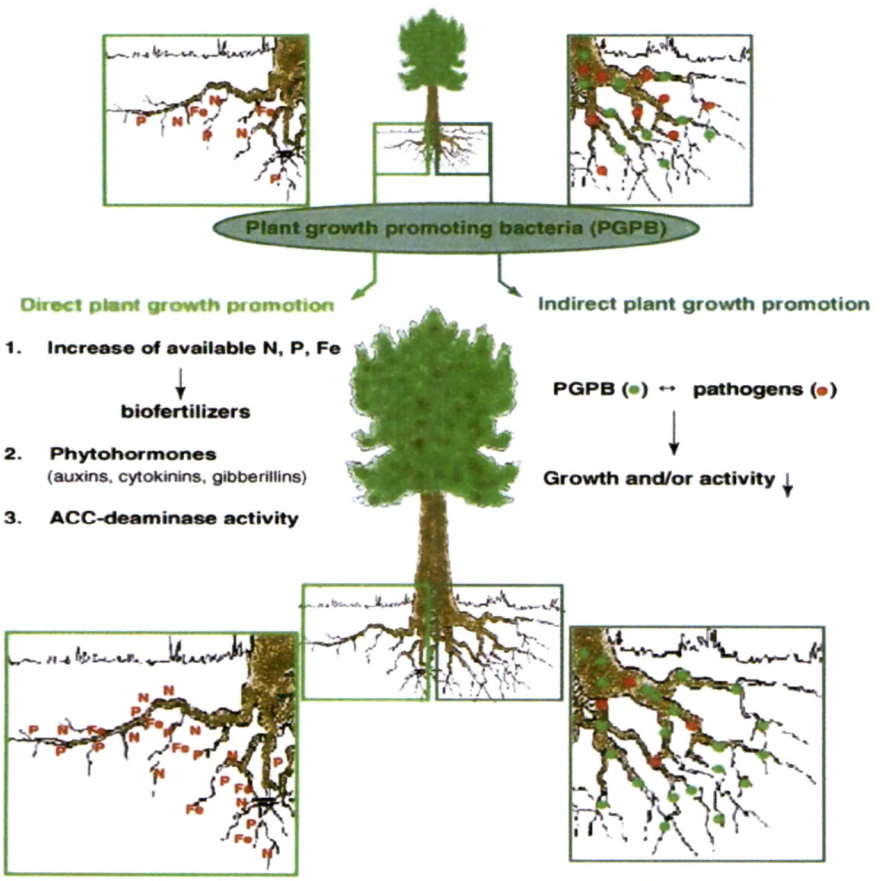
Chapter 1



Review of Literature and Introduction

1.1: Introduction

Demand on agriculture for supply of food to the increasing human population in the future is one of the greatest challenges of 21st century. The problem gained major focus as the total cultivable land has reached the maximum levels and existing land is eroding due to excessive irrigation. Thus, instead of enhancing the crop yields, sustainability is also becoming very difficult. The effort to focus on the soil biological system and agro-eco system as a whole has become a necessity in order to understand complex interactions governing the stability of agricultural land so as to meet the problem of this food insecurity. Green revolution especially in developing countries like India had led to excessive use of chemical fertilizers and other chemicals which have resulted in reduction in soil fertility and to environmental degradation (Brar et al., 2012). The chemical fertilizers usage has gone to theoretical maximum beyond which there will be no further increase in yields (Ahmed, 1995). According to some reports 8-9 billion people will be added by 2040 necessitating the need for second green revolution since food production will not keep pace with the growing population demand (Leisinger, 1999; Vance et al., 2000). After nitrogen, phosphorous is the chief macronutrient required for plant growth and crop yield (Bieleski, 1973; Vance et al., 2000). Phosphorus is required for the synthesis of key molecules such as nucleic acids, phospholipids, ATP and several other biologically active compounds. 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.

1.2: Global Phosphate Status.

The concentration of Phosphorus in many rocks is very less. Total phosphorous concentration in soil ranges from 0.02 to 0.5% (400–1200 mg-1 kg of soil) depending upon the weathering of rocks and other environmental factors (Kucey et al., 1989). P in soil exits in inorganic and organic forms. More than 60% of the soils are highly deficient in phosphorus (Fairhurst et al., 1999). Although 40% arable soils are not

limited by P abundance, they do not support optimal plant growth due to P limitation which is evident from increase in plant growth and crop yields upon supplementation of soluble P in terms of chemical fertilizers. Inorganic P in soil is present as salts of calcium in case of calcareous alkaline soils while Al and Fe form predominant in acidic soils (Gyaneshwar et al., 2002). Most inorganic soil P is made up of poorly soluble mineral phosphate precipitates. Soluble P also gets adsorbed to the soil particles making them in accessible to plants. Recent IFDC survey estimated phosphorus reserve of Moracco and Western Sahara territory from 15 billion ton to 65 billion ton (Cooper et al, 2011). Overall, three countries have more than 85% of known phosphorus reserves **(Fig. 1.1).**

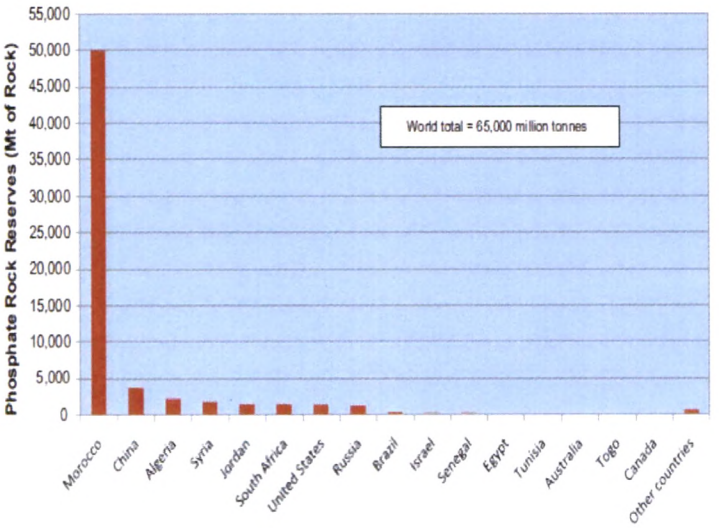


Fig. 1.1: Global distribution of phosphorus reserve

(Cooper et al., 2011).

