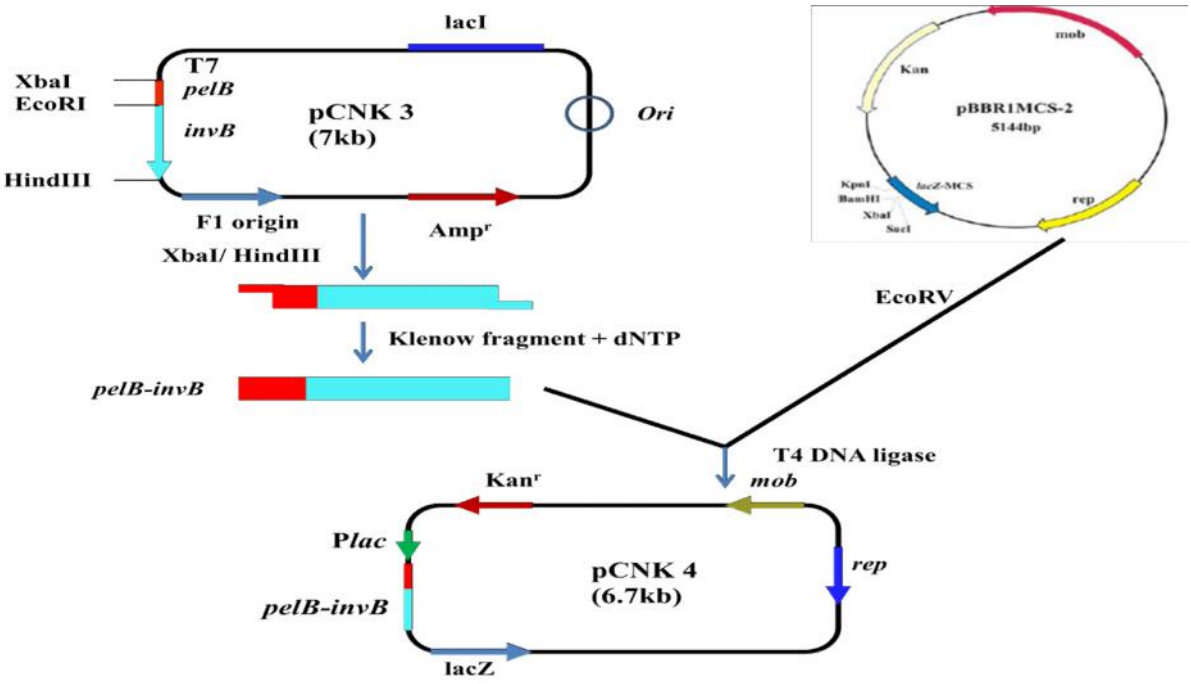


Fig.4.10 Schematic representation of cloning strategy of pCNK4.

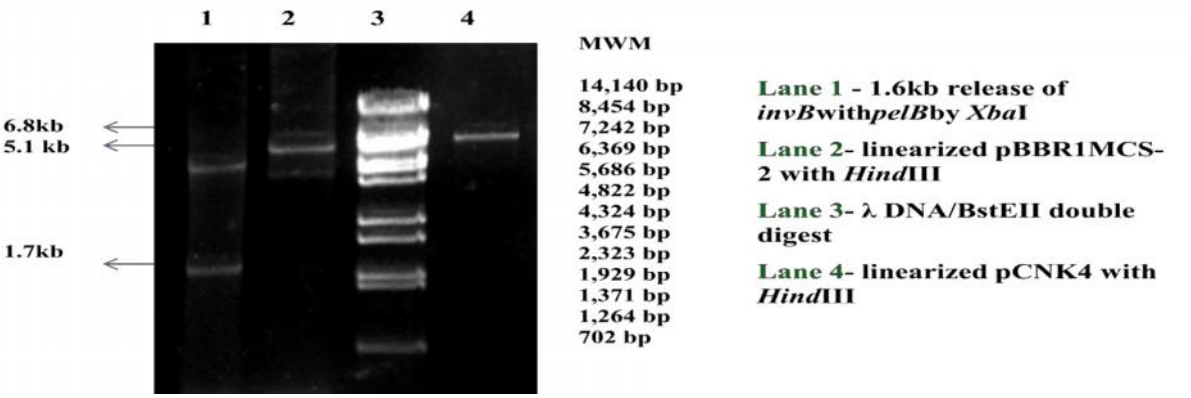


Fig. 4.11: Restriction digestion pattern of pCNK4.

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**).

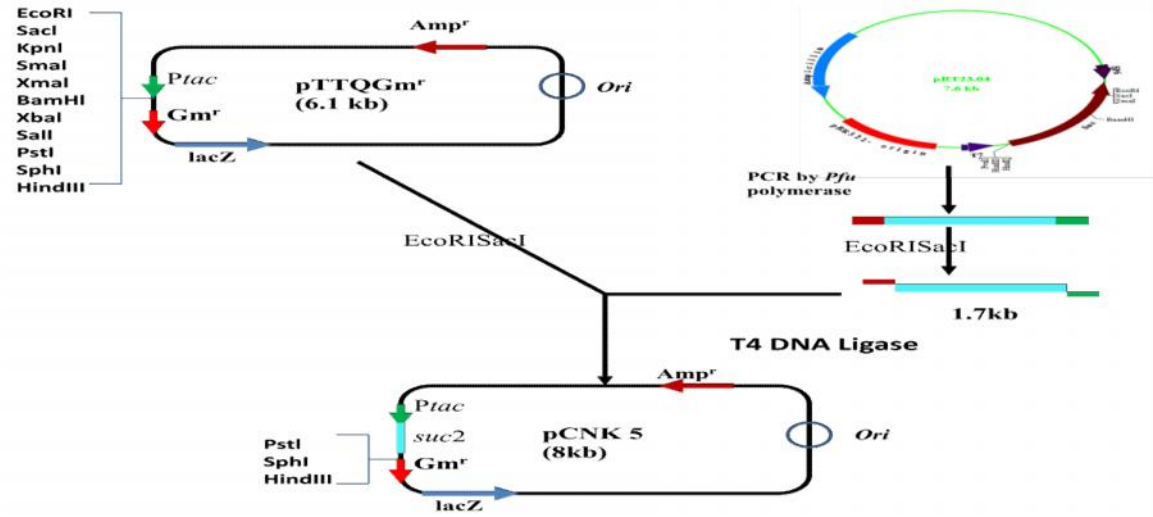


Fig.4.12 Schematic representation of cloning of *suc*2 gene in pTTQGm^r.

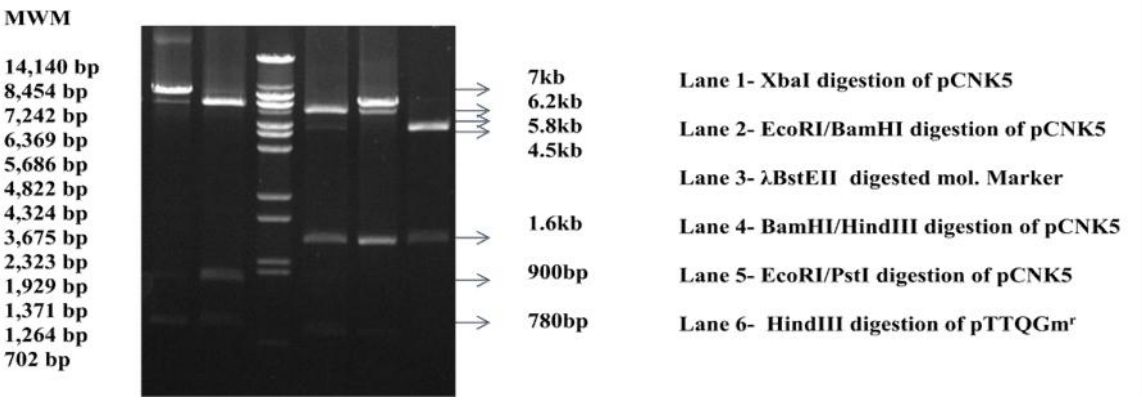


Fig. 4.13 Restriction digestion pattern of pCNK5.

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**).

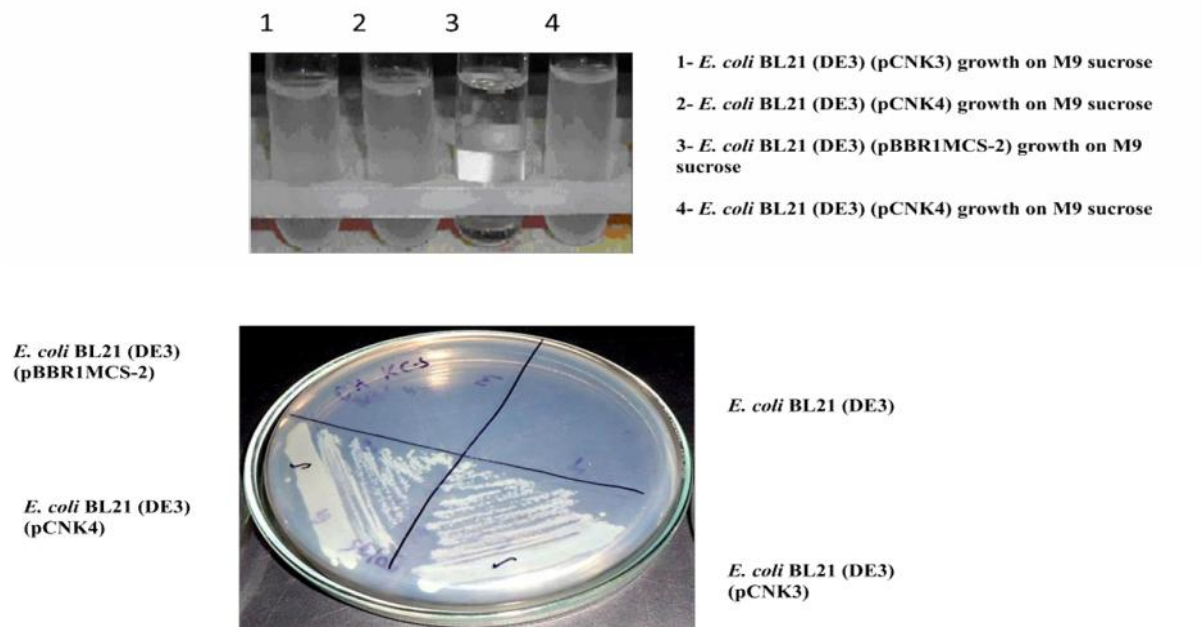


Fig. 4.14 Growth of *E. coli* transformant pCNK3 and pCNK4 on M9 minimal medium containing sucrose as carbon source.

