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 (Kauwenbergh, 2010). Overall, three countries have more than 85% of known phosphorus reserves.

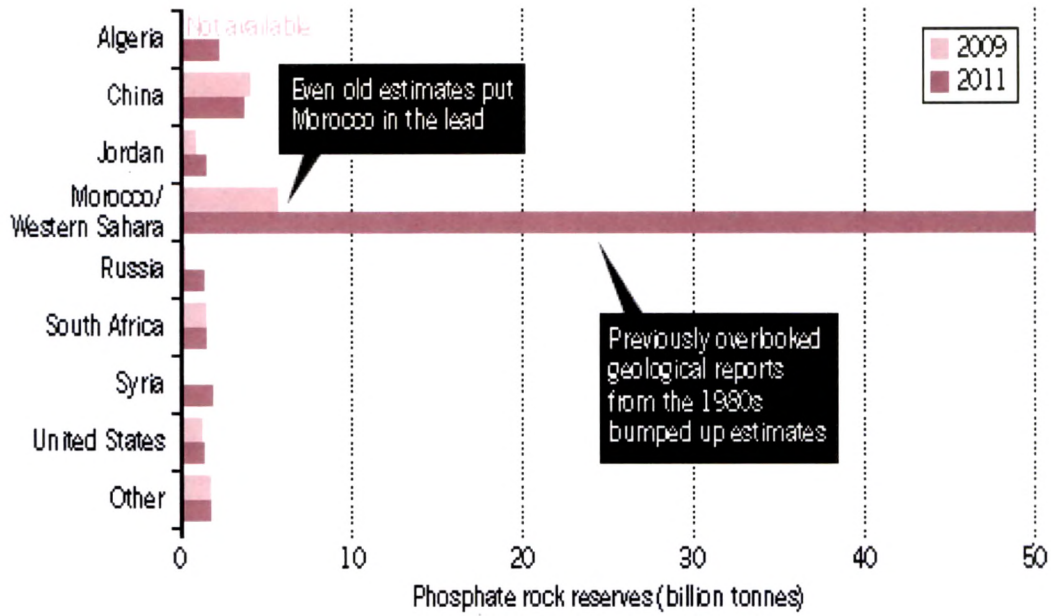

Fig. 1.1 Global distribution of phosphorus reserve (Elser and Bennett, 2011).

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 (Fuentes et al, 2008; Richardson 2001; Turner, 2007), 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; Henri et al.,2008; Hameeda 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. (Wani et al., 2005; 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 (Heijnen et al., 1993; 1995; Bashan et al., 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., 1998).

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., 2010). 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., 1998; Srivastava et al., 2007).

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 (Konietzny and Greiner, 2002).

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/	Ins(1,2,3)P3		

		D-Ins(1,2,3,6)P4	
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, 2001[a]). 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) has 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, *Ensifer meliloti* (ATCC9930), *Rhizobium* sp. ST1, *Rhizobium* sp. IC-3109 (Transgenic expression)	Mung Bean (*Vigna radiata*)	Patel et al., 2011
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.,(1998)
*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., 1998b). 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., 1998b).

Overexpression of *E. coli gcd* gene in *Azotobacter vinelandii* showed MPS ability by production of GA (Sashidhara and Podille, 2009). DNA fragment of non

PSM *Synechocystis* PCC 6803 expressed in *E. coli* showed MPS ability on different carbon sources (Gyaneshwer et al., 1998). Overexpression of *E. coli* citrate synthase gene (*glt*A) in *P. fluorescens* ATCC 13525 results in secretion of citric acid which is confers MPS phenotype (Buch et al., 2009).

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*	DCP	Citric acid	Buch et al (2009)

		ATCC 13525			
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 radiate*) 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 (Frey and Kallio, 2003).

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).