P fertilizers are essential for maintaining and increasing the world food production (Koning et al., 2008). Phosphorus is completely produced from non-renewable resources, phosphate rocks, and the current extraction rates would lead to a rapid depletion of these resources.

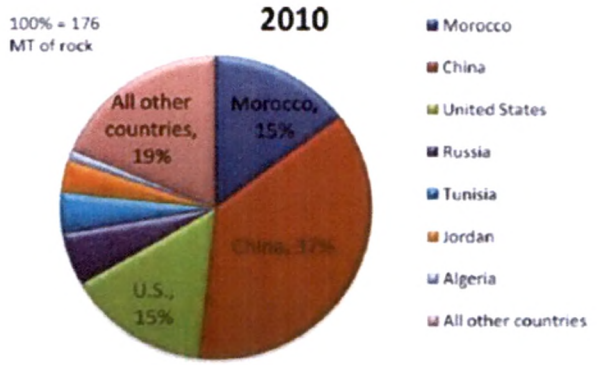


Fig. 1.2: Share of Rock phosphate production in 2010 (Cooper et al., 2011).

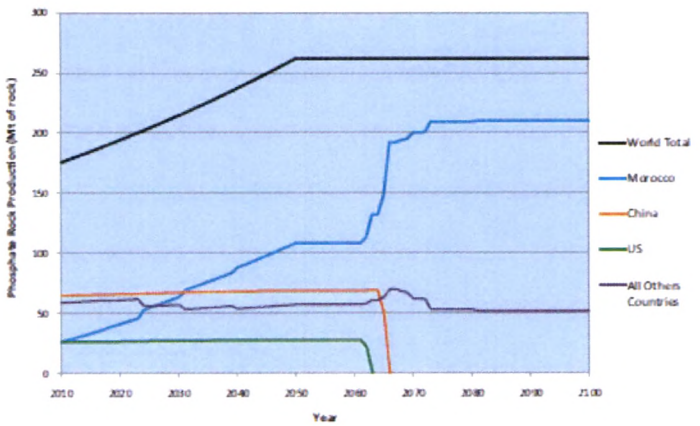


Fig. 1.3: Total global P consumption (Cooper et al., 2011).

The main producers are Africa, China, the United States, the Middle East and Russia (**Fig. 1.2**) and according to the model results, by 2050 Africa would totally dominate the market, with more than half the global production. In contrast, production in United States would decrease. Most studies expect a further increase in the use of P fertilizers in the coming decades (**Fig. 1.3**) (USGS, 2008; Cordell et al., 2009). In developing countries, P fertilizer use for the production of food crops is increasing while in industrialized regions the P fertilizer use is declining. For 2100, the resulting range in P use would be 65–115Mt P_2O_5. P production is dominated by the United States, Africa, and China (**Fig. 1.4**) and rock phosphate reserves (**Fig. 1.5**)

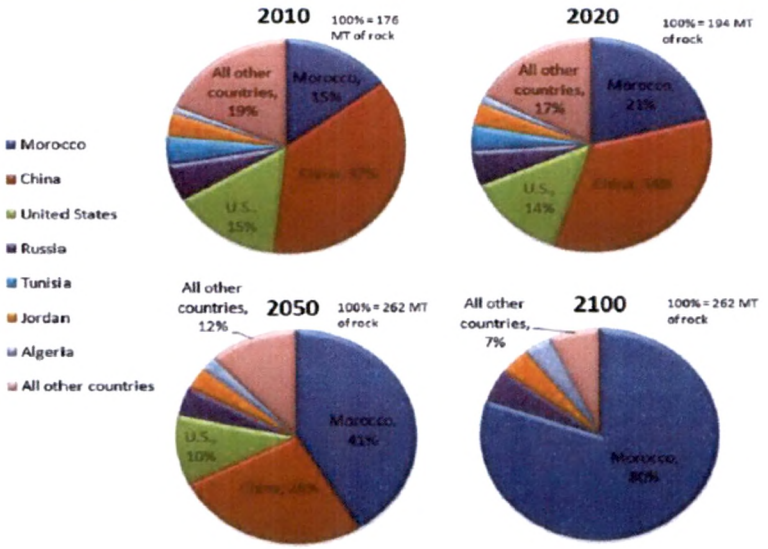


Fig. 1.4: Changing share of rock phosphate production (Cooper et al., 2011).

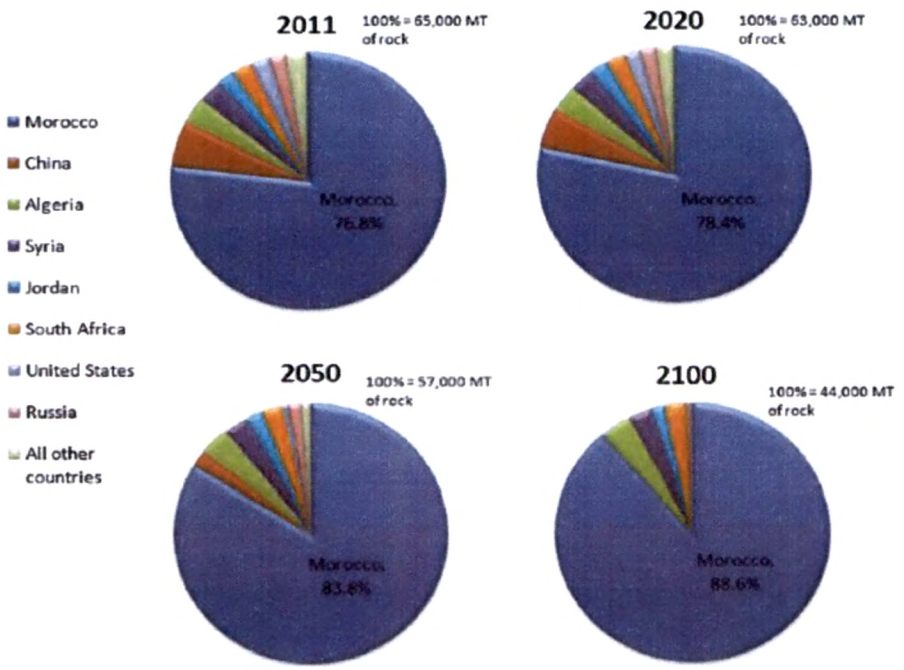


Fig. 1.5: Changing share of rock phosphate reserves (Cooper et al., 2011).