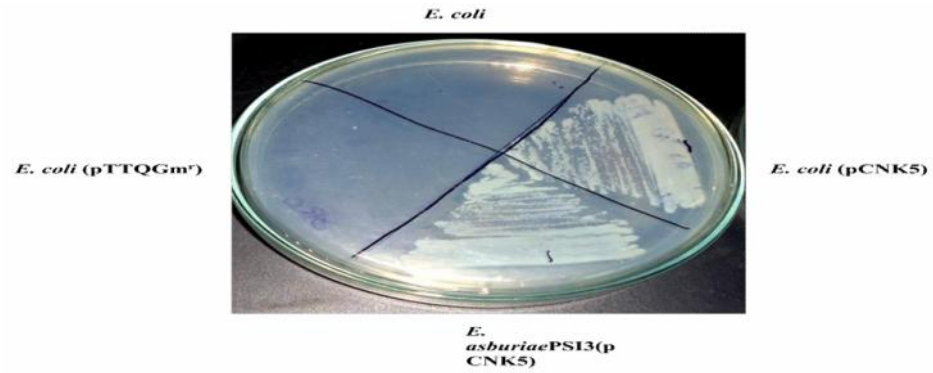
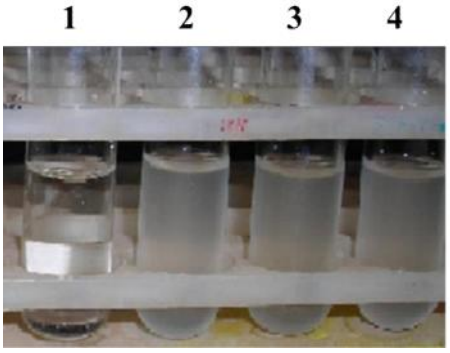


Fig. 4.15 Growth of *E. coli* (pCNK5) on M9 minimal medium agar plate with sucrose as c source.



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

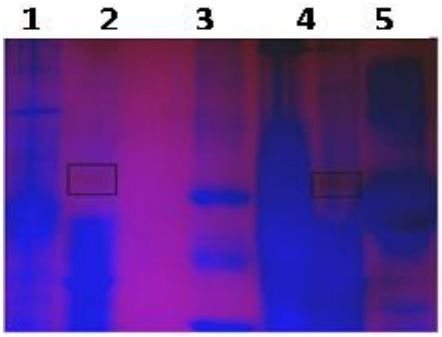
2- *E. coli* (pCNK5) growth on M9 sucrose

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.

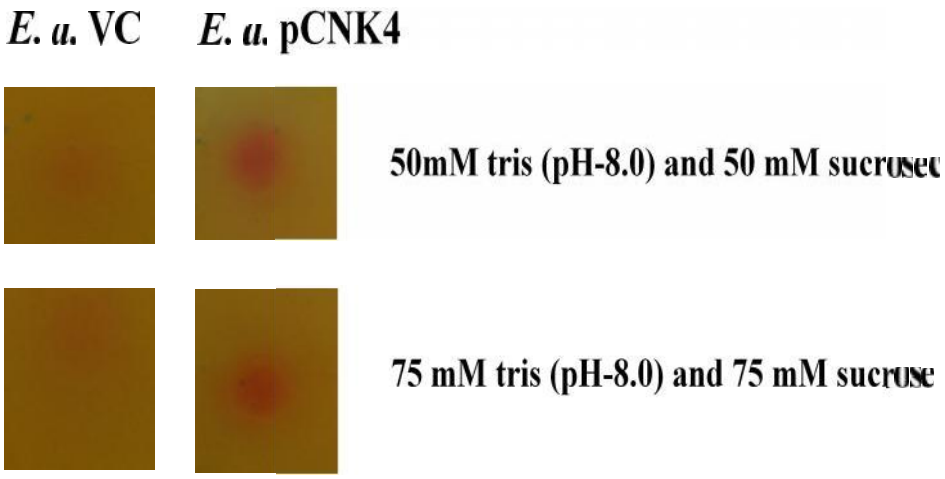


Fig.4.18: MPS ability of *E. asburiae* PSI3 VC (pTTQGm) and *E. asburiae* PSI3 (pCNK4) in TRP agar medium with sucrose as carbon source.

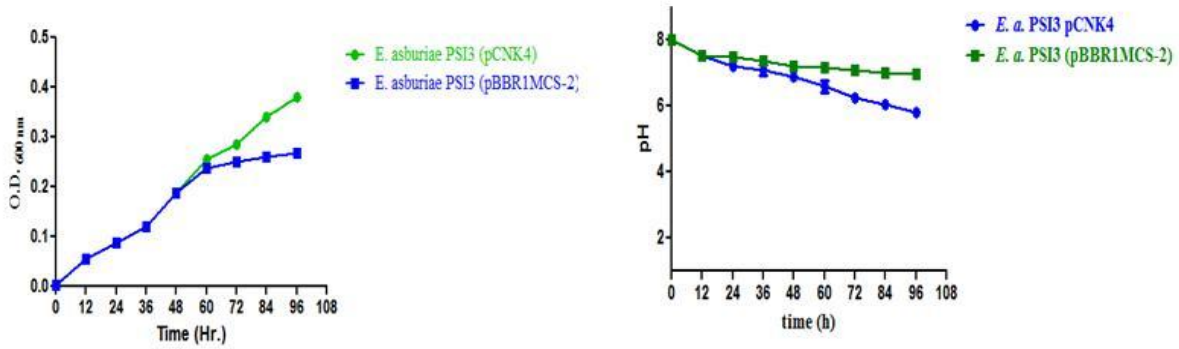


Fig.4.19: Growth and pH profile of *E. a.* PSI3 (pBBR1MCS-2) and *E. a.* PSI3 (pCNK4) in TRP containing 75mM Tris and 75mM sucrose.

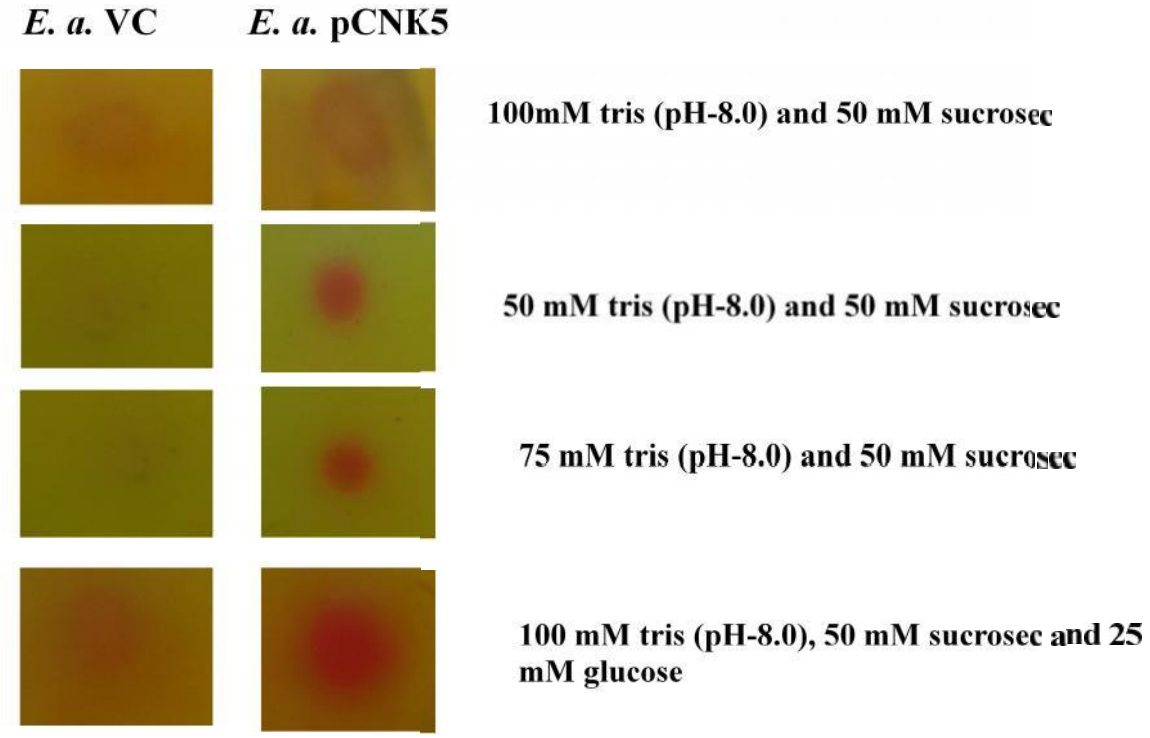


Fig. 4.20: MPS ability of *E. a.* PSI3 (pTTQGm^r) and *E. a.* PSI3 (pCNK5) in TRP containing varied Tris and carbon source.

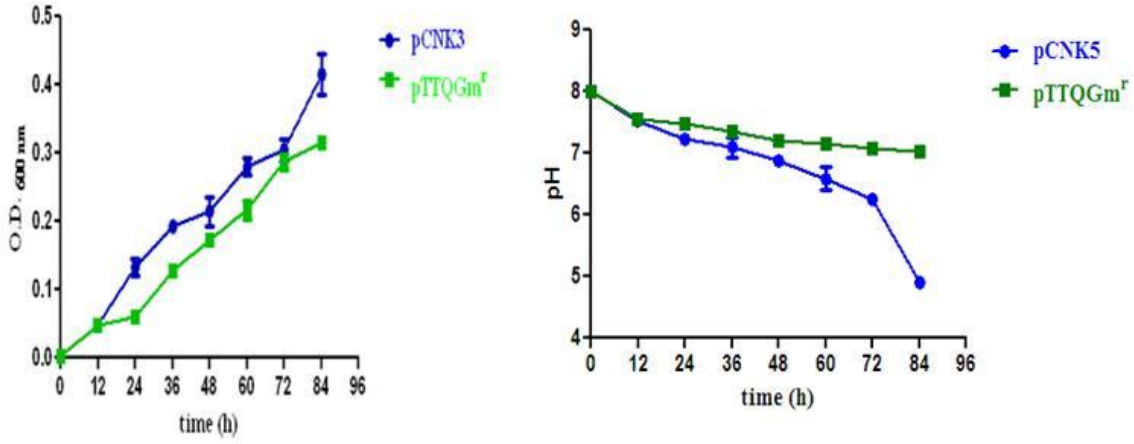


Fig. 4.21: Growth and pH profile of *E. a.* PSI3 (pTTQGm^r) and *E. a.* PSI3 (pCNK5) in TRP containing 75mM Tris and 50mM sucrose.