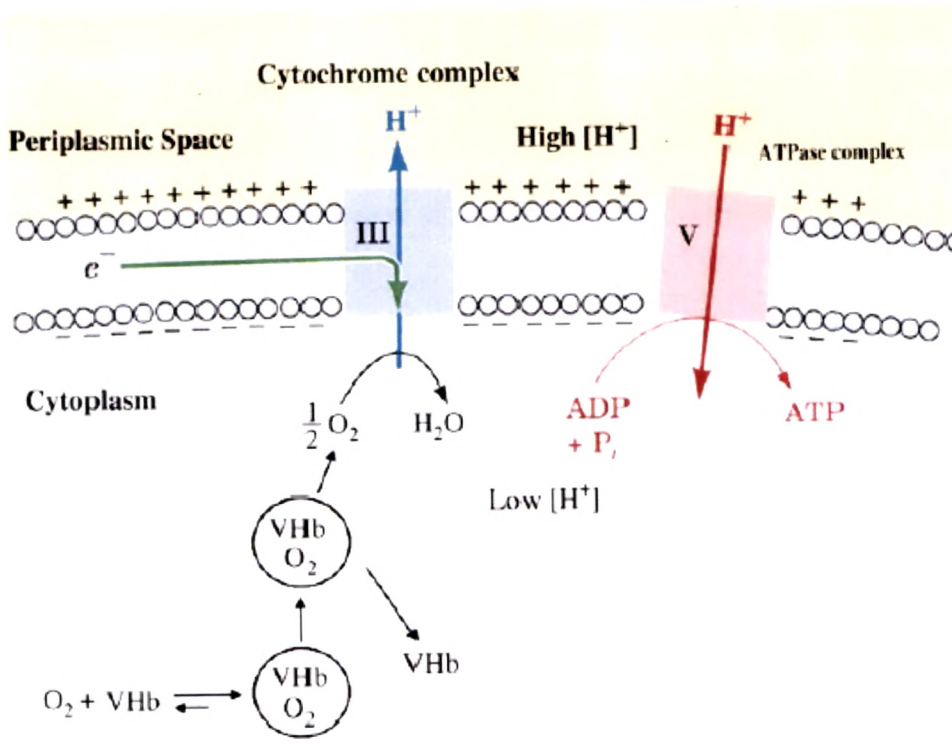

Fig. 1.2: Proposed Energy generation model in presence of VHb (Zhang et al., 2007).

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 (Kaur et al., 2002; Frey 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 et al., 1990, Khosla and Bailey, 1988a,b). Since then VHb was expressed in many organisms for enhanced growth and metabolite production (**Table 1.7**).