Under the Geological orchestration scenarios (medium resource estimates), about half of the current reserves is projected to be depleted by 2050, and the other half before the end of the century (**Fig. 1.6**). As a result of the associated increase in prices, at the same time a larger part of the reserve base would become economic for exploitation and by the end of the century about 40% of the reserve base would also have been exploited.

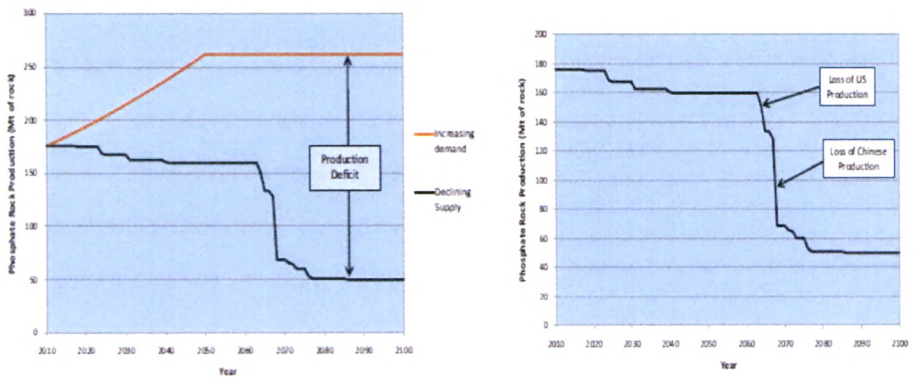


Fig. 1.6: Depletion of resource base of phosphate rock under the Global Orchestration (GO) scenario (Cooper et al., 2011).

For the total resource base, this implies a depletion of 35% by the end of the century. Thus, scarcity of P resources would become a more important issue by the end of the century, with only 65% of the resource base being left. Hence, current reserves or resources with a very high probability of being available would be totally depleted by the end of the century

1.3: Status of phosphorus in soil.

After N, phosphorus (P) is the major plant growth limiting nutrient despite being abundant in soils in both inorganic and organic forms. However, many soils throughout the world are P-deficient and the free phosphorus concentration (the form available to plants) even in fertile soils is generally not higher than 10 μM even at pH 6.5 where it is most soluble (Arnou, 1953). On an average, most mineral nutrients in

soil solution are present in millimolar amounts; however, phosphorus is present only in micromolar or lesser quantities (Ozanne, 1980). These low levels of P are due to high reactivity of soluble P with Calcium (Ca), iron (Fe) or aluminum (Al) that lead to P precipitation. Inorganic P in acidic soils is associated with Al and Fe compounds (Sharpley et al., 1984) whereas calcium phosphates are the predominant form of inorganic phosphates in calcareous soils. Organic P contributes to a large fraction of soluble P, as much as 50% in soils with high organic matter content (Barber, 1984). Depending upon the weathering of rock and other environmental factors, the amount of P in soil varies from 0.02 to 0.5% (Kucey et al., 1989).

1.3.1: Alkaline Vertisol.

Alkaline vertisols, also called as calcareous soils, have high buffering capacity and mainly contains P in bound form with Ca (Ca-P complexes) which could be solubilized by acidification of soil. Thus, organic acids which lower the pH of the medium could efficiently release P form vertisols. However, the amount of organic acid required could vary from 10-100 mM depending on the nature of organic acid (Gyaneshwer at al., 1998).

1.3.2: Acidic Alfisols.

Acid soils such as alfisols, aridisols, entisols and ultisols occupy about 30% of world's ice free land area (von Uexull et al., 1995). Deficiency of nutrients such as P and toxicity of metals such as aluminium (Al) chiefly mark the crop growth in acid soils. Of the total land area of the world, about 24.2% is considered potentially arable land and most of this belongs to acid soils. In alfisols, phosphate is predominantly present in iron (Fe) and Al bound forms which are difficult to be solubilized by even strong organic acids (Ae et al., 1991; Jones and Darrah 1994; Srivastava et al., 2006). Low molecular weight organic acids effectively chelate Fe and Al from ferric phosphate and aluminium phosphate; thereby solubilize P (Kpomblekou & Tabatabai 1994; Gadd 1999). However, addition of organic acids at high concentrations (10

mM-50 mM) did not result in the release of detectable amount of P from alfisol although pH of the soil suspension dropped below 4.0 (Srivastava et al., 2006).

1.4: Phosphorous availability to plants.

Phosphorous being the 10th most abundant element on earth and the agricultural soils are postulated to contain enough amount to sustain the agriculture for 100years (Goldstein, 1993). In majority of soils, including the soils categorized as highly fertile, P limits plant growth. Soluble P even in fertile soil is in micro molar ranges as compared to other elements which are present in millimolar concentration (Khan et al., 2006). Soluble P in the form of $H_2PO_4^-$ and HPO_4^{2-} is only taken up by the plants. Hence, inorganic and organic P complexes need to be converted to either soluble orthophosphate anions or low molecular-weight organic phosphate (Rodriguez and Fraga, 1999). Only 10% of the applied chemical fertilizer is available to the plants rest gets fixed due to interaction with metals such as Al, Ca and Fe (Vig and Dev, 1984; Stevenson, 1986). The amount of P in plants ranges from 0.05% to 0.30% of total dry weight. The concentration gradient from the soil solution to the plant cell exceeds 2,000- fold, where the concentration of P in plants cells is around 10mM. Phytate has dense negative charges, hence they are precipitated as insoluble salts with positive charged metal ions (e.g. Fe, Al and Ca phytate) or adsorbed into soil colloids, thus rendering them highly invulnerable to biodegradation (Celi et al., 1999; Tang et al., 2006). In fact, only a limited number of soil bacteria can utilize soil phytate as a sole source of C and P (Richardson and Hadobas, 1997). Impact of pH on phosphorous levels in soil and Conversion of unavailable to available forms of soil P usually occurs too slowly to meet crop P requirements **(Fig. 1.7 and Fig. 1.8).**