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	0.24±0.02	100 + 50 + 0	ND	UD
pCNK5	0.415±0.03	100 + 50 + 0	8.3	UD
pTTQGm^r	0.316±0.007	75 + 50 + 0	ND	UD
pCNK5	0.44±0.003	75 + 50 + 0	22	0.438± 0.073
pTTQGm^r	0.34±0.008	100 + 50 +25	9.4	UD
pCNK5	0.52±0.02	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

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).

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., 2011). 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., 2011). 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., 1999; 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., 1998
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 pCNK13. 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 º 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 37º 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.

5.2.7 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.

5.2.8 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.

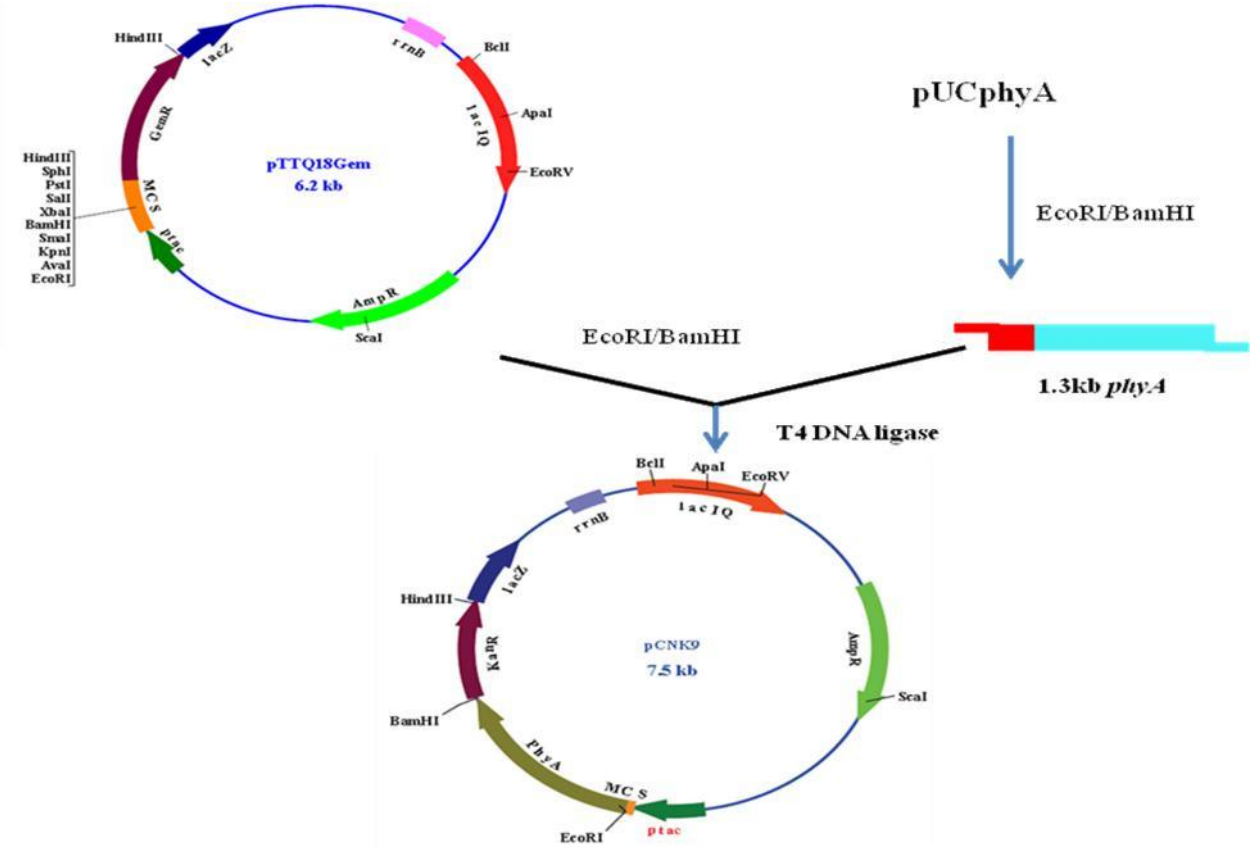


Fig. 5.1 Schematic representation of pCNK9 cloning strategy

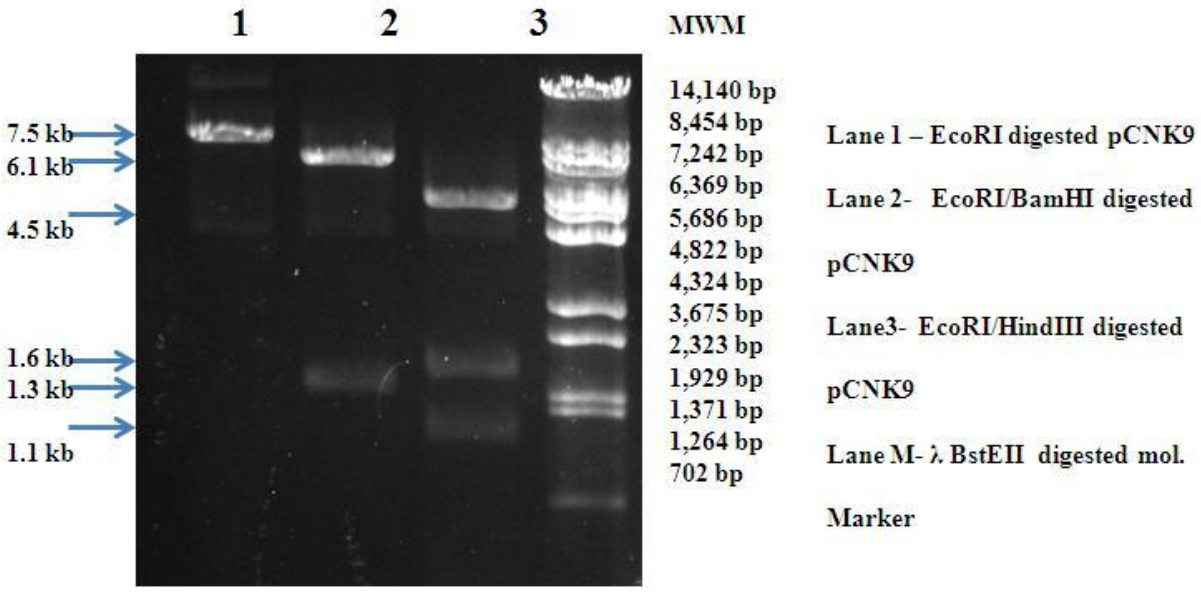


Fig. 5.2 Restriction digestion pattern of pCNK9

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.

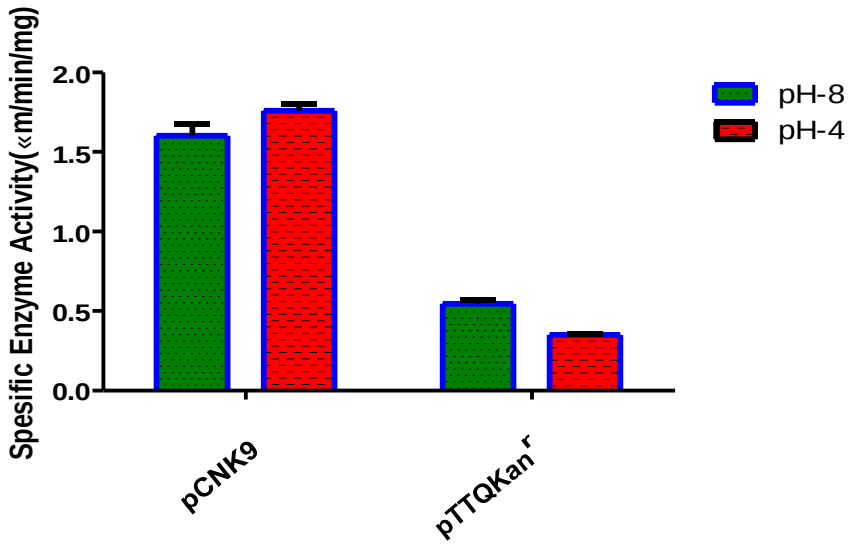


Fig.5.3 Enzyme activity of pCNK9 at pH-8.0 and 4.0

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**).