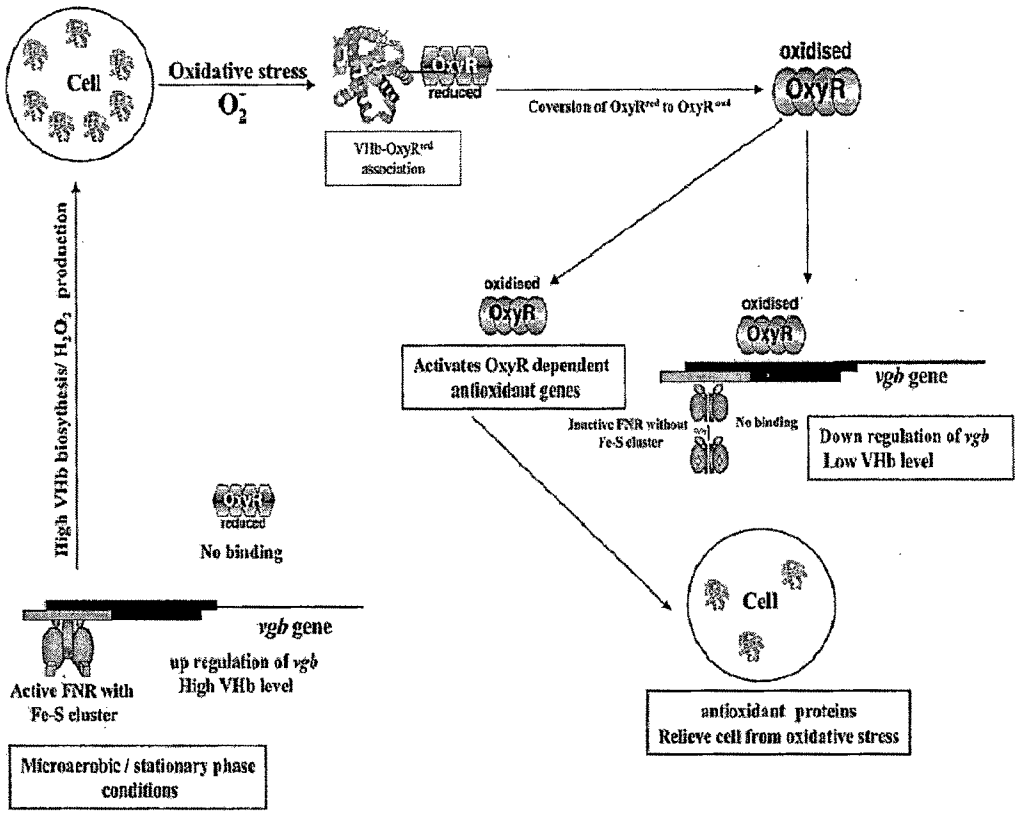

Fig. 1.4: Proposed role of *vgb* during oxidative stress (Anand et al., 2010)

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 Bioremediati	*Pseudomonas delafieldii*	Sulfur (from diesel oil)	Li et al.,(2009)

on			
	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, Urgun-Demirtas et al., 2004
Physiological improvement	*Tremella fuciformis*	Enhanced growth	Zhu et al.,(2006)
	E. coli	Increased copper uptake	Khleifat,(2006)
	E. aerogenes	Increased sensitivity to mercury and cadmium	Geckil et al.,(2004a,b)
	E. aerogenes	Improved growth and oxygen uptake rates	Erenler et al.,(2004)
	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; Urgun-Demirtas et al., 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., 1999) 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**)

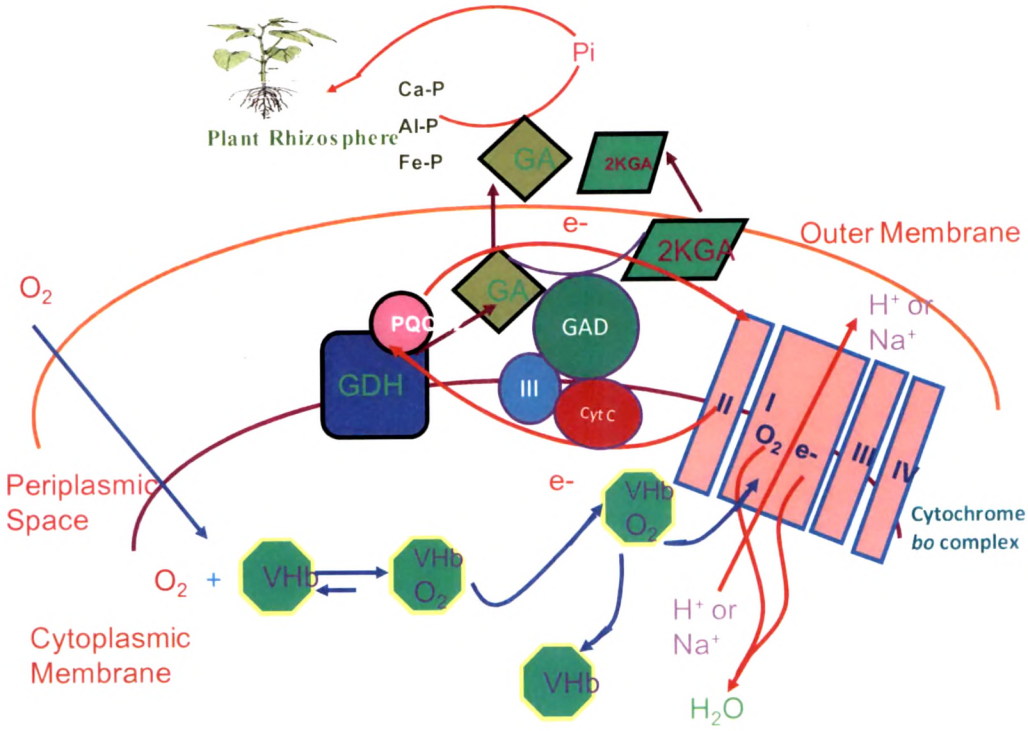

Fig. 1.4 Proposed model for genetic manipulation in *E. asburiae* PSI3

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 solubilisation 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.