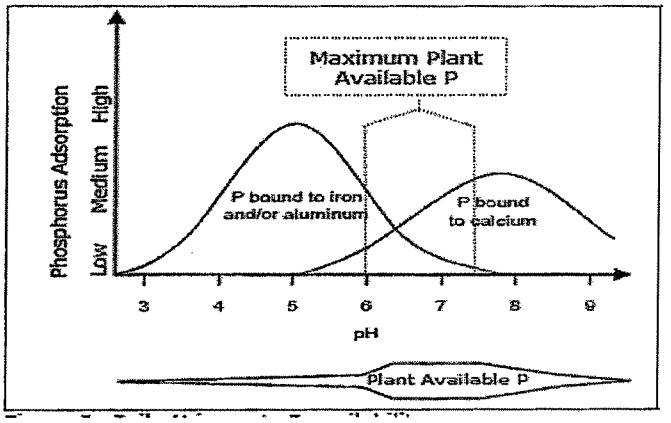


Fig. 1.7: Soil pH impacts P availability (Hyland et al., 2005 Agronomy Fact Sheet Series, Fact Sheet 12).

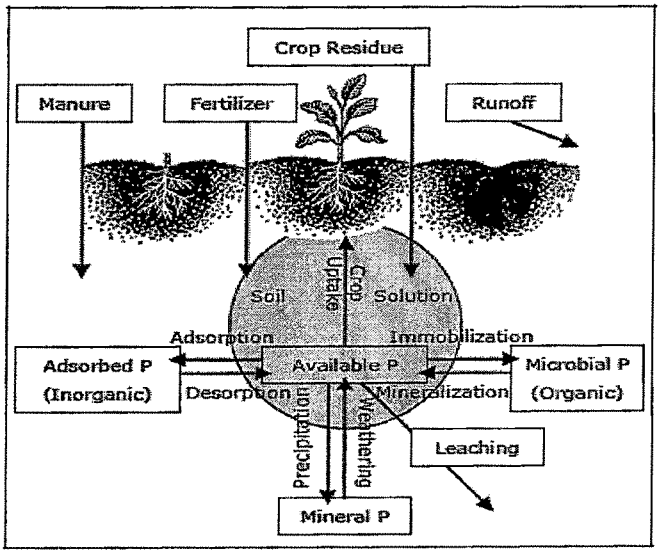


Fig.1.8: Phosphorous cycle

(Hyland et al., 2005 Agronomy Fact Sheet Series, Fact Sheet 12).

1.5: Need for biofertilizers in plant phosphate nutrition

Excessive addition of phosphate to sustain the plant growth and crop yield has resulted in degradation in health of the soil and other environmental problems such as eutrophication, and hypoxia of lakes and marine estuaries (Bennett et al., 2001). Along with environmental hazards and soil health, economical burden has also led the agronomists to find an alternate for chemical fertilizers which are environmental friendly, economical and can sustain agriculture for long time (Khan et al., 2006). Therefore the use of microbial inoculants (biofertilizers) which included phosphate solubilizing microorganisms (PSMs) in agriculture represents an environmentally friendly alternative to the conventional chemical fertilization. Phosphorus biofertilizers in the form of microorganisms can help in increasing the availability of accumulated phosphates for plant growth by solubilization (Subba Rao, 1982; Tandon, 1987; Kucey et al., 1989; Goldstein, 1993; Richardson, 1994). In addition, the microorganisms with P solubilizing potential can enhance plant growth by increasing the efficiency of biological nitrogen fixation, enhancing the availability of other trace elements such as Fe, Zn etc. and by production of plant growth promoting substances (Kucey et al., 1989; Vessey, 2003).

1.6: Phosphate Solubilizing Microorganisms.

PSMs are ubiquitous, and their numbers varies depending upon the soil. In soil, P-solubilizing bacteria (PS bacteria) out numbers P solubilising fungi (PS fungi) with a factor of 2-150 fold (Banik and Dey, 1982; Kucey, 1983, 1989). P solubilising bacteria constitute 1–50% and fungi 0.5%–0.1% of the total respective population. However, these PSMs may be effective even in these soils when supplemented with rock phosphate (RP) (Kucey, 1983). Most P-solubilizing bacteria were isolated from the rhizosphere of various plants and are known to be metabolically more active than those isolated from bulk soil (Katznelson and Bose, 1959; Baya et al., 1981). Among the bacterial genera with P solubilising capacity are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus,*

Aereobacter, Flavobacterium, Rahnella, Enterobacter, Azospirillum and *Erwinia.* In general, fungal isolates exhibit greater P-solubilizing ability than bacteria in both liquid and solid media (Kucey, 1983; Gyaneshwar et al., 2002; Khan et al., 2006). Among the fungal genera with this capacity are *Aspergillus, Penicillium,* and *Rhizopus.* The P-solubilizing ability of PSMs also depends on the nature of N source used in the media, with greater solubilization in the presence of ammonium salts than when nitrate is used as N source. This has been attributed to the extrusion of protons to compensate for ammonium uptake, leading to a lowering of extracellular pH (Roos and Luckner, 1984). In some cases, however, ammonium can lead to decrease in P solubilization (Reyes et al., 1999). In addition, other media components were also found to affect the P solubilization ability (Cunningham and Kuiack, 1992). PSMs are integral component of the soil which controls the biofertility of the soil. 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). Many other genera also show MPS ability including *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 improvement in plant P content by mobilizing inorganic and organic P from the soil.

1.7: Mechanism of mineral phosphate solubilization.

1.7.1: Acidification.