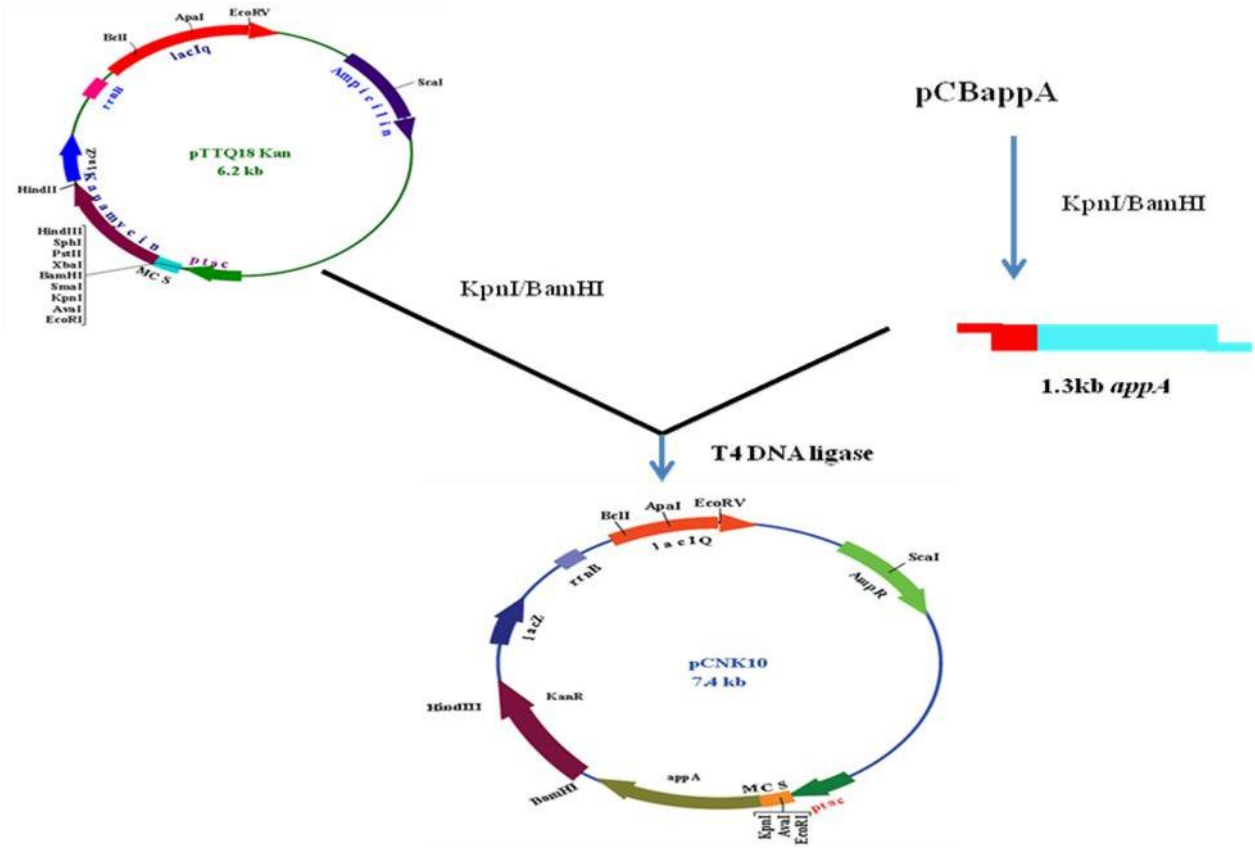


Fig. 5.4 Schematic representation of pCNK10 cloning strategy

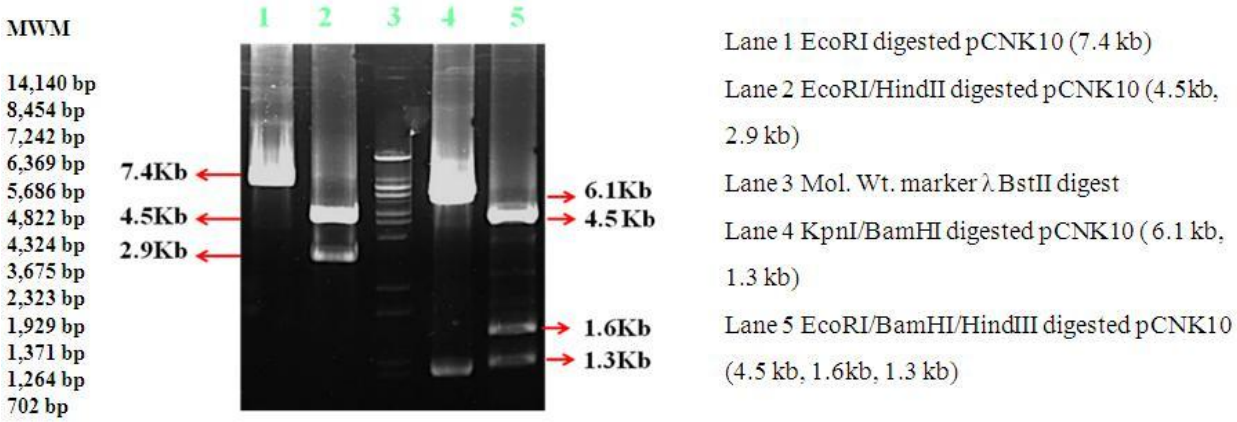
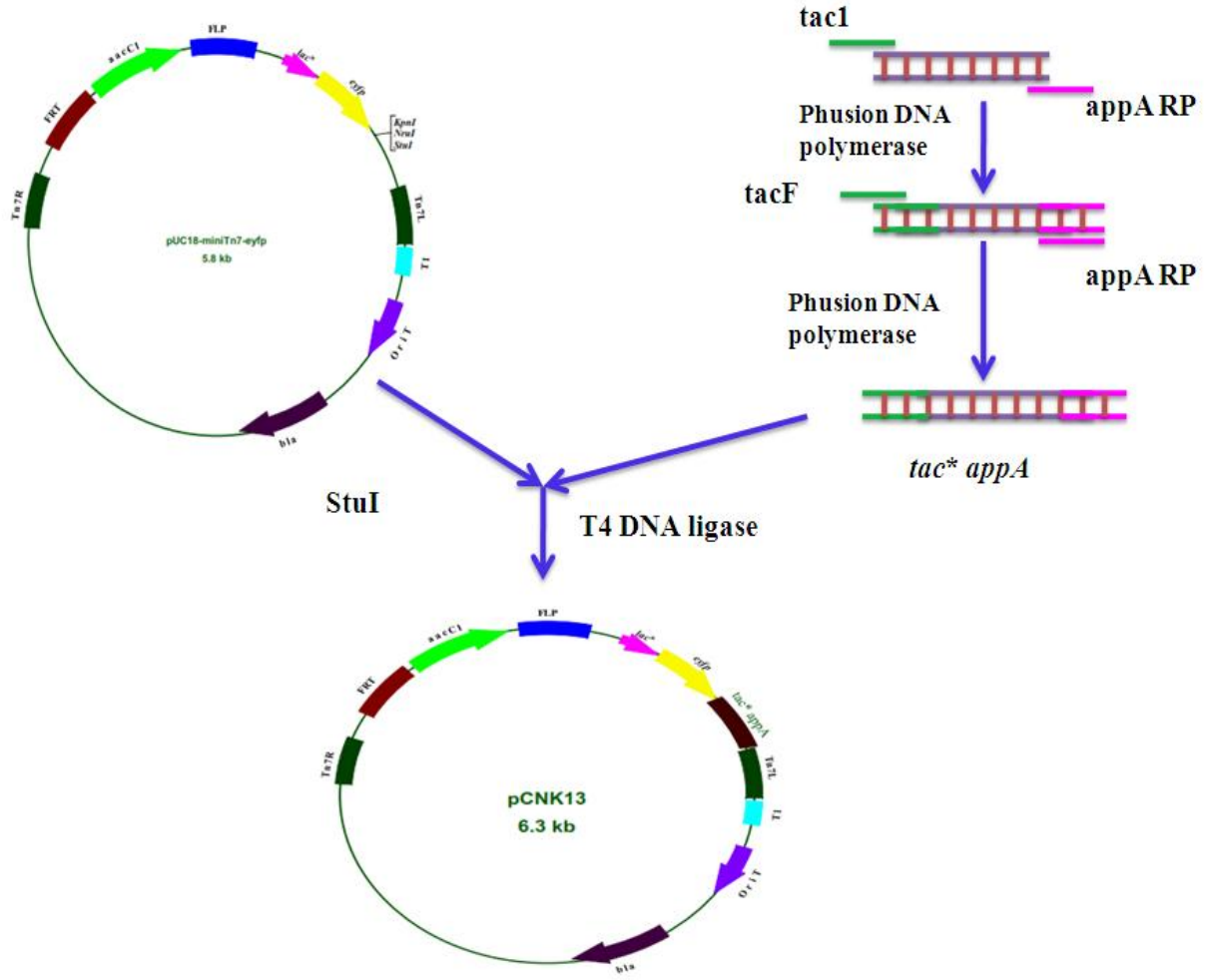


Fig. 5.5 Restriction digestion pattern of pCNK10.



Schematic representation of pCNK13 cloning strategy

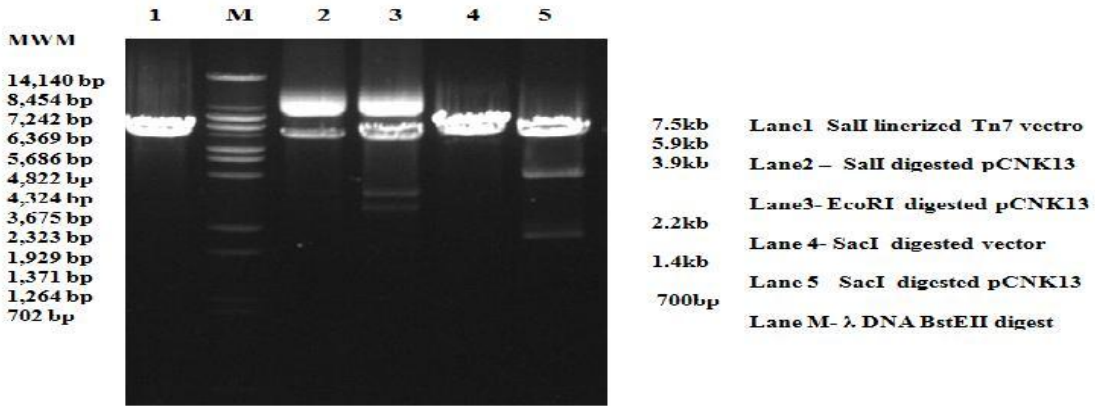
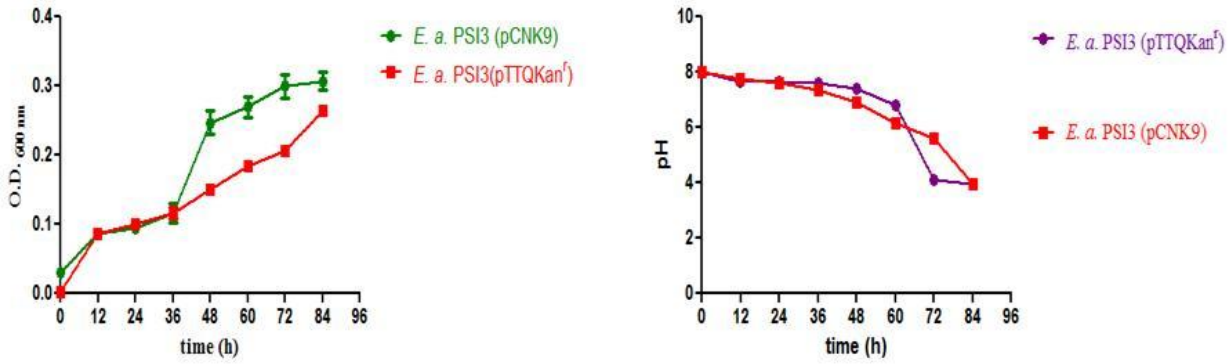


Fig. 5.6 Restriction digestion pattern of pCNK13.