PSM ability to solubilize insoluble phosphates has been attributed to reduce the pH of their surroundings either by the release of organic acids or protons (Gyaneshwar et al., 1999; Whitelaw, 2000; Patel et al., 2008) (**Fig. 1.9**). The organic acids secreted can either directly dissolve the mineral phosphate as a result of anion exchange of PO_4^{2-} by acid anion i.e. acid hydrolysis, chelation of both Fe and Al ions associated with phosphate or by competing with phosphate for adsorption sites in soil (Gyaneshwar et al., 2002; Khan et al., 2006). PSMs secrete variety of organic acids when grown on simple carbohydrates such as gluconic acid (GA), oxalic, citric, malic, etc. (**Table 1.1 A and B**). In certain cases phosphate solubilization is induced by phosphate starvation (Gyaneshwar et al., 1999). Among nodule bacteria (e.g., *Rhizobium/ Bradyrhizobium*), the phosphate-solubilizing activity of *Rhizobium* was associated with the production of 2-ketogluconic acid which was abolished by the addition of NaOH, indicating that the phosphate-solubilizing activity of this organism was entirely due to its ability to reduce the pH of the medium (Halder and Chakrabarty, 1993). Inorganic acids such as hydrochloric acid can also solubilize phosphate but they are less effective compared with organic acids at the same pH (Kim et al., 1997).

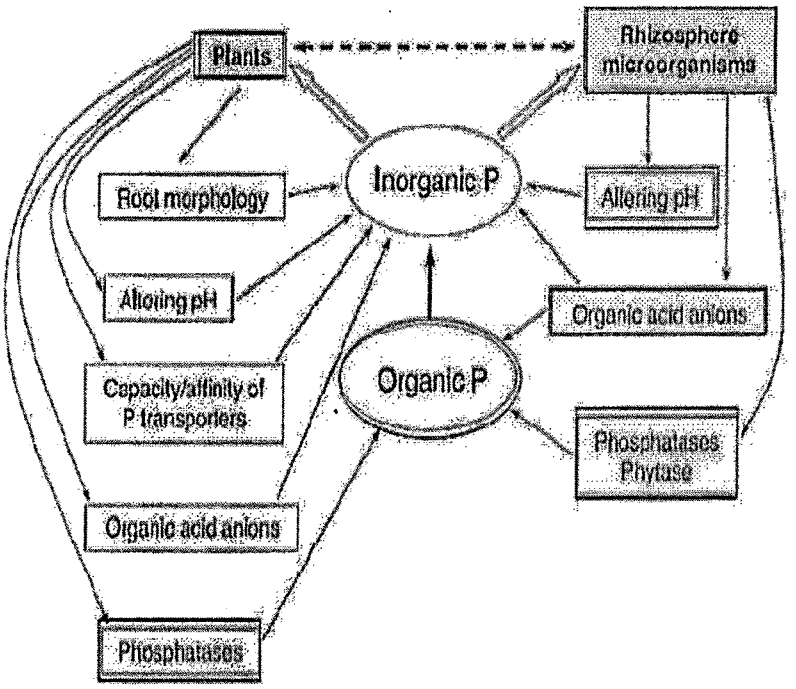


Fig.1.9. Plant- microbe mechanism to increase Phosphorus availability in rhizosphere (Richardson, 1994).

1.7.2: Chelation.

Acidification, however, does not seem to be the only mechanism of solubilisation. The chelating ability of organic acids is also important, as it has been shown that the addition of 0.05M EDTA to the medium has the same solubilizing effect as inoculation with *Penicillium bilaii* (Kucey, 1988). The formation of complexes between chelator and cations such as Al^{3+} and Ca^{2+} depends on the number and kind of functional groups involved as well as the specific cation. It has been found that acids with an increased number of carboxyl groups are more effective at solubilizing RP (Kpomblekou and Tabatabai 1994; Xu et al. 2004). Ca^{2+} was found to form complexes more readily with tricarboxylic acids such as citric acid, over dicarboxylic acids such as malic and tartaric acids (Whitelaw, 2000). *P. bilaii*, a citric acid producer and *Aspergillus niger*, an industrial citric acid producing strain, have

been effective P solubilizers (Sperber 1958; Agnihotri, 1970; Kucey, 1988; Cunningham and Kuiack, 1992; Omar, 1998; Abd-Alla and Omar, 2001). Apart from tricarboxylic acids, dicarboxylic acids show potent RP solubilizing ability. Oxalic and tartaric acids release more P into solution than citric acid which was attributed to the fact that oxalic and tartaric acids form poorly soluble precipitates with Ca^{2+}, effectively lowering the solution saturation point (Sagoe et al., 1998). Oxalic acid is also better in releasing P from alfisols than citric acid (Srivastava et al., 2006).

1.7.3: H^+ excretion.

Microbial excretion of H^+ occurs in response to the assimilation of cations, has also been attributed to solubilisation of insoluble P. This phenomenon is well documented in fungi which excrete H^+ in exchange for NH^{4+} (Beever and Burns 1980; Banik and Dey, 1982; Asea et al., 1988). This is also been shown under laboratory conditions, where it has been observed that more RP is solubilized using NH^{4+} rather than NO^{3-} as a source of N and therefore pH was generally lower but higher titratable acidity, on NH^{4+} (Whitelaw et al., 1999). Similarly, ammonium sulphate was found to promote RP solubilization for bacterial species *Bacillus circulans*, *Bacillus brevis*, and *Bacillus coagulans* on a variety of N sources (Vora and Shelat, 1998). For some microorganisms, the release of H^+ ions due to the assimilation of NH^{4+} seems to be the sole mechanism promoting RP dissolution. *Penicillium bilaii* and *Penicillium fuscum* could decrease pH and solubilize RP when media contained NH^{4+} compared to *P. billai* which could maintain the ability to lower the pH and solubilize RP when there was no N in the media (Asea et al., 1988). This result suggested that different mechanisms were utilized by the different species; one mechanism required the presence of NH^{4+} in the medium and the other did not.

Table 1.1: Nature of organic acids secreted by PSMs (Khan et al., 2006).

A: Nature of organic acids secreted 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)

B: Nature of organic acids secreted by PS microorganisms (Patel et al., 2008)

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,	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, 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)

1.8: Necessity of genetic modifications for P solubilization.

P fertilizers are essential for maintaining and increasing the world food production. Phosphorous is completely produced from large scale mining of rock phosphate which is non-renewable resources. In ancient times P was applied to agricultural soil by recycling animal manure, crushed animal bones, human and bird excreta, city waste and ash. Industrial revolution replaced this by phosphate material from non-renewable resources. According to the International Fertilizer Industry Association (IFA), in 2008, close to 53.5 million tonnes (Mt) of P_2O_5 (i.e. 175 Mt of phosphate concentrates, averaging 30.7% P_2O_5 content) was mined (IFA, 2009a). Hence there is a gradual depletion of global high-grade P resources (USGS, 2008), (Vuuren et al., 2010; Childers et al., 2011).

The soil P is in a dynamic equilibrium involving the geochemical and biological P transformations. Predominantly P is uptaken by plants in the form of H_2PO_4 - (Vance et al., 2003). But a greater part of total soil P, almost 85-90%, is fixed in the form of insoluble phosphates mainly mineralized as Ca-P, Fe-P and Al-P or as organic bound forms like phytates, RNA etc. and hence cannot be utilized by plants (Holford, 1997; Khan et al., 2006). The degree of preicipitation and solubilization of Ca-P (predominant in vertisols), Fe-P and Al-P (predominant in alfisols) depends on the buffering capacity of a particular soil (Gyaneshwar et al., 2002). The soil P solution is in equilibrium with relatively labile form of P (present as very small portion) which is adsorbed to clay, calcium carbonates, organic matter etc. (Holford, 1997; Whitelaw, 2000; Arcand and Schneider, 2006). Soil P fixed as organic pool could supply P to the plants depending on the prevailing plant-microbial interactions (Arcand and Schneider, 2006). P deficiency is one of the main biophysical constraints to crop production globally which engraved due to soil weathering and intensive nutrient-extracting agricultural practices (Akinrinde, 2006; Arcand and Schneider, 2006; Khan et al., 2006). Efficiency of the chemical fertilizers is low as the supplemented free Pi gets fixed into the soil due to pH dependent precipitation with cations and adsorption to soil particles (Rodríguez and Fraga,

1999). The use of conventional P fertilizers is highly limited in developing regions due to involvement of high expenditure and is prohibited for use by organic farmers as it harms the soil quality in long run (Khan et al., 2006; Harris et al., 2006). Additionally, soluble P in soil moves to plant roots mainly by diffusion which rate of which (10-12 to 10-15 m^2 s^{-1}) may be too low to meet the high plant P-uptake rates thereby creating a zone of P-depletion around the roots (Arcand and Schneider, 2006). According to the reports, about 40–60% of the current resource base would be extracted by 2100. At the same time, production will concentrate in Asia, Africa and West Asia, and production costs will likely have increased. Most of the current resource base is concentrated in Africa (Morocco) The depletion of resources outside Africa may lead to a high share of African production in world phosphate rock supply which is more than half the global production, unless new important resources are identified and exploited in other regions. Short-term focus on domestic resources may lead to higher prices and in the long run to even higher imports. Depletion is also likely to increase phosphate production costs by about a factor of 3–5, during this century (**Fig.1.10**). Increasing the efficiency of P application seems to be an urgent need to mitigate the increasing global P imbalance and fulfill the phosphorous requirement of crop and livestocks. In this context mineral phosphate solubilization by microorganisms by genetic modification could hold tremendous importance.

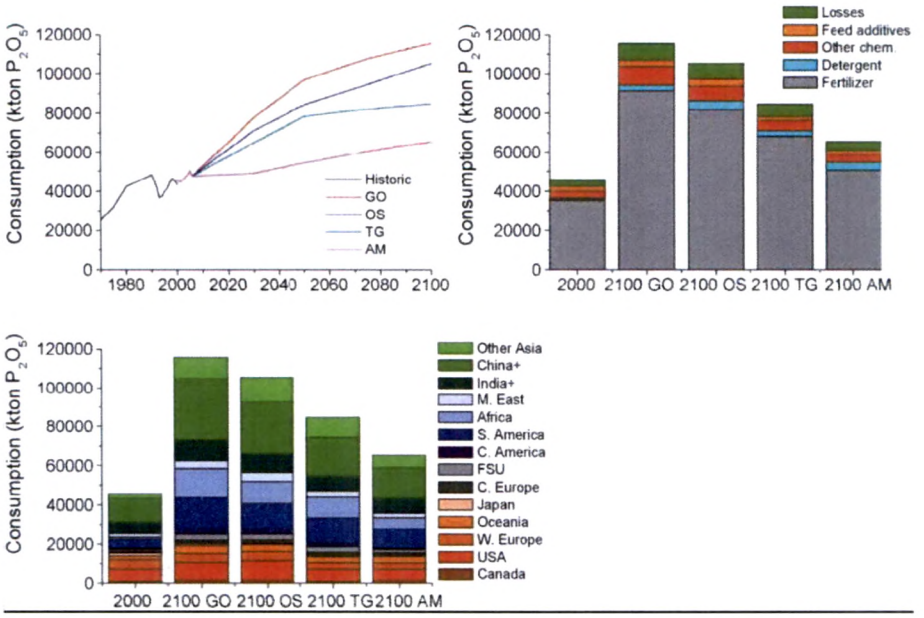


Fig. 1.10. Total global P consumption (a), P use per use category (b), and P use per world region (c), for the four Millennium Ecosystem Assessment (MA) scenarios (Vuuren et al., 2010).

These PSMs reportedly produce organic acids such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate, glycolate, etc (Rodriguez and Fraga, 1999; Khan et al., 2006). Some PSMs have proved even more advantageous as they not only increase the availability of soluble phosphate but also possess additional plant growth promoting abilities (Vessey, 2003; Haas and Defago, 2005; Ponmurugan and Gopi, 2006; Vassilev et al., 2006). With the increasing need of efficient PSMs, apart from isolation, identification of the organic acids responsible for P-solubilization and elucidation of mechanisms underlying production of these organic acids has been of prime interest.

1.8.1: Status of genetic modifications for developing P solubilizing ability.