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

E. asburiae PSI3 (pCNK9,10 and 13) 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. :**).



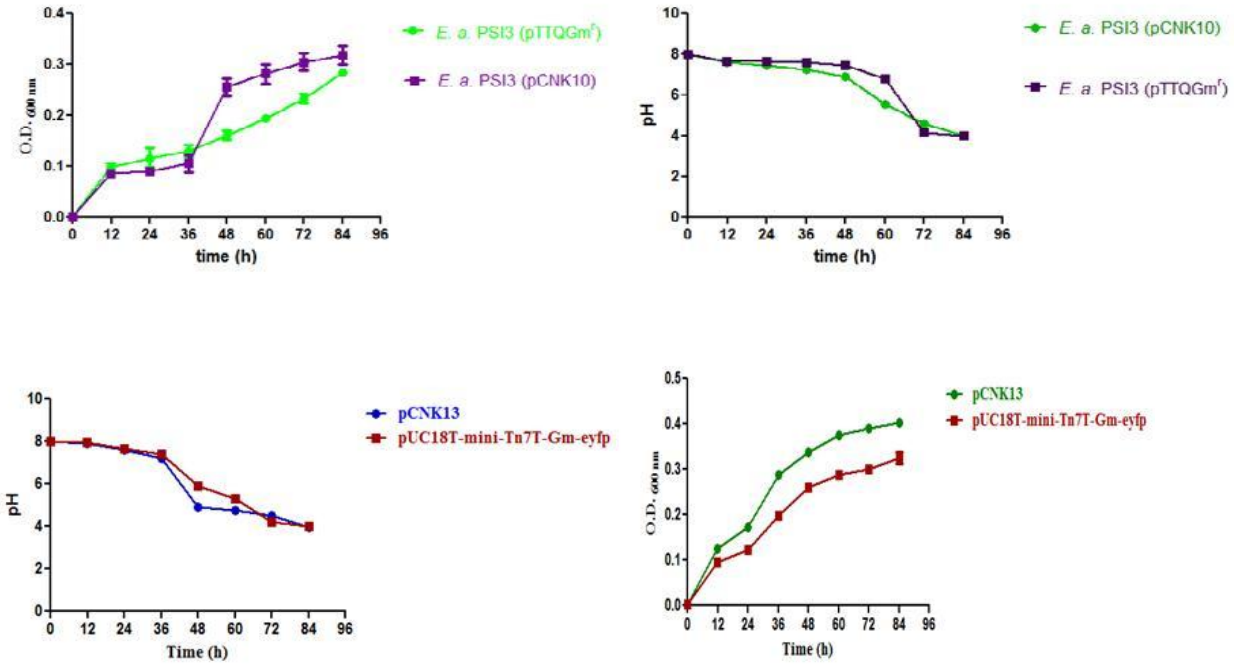


Fig.5.7: Growth and pH profile of *E. a.* PSI3 (pCNK9, pCNK10 and pCNK13) in 100 mM Tris buffered pH-8.0 phytate minimal medium containing 75 mM glucose.

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**).

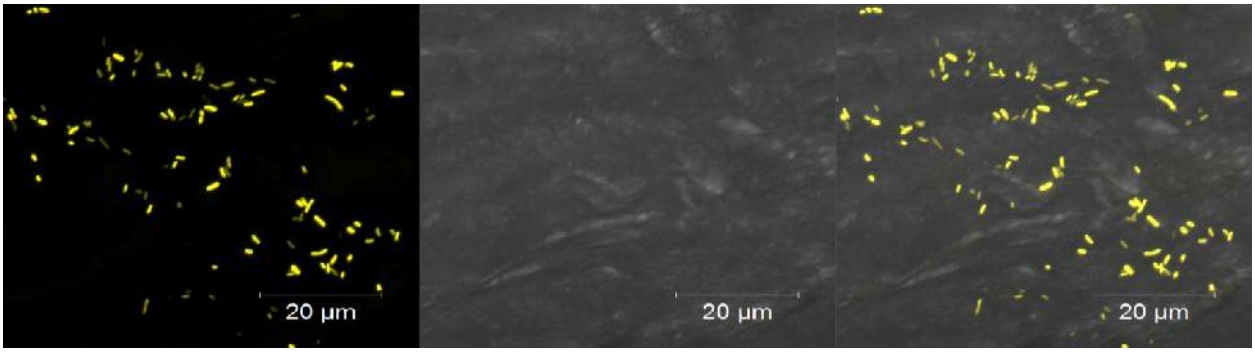


Fig. 5.8: Colonization of *E. asburiae* PSI3 (pCNK13) in non sterile soil after 5 days of Inoculation.

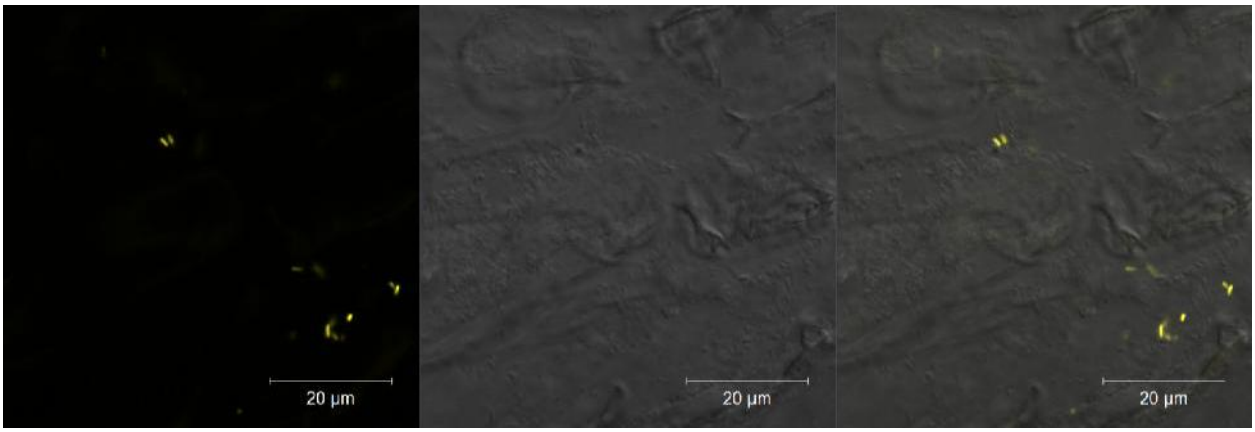


Fig. 5.9: Colonization of *E. asburiae* PSI3 (pCNK13) in non sterile soil after 10 days of inoculation.

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., 2006). 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., 2007). 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.

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, 2011). 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., 1998). 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 glucose/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.

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		
pUC8-16	Amp^r, pUC8 backbone	Liu et al 1994
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

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

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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.

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 pUC8-16 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 to obtained pCNK15 for selection in *E. asburiae* PSI3 (**Fig. 6.1, 6.2, 6.3**).

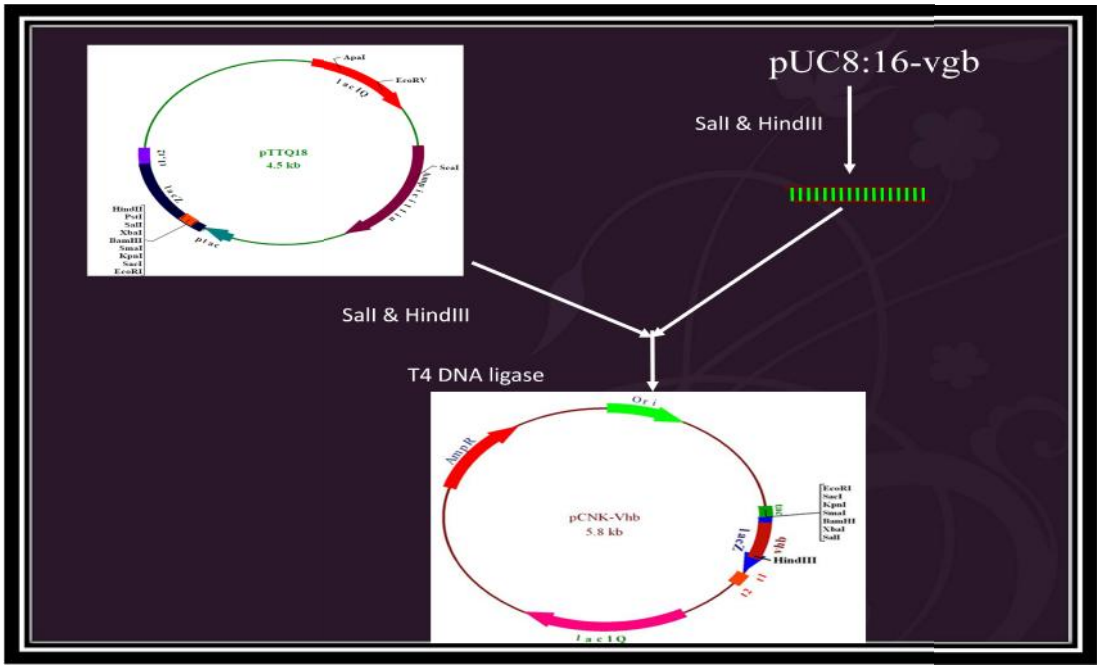

Fig.6.1: Schematic representation of cloning of *vgb* gene in pTTQ18 (pCNK11)

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. 6.3**).