In gram negative bacteria, direct oxidation of glucose is major pathway for glucose utilization, in which glucose gets converted into gluconic and 2-ketogluconic acid (Goldstein, 1995). These acids play a major role in MPS ability of

microrganisms and their secretion has been incorporated to achieve and improve the MPS ability of non- or weak-PSMs. Gene responsible for MPS ability were cloned and expressed in different hosts which imparts or improves the MPS phenotype of genetically modified organisms (**Table 1.2**). Mostly genetic modifications were done for enzymes or cofactors of direct oxidation pathway including the genes responsible for gluconic, 2-ketogluconic acid, and cofactor for glucose dehydrogenase. However, previous studies shows that gluconic acid is required in very high amount (50 mM) for phosphate solubilization while citric and oxalic acid are required in very low amount (5-10 mM) and shows efficient P release (Gyaneshwar et al., 1998). Thus, rhizobacteria secreting citric or oxalic acid could be most efficient in P release from both alkaline vertisol and acidic alfisols. Genetic modification in the central carbon metabolism of rhizobacteria for secretion of citric and oxalic acid could be a great approach for converting rhizobacteria into potential phosphate solubilizing bacteria (PSB). Overexpression of *E. coli* citrate synthase gene (*glt*A) in *P. fluorescens* ATCC 13525 results in secretion of citric acid however the secreted citrate was not sufficient to release P (Buch et al., 2009).

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 and strength of organic acid seems to be effective in P solubilization. Oxalic acid is more effective as compared to citric acid in acidic alfisols supplemented with RP but in alkaline vertisol both the acids efficiently release P from RP (Gyaneshwar et al., 1998; Srivastava et al., 2006). Phosphorus biofertilizers in the form of micro-organisms can help in increasing the availability of accumulated phosphates for plant growth (Subba Rao, 1982; Goldstein, 1986; Tandon, 1987; Kucey et al., 1989; Richardson, 1994). In addition, the micro- organisms involved in P solubilization as well as better scavenging of soluble P (P biofertilizers) can enhance plant growth by increasing the efficiency of biological nitrogen fixation, enhancing the availability of other trace elements such as Fe, zinc (Zn), etc. and by production of plant growth promoting substances (Kucey et al., 1989).

The involvement of micro-organisms in solubilization of inorganic phosphates was known as early as 1903 (Kucey et al., 1989). Since then, there have been extensive studies on the solubilization of mineral phosphates by micro-organisms. These have been reviewed by Subba Rao (1982), Goldstein (1986), Tandon (1987) and Kucey et al. (1989). Phosphate solubilizing microorganisms (PSMs) are ubiquitous, and their numbers vary from soil to soil. In soil, P-solubilizing bacteria constitute 1–50% and fungi 0.5%–0.1% of the total respective population. In general, P solubilizing bacteria generally out number P solubilizing fungi by 2–150 fold (Banik and Dey, 1982; Kucey, 1983; Kucey et al., 1989). The majority of the PSMs solubilize CaP complexes and only few are effective in solubilizing FeP and AlP (Banik and Dey, 1983; Kucey et al., 1989). Hence, these PSMs could be effective in calcareous soils in which CaP complexes are present, but not in other soils such as alfisols in which phosphates are complexed with Fe and Al ions.

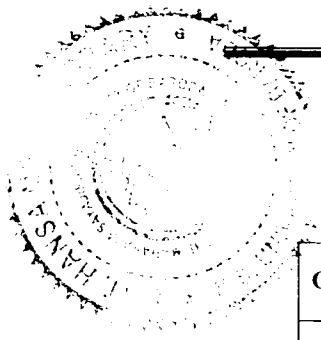


Table 1.2: Genetic modifications of microorganisms for phosphate solubilization

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	*E. 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*	*A. 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	*P. fluorescens* ATCC 13525	DCP	Citric acid	Buch et al., 2009
Unknown	*Synechocystis* PCC 6803	*E. coli* DH5α	RP	Unknown	Gyaneshwar et al., 1998
gad / Gluconate dehydrogenase	*P. putida* KT2440	*E. asburiae* PSI3	RP	GA and 2KG	Kumar et al., 2013

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

1.8.2: Direct oxidative pathway.

1.8.3: Gluconic and 2-ketogluconic acid secretion.

Plant requires phosphorus (P) for the growth and development, phosphorous is second macronutrient required by plant.phosphorous present in Soil is in form of (organic and inorganic forms) (Zou et al., 1992; Vance, 2001). This P deficiency is limiting factor for crop production worldwide (Arcand and Schneider, 2006). Phosphorous gets re fixed in large proportion and rapidly precipitated , in the form of Fe–P and Al–P complexes, which becomes unavailable to plants (Johnson and Loepper, 2006; Rengel and Marschner, 2005). As an alternative strategy, to make free P available to plants is the mineral phosphate solubilizing (MPS) by microorganisms. Microorganisms are known to solubilize mineral phosphate by secreting variety of low molecular weight organic acids such as gluconic, 2-ketogluconic, citric and oxalic.

In many Gram-negative bacteria's the MPS mechanism is well characterized, as it involves gluconic acid secretion by a direct oxidation pathway, which is mediated by the membrane-bound glucose dehydrogenase (GDH) (Kim et al., 1997; Lin et al., 2006; Patel et al. 2008). GDH enzyme requires pyrroloquinoline quinone (PQQ) as a cofactor, for glucose oxidation to convert glucose to gluconic acid. Katznelson et al. (1962) provided first evidence of Direct Oxidative pathway in MPS. Gluconic acid in periplasmic space undergoes for oxidations to produce 2-ketogluconic acid which is mediated by gluconate dehydrogenase (GADH) (Anderson et al. 1985). Amongst the organic acids secreted into the extracellular medium by bacteria, gluconic and 2-keto gluconic acids are strongest (Duine 1991). These acids act as Ca^{2+} chelators under suitable conditions and attribute for acidification and make free P available, from calcium phosphates such as tri calcium phosphate (TCP) or hydroxyapatite (HAP) (Krishnaraj and Goldstein 2001).

Pseudomonads are significant due to their plant growth promoting and phosphate solubilizing abilities. Under P-limitation, due to compromised metabolic status, intracellular phosphorylative pathway of glucose oxidation in both the pseudomonads was subdued while enhanced direct oxidative pathway which could benefit the metabolic status since GDH activity is directly coupled to electron transfer and generation of proton motive force (van Schie et al, 1985). This may also explain why most of the rhizospheric MPS bacteria employ direct oxidation pathway mediated gluconic or 2-ketocgluconic acid secretion for P-solubilization. Compromised metabolic status of *P. fluorescens* 13525 under P-deficient conditions was overcome in *Pseudomonas* P4 by shifting the metabolism towards direct oxidation pathway producing high gluconic acid levels which facilitated ATP generation as a consequence of improved Pi availability. Metabolic flexibility/rigidity behind gluconic acid secretion in P-solubilizing pseudomonads could facilitate metabolic engineering strategies for enhancing the MPS ability of *Pseudomonas* strains.