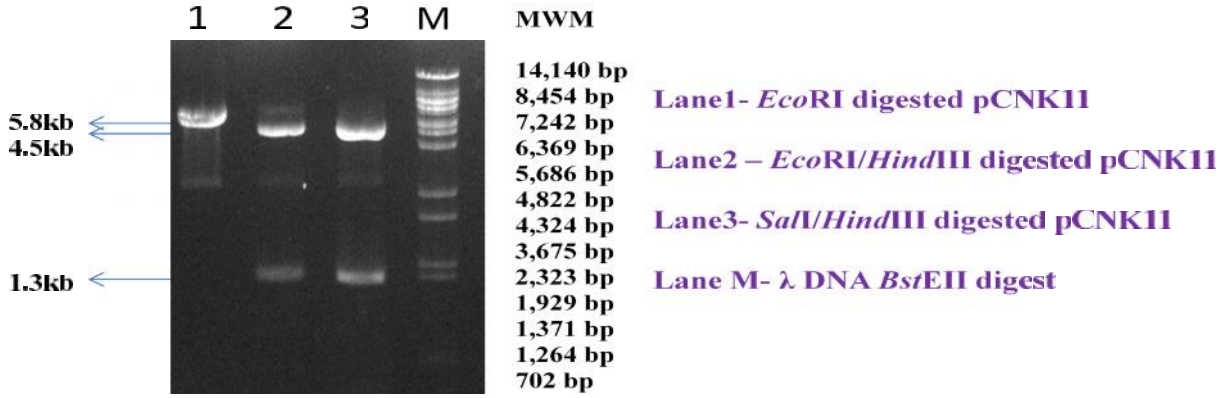

Fig.6.2 Restriction digestion pattern of pCNK11.

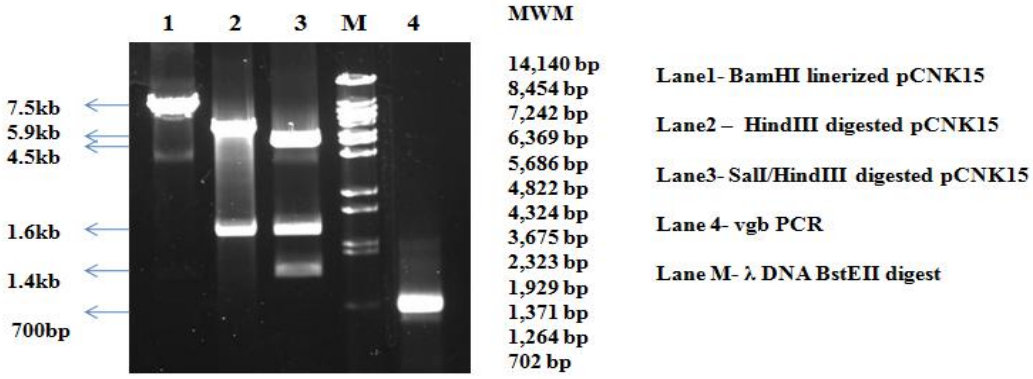

Fig.6.3 Restriction digestion pattern of pCNK15.

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 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**).

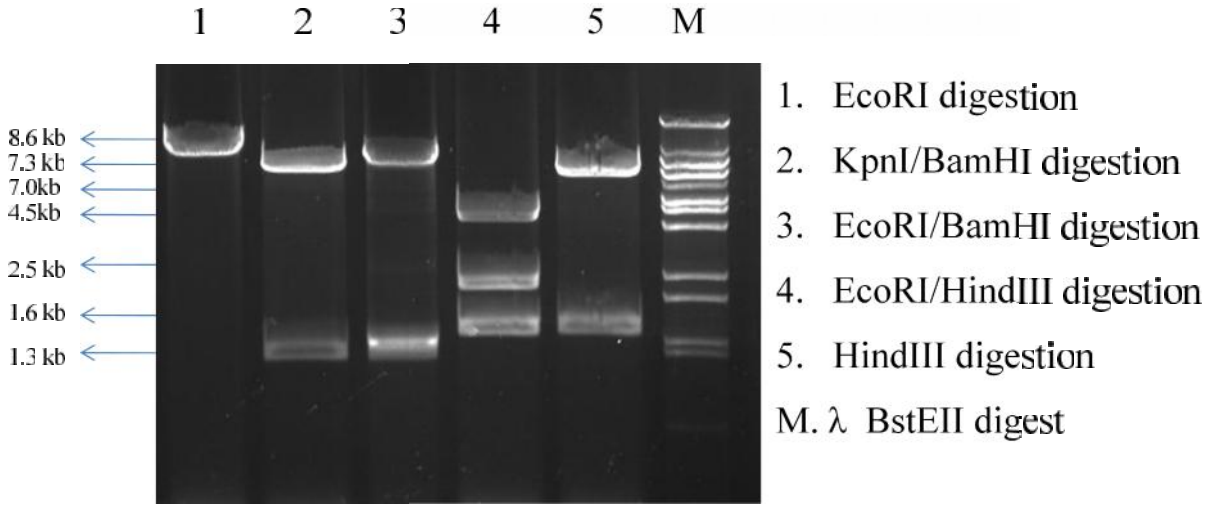

Fig.6.4 Restriction digestion pattern of pCNK21.

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 to obtained pCNK22. 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 further confirmed PCR amplification. Functionality of the gene was confirmed by *E. coli* transformant grown on sucrose M9 minimal medium and enzyme activity (**Fig. 6.5**). Invertase

enzyme activity was 0.3mU. Phytase and acid phasphatase activity was similar to *E. asburiae* PSI3 (pCNK21).

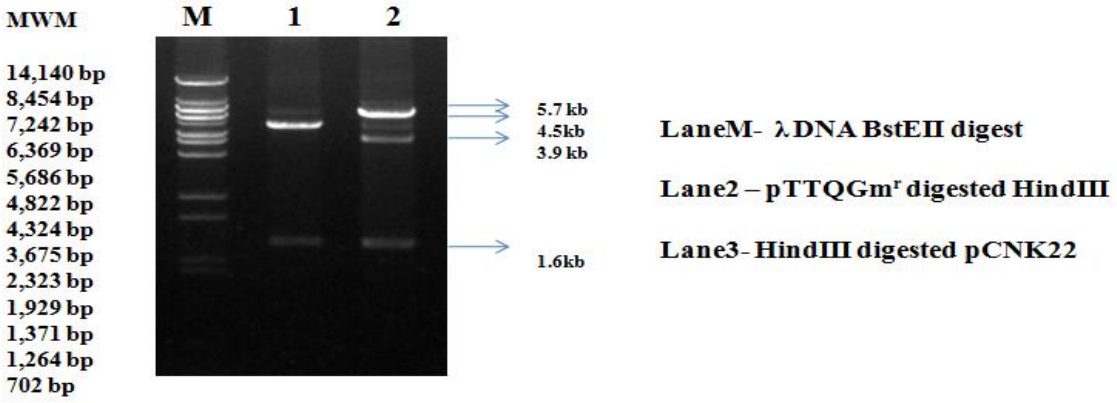

Fig.6.5 Restriction digestion pattern of pCNK22

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, 6.7, 6.8**).

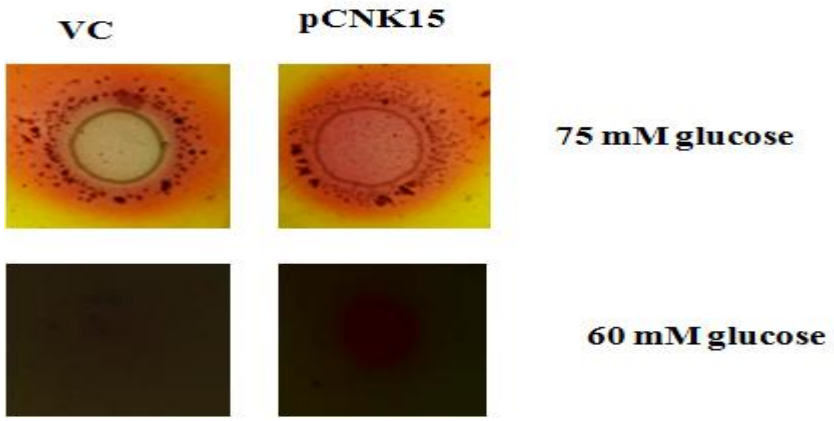

Fig. 6.6 MPS ability of *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK15) in TRP agar medium with glucose as carbon source.

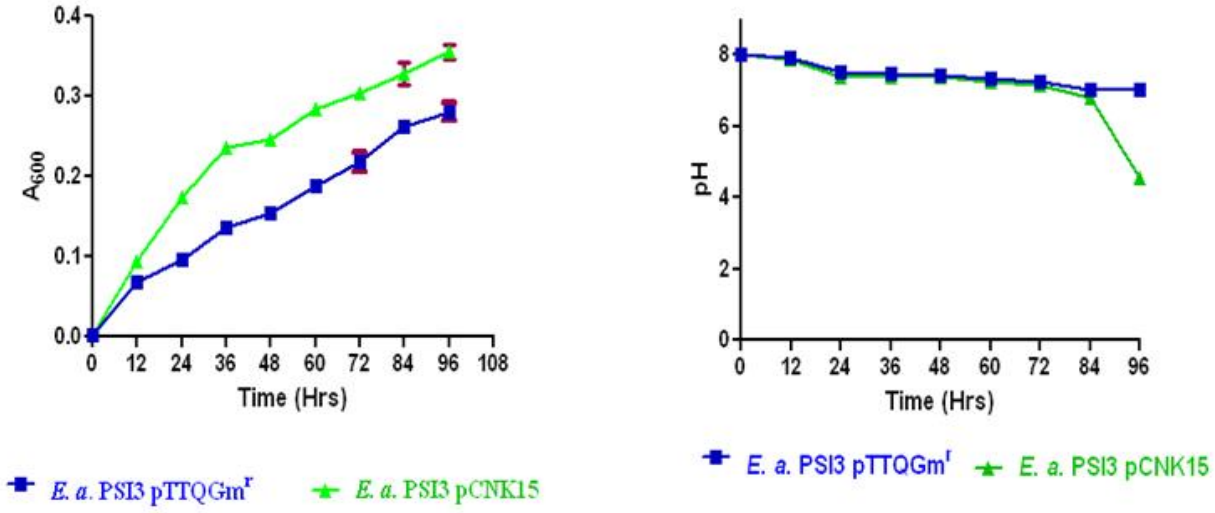

Fig. 6.7 Growth and pH profile of *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK15).

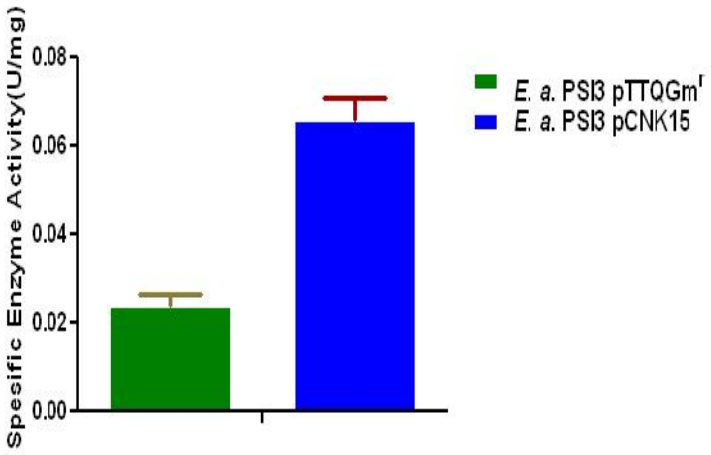

Fig. 6.8 GDH activity of *E. asburiae* PSI3 pTTQGm^r and *E. asburiae* PSI3 pCNK15.

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. 6.10**). 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).

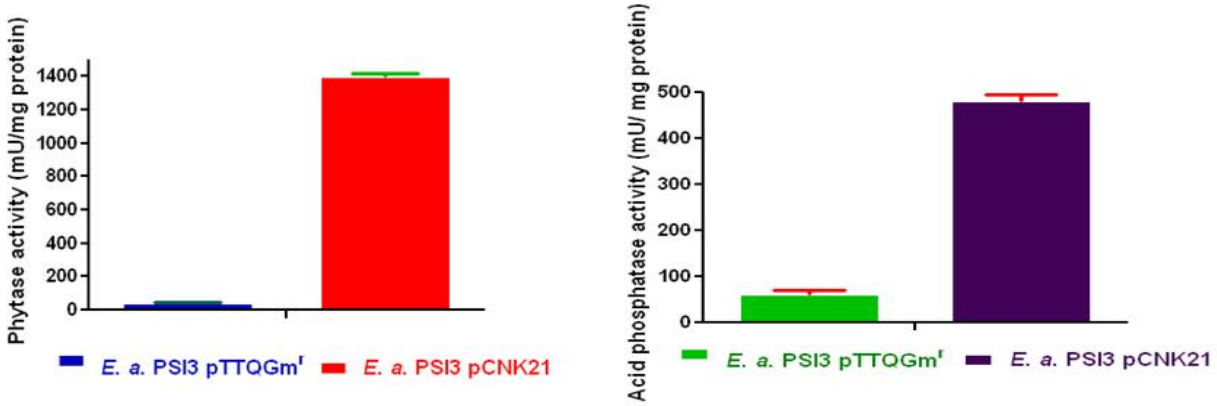

Fig. 6.9 Phytase and acid phosphatase activity of *E. asburiae.* PSI3 (pCNK21) and *E. asburiae* PSI3 (pTTQGm^r).

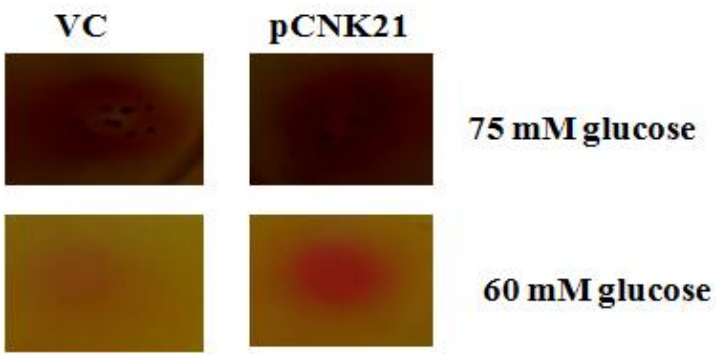

Fig. 6.10 MPS ability of *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK21) in Tris phytate agar medium with glucose as carbon source.

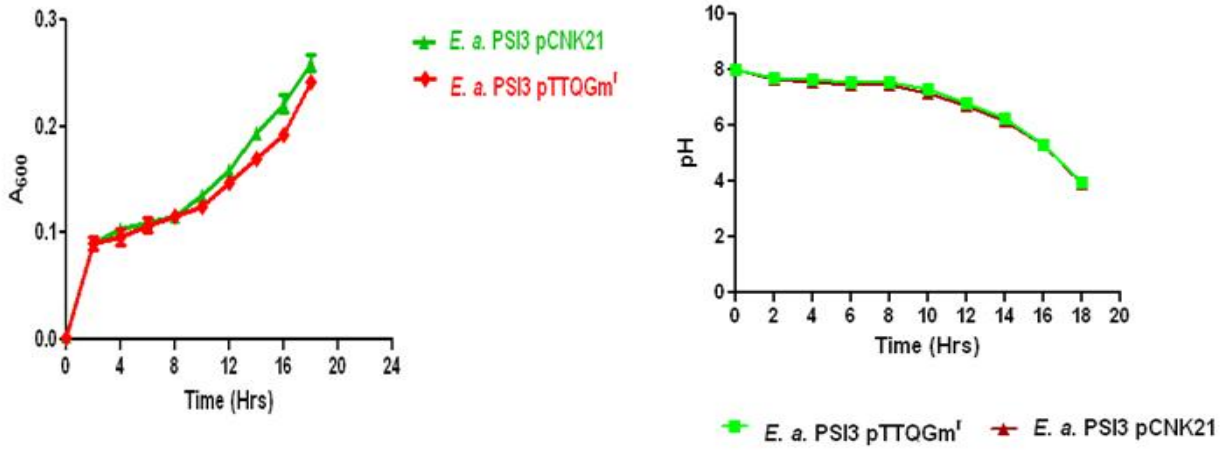

Fig. 6.11 Growth and pH profile of *E. asburiae* PSI3 (pCNK21) and vector control in unbuffered condition.

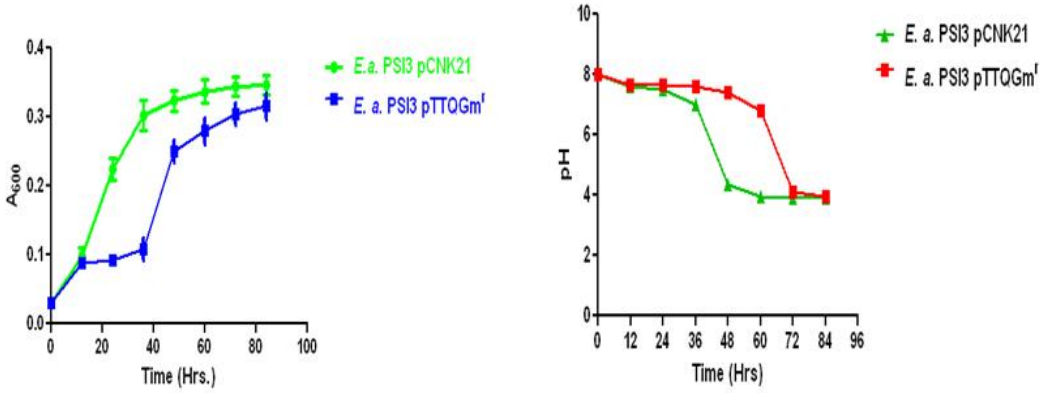

Fig. 6.12 Growth and pH profile of *E. asburiae* PSI3 (pCNK21) and vector control in buffered condition.

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**).

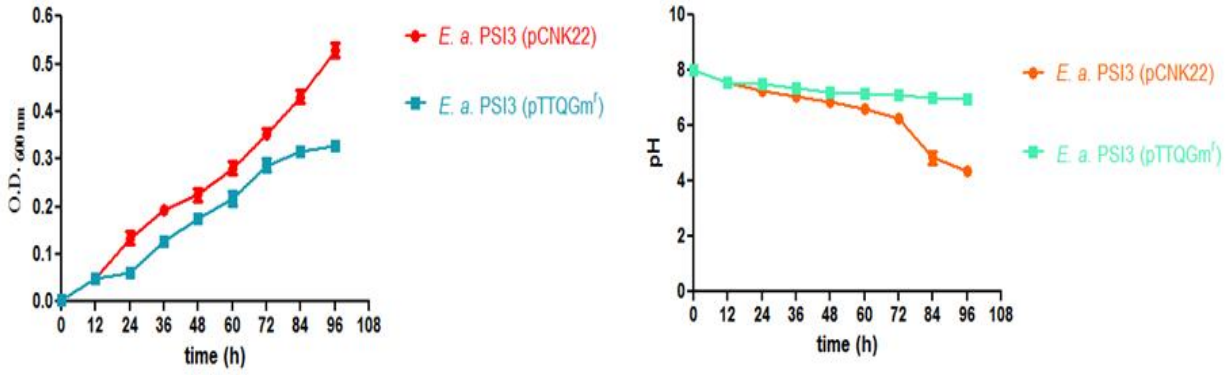

Fig. 6.13: Growth and pH profile of *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK22) in Tris phytate minimal medium containing 75mM Tris and 50mM sucrose.

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, 6.15**).

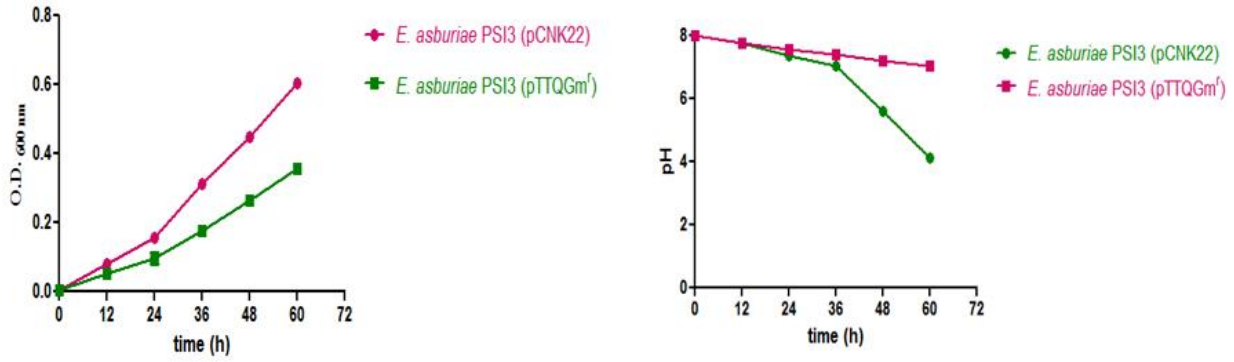

Fig. 6.14 Growth and pH profile of *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK22) in Tris phytate minimal medium containing 100 mM Tris and mixture of sugars (each at 10 mM).