Pseudomonads catabolize glucose by two different routes: the direct oxidative pathway which acts on glucose extracellularly and the simultaneously operating intracellular phosphorylative pathway. Early glucose dissimilation studies showed that in most of the pseudomonads glucose oxidation occurs in two successive reactions forming D-gluconate and 2-keto-D-gluconate (2-KG) catalyzed by a membrane-bound PQQ-GDH and gluconate dehydrogenase (GADH) respectively, in the periplasm (Lessie and Phibbs, 1984). Since then direct oxidative pathway has been demonstrated to occur in *Pseudomonas fluorescens* 52-1C (Fuhrer et al., 2005), *P. putida* U (Schleissner et al., 1997), *P. putida* KT2442 (Basu and Phale, 2006) and *P. aeruginosa* 2F32 (Midgley and Dawes, 1973). Although the direct oxidation pathway was not found in *P. putida* KT2440 (Lessie and Phibbs, 1984; Fuhrer et al., 2005), its genome sequence showed the presence of the PQQ-GDH encoding *gcd* gene (Nelson et al., 2002).

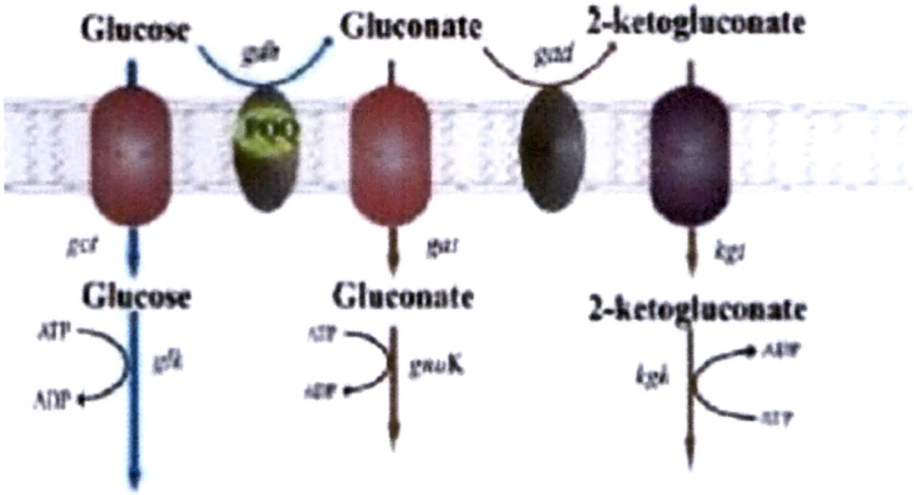


Fig. 1.11: Direct oxidative pathway in pseudomonads

(Archana et al., 2012)

1.8.4: Importance of pyrroloquinoline quinone in abiotic stress.

Hauge (1964) predicted pyrroloquinoline quinone (PQQ) as a bacterial cofactor which was confirmed as methoxatin by Salisbury (1980; 1981) **(Fig. 1.12).** Under proper conditions, PQQ is water soluble and heat stable and can catalyze ~20000 redox cycling (continues oxidation and reduction reactions) events (Rucker et al., 2009) **(Fig. 1.13).**

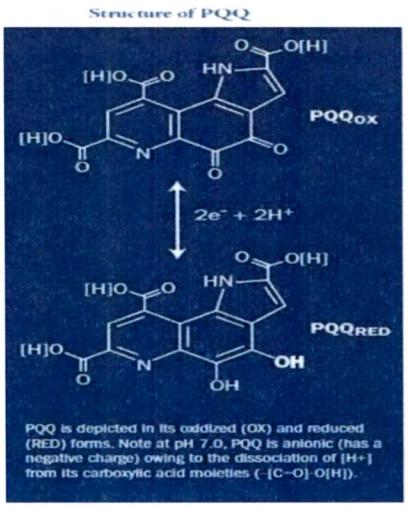


Fig. 1.12: Structure of PQQ (Rucker et al., 2009).

Compound	Potential Number of Catalytic Cycles
PQQ	20,000
Quercetin	800
Catechin	75
Epicatechin	700
Norepinephrine	200
Epinephrine	100
DOPA	20
6-OH-DOPA	20
Ascorbic Acid	4

Fig. 1.13: PQQ as a redox cycling agent.

PQQ biosynthesis in bacteria involves varying number of genes present in clusters (**Fig.1.10**) (Choi et al., 2008). PQQ biosynthesis is not completely understood but a putative biosynthetic pathway has been proposed on the basis of the functions of conserved genes in bacteria (Puehringer et al., 2008). *Klebsiella pneumonia* possesses *pqqABCDEF* genes which are involved in PQQ biosynthesis (Meulenberg et al., 1992). *pqqA* gene encodes for 23-24 amino acid polypeptide which a substrate for a set of enzymes modifying the glutamate and tyrosine residues leading to PQQ formation

(Goosen et al. 1992; Meulenberg et al. 1992; Velterop et al. 1995). PqqB belongs to metallo-β-lactamases and has been suggested to help in the transport of PQQ into periplasm (Velterop et al., 1995). PqqC is a cofactor less oxidase, activates oxygen, and catalyzes the final step of ring closure reaction (Magnusson et al., 2004). PqqD has been shown to interact with PqqE possesses reductively cleavage activity of S-adenosyl methionine to form 5' deoxyadenosine and methionine (Wecksler et al., 2010). PqqE catalyzes the first step of linking glutamate and tyrosine residues of PqqA peptide. Additionally, PqqF, G, H, I, J, K and M are found in some bacteria possessing putative Zn dependent peptidase, non-catalytic subunit of peptidase, transcriptional regulator, aminotransferase, cytosolic protein, DNA binding and prolyl oligopeptidase, respectively (Choi et al., 2008).

Sr/no	Genes	Function
1	*pqqA*	Serves as the necessary precursor substrate for PQQ
2	*pqqB*	Carrier for PQQ