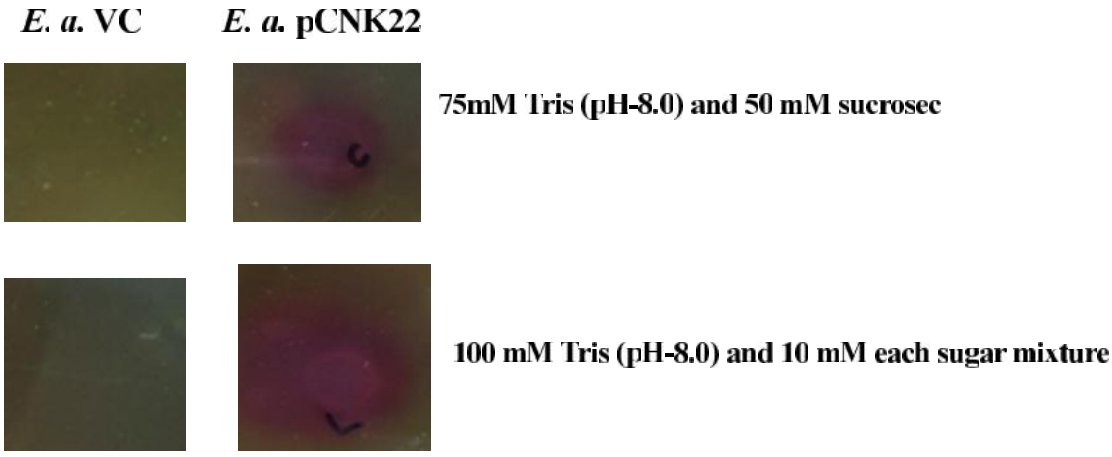

Fig. 6.15 Ca- phytate mineralization by *E. asburiae* PSI3 (pTTQGm^r) and *E. asburiae* PSI3 (pCNK22) in buffered phytate minimal medium containing 50 mM sucrose and mixture of sugars (each at 10 mM).

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.

SUMMARY

Phosphorus deficiency is a major problem for plant growth and grain yield. This problem persists in whole world over the century but very little progress has been done towards meeting the demand of food supply required into next 50 years. Multiple factors are responsible for this problem including depletion of natural rock phosphate (RP) ore reserve, pollution generated during the chemical fertilizers production; expensive chemical fertilizers along with its strong refixation ability of free P in the soil limit the use of chemical fertilizers. Disadvantage of chemical fertilizers can be overcome by applying biofertilizers in the field. Various phosphate solubilizing microorganisms (PSMs), including fungi and bacteria, have been isolated from different soil and environmental conditions. In soil, PSBs dominated over PS fungi in numbers which account 1- 50 and 0.5% of total population respectively. PSB isolated from rhizosphere are more metabolically active compared to bulk soil. However, drastic variations were observed in the performance of PSMs under laboratory and field condition as a result of variations in soil, environment and plant rhizosphere. Alternative method to select better PSMs involves isolation of organisms mimicking natural conditions such as buffering of alkaline vertisols, salinity and high or temperatures, nutrient availability and colonization ability. PSMs are integral part of soil and control the soil fertility throughout biogeochemical cycle.

Organic acids secreted by PSMs play a major role in P solubilization which chelate mineral ions and drop pH. Most of the PSMs secrete gluconic acid or 2-ketogluconic acid in the periplasm of Gram negative bacteria *via* direct oxidation pathway. In direct oxidation pathway glucose gets converted into gluconic acid by glucose dehydrogenase (GDH) while gluconate converted into 2-ketogluconic acid by gluconate dehydrogenase (GAD) enzymes. However, in

plant rhizosphere where PSBs colonize, glucose might not available in sufficient amount to solubilize bound P. Root exudates are major carbon and energy sources for rhizobacteria and are known to contain high amount of sucrose and fructose along with small molecular weight organic acids. PSMs ability to use multiple substrates for organic acid production will be more effective in field conditions. Most of the rhizobacteria produce gluconic acid is required in high amount but 2-ketogluconic acid is stronger acid than gluconic acid. Most of the PSMs do not possess GAD required for the conversion of gluconic acid into 2-ketogluconic acid. Rhizobacteria having the ability to use multiple carbon sources along with 2-ketogluconic acid secretion may perform better in field condition.

Organic bound phosphates constitute 50-70% of total bound phosphate of soils. Most plants produce phytase but they are not able to mineralize the bound phytates. Although many rhizobacteria secrete phytase, several factors prevent their application for plant growth promotion. Phytate remain recalcitrant in soil due to precipitation and adsorption on soil particle which are resistant to solubilize by three major classes of phytaes *viz* histidine acid phosphatases (HAPs), purple acid phosphatases (PAPs) or β-propellor phytases (BPPs). Addition of gluconic acid, citric acid and malic acid in surrounding stimulate the phytate mineralization. Hence organic acid secreting rhizobacteria which express phytase could efficiently mineralize the phytate and release P in the surrounding.

Availability of oxygen plays an important role in the colonization and survival of PSMs as oxygen levels modulate the energy metabolism. Oxygen and cognate oxygen radicals can affect bacterial cell either by altering the function or biosynthesis of specific proteins mediated

by global transcription regulation. *Vitreoscilla* hemoglobin (VHb) encoded by *vgb* gene gets expressed when dissolved oxygen is less than 2% air saturation. VHb protein known to significantly increases energy metabolism, protein biosynthesis, metabolite production and prevents from oxidative and nitrasive stress under hypoxic conditions. Hence present study deals with genetic manipulation of *E. asburiae* PSI3 for enhance P nutrition.

E. asburiae PSI3 exhibits MPS ability on Tris buffered rock phosphate minimal medium by secretion of high amount of organic acids in TRP medium by phosphate starvation inducible GDH enzyme on glucose or mixture of sugars. Incorporation of *gad* operon of *Pseudomonas putida* KT 2440 in *E. asburiae* PSI3 resulted in 438U of enzyme activity and secretion of 21.65 mM gluconic and 11.63 mM 2-ketogluconic acid in the medium. Pi release from rock phosphate (RP) in the medium was 0.84 mM in presence of 100mM Tris pH 8.0 and 45mM glucose while wild type could only solubilize RP in presence 75mM glucose. Improvement in the phosphate solubilization was found in Mung bean (*Vigna radiata*) plant inoculation study which showed improvement in plant dry shoot weight, shoot/root dry weight and P content as compared to wild type vector control.

Incorporation of periplasmic invertase (*invB*) of *Zymomonas mobilis* and periplasmic invertase (*Suc*2) of *S. cerevisiae* showed 438 mU and 29mU enzyme activity, respectively. Periplasmic localization of invertase in *E. asburiae* PSI3 resulted in 18.65 mM gluconic acid and 0.180mM P in the medium when 75mM sucrose and 75 mM Tris was used. Expression of *suc*2 showed much better MPS phenotype by producing 22 mM gluconic acid and 0.438 mM P in presence of 75mM Tris and 50 mM sucrose. MPS phenotype was further improved when

mixture of 50mM sucrose and 25 mM glucose in 100 mM Tris buffer was used by secretion of 34 mM gluconic acid and resulted in 0.479 mM P release into the medium. Thus, invertase incorporation in *E. asburiae* PSI3 improved MPS ability by producing gluconic acid on sucrose.

Aspergillus fumigatus phyatse (*phyA*) gene and *Citrobacter braakii* phytase (*appA*) gene were incorporated in *E. asburiae* PSI3 for phytase mineralization. Expression of both genes under *tac* promoter resulted in 1.7 and 1.5U of enzyme activity, respectively. In 100mM Tris buffered Ca-phytate medium, *E. asburiae* PSI3 (*phyA*) produced 43 mM gluconic acid and released 3.93 mM P in the medium on 75mM glucose while *E. asburiae* PSI3 (*appA*) produced 47 mM gluconic acid and released 4.46 mM P in the medium.

Incorporation of *vgb* gene in *E. asburiae* PSI3 increased GDH activity (by ~3 fold), growth and MPS ability in TRP medium. *vgb* expression reduced the glucose requirement for MPS ability to 60 mM in 100 mM Tris buffer medium by secretion of 38 mM gluconic acid and released of 0.53 mM P. When *vgb* gene was coexpressed with *appA* no significant increase was found in phytase activity but improved the phytase mineralization. In 100mM Tris buffer and 60 mM glucose, *E. asburiae* PSI3 (*appA, vgb*) secreted 39.7 mm gluconic acid and released 3.1 mM P into the medium. Coexpression of *appA, vgb* and *Uinv* genes further improved phytase mineralization in presence of sucrose and mixture of sugars. In presence of 50 mM sucrose and 75 mM Tris, *E. asburiae* PSI3 (*appA, vgb, Uinv*) produced 21 mM gluconic acid and released 2.8 mM P in the medium. In mixture of sugars, the amount of each of the sugar required for P release was reduced to 10 mM from 15 mM. In presence of 100 mM Tris and 10 mM mixture of each sugar resulted drop in pH up to 4.0 and released 3.3 mM P in the medium.

Overall, genetic manipulation with a set of genes consisting of *appA, vgb, Uinv* and *gad* enabled *E. asburiae* PSI3 to significantly improved qualitative as well as quantitative phosphate solubilization and mineralization leading to much improved P mobilization. These results could significantly improve the efficacy of *E. asburiae* PSI3 in field conditions. Additionally, similar genetic manipulations could incorporate very good PSM ability in plant growth promoting rhizobacteria (PGPR).

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PRESENTATIONS

List of posters presented

Poster presentation:-

1. Chanchal Kumar and **G. Naresh Kumar (2009)** Enhancement of mineral phosphate solubilization (MPS) abilities of *Enterobacter asburiae* PSI3 by incorporation of *Pseudomonas putida* KT 2440 gluconate dehydrogenase (*gad*) gene. Presented at the *First Asian PGPR Congress for Sustainable Agriculture* held at Acharya N. G. Ranga Agricultural University, Hyderabad during June 21-24, 2009.

2. **Publication:**

   Incorporation of *Pseudomonos putida* KT 2440 gluconate dehydrogenase (*gad*) operon improves the efficacy of phosphate solubilization by *Enterobacter asburiae* PSI3. Chanchal Kumar[1], Kavita Yadav[1], G. Archana.[2] and G. Naresh Kumar [*1]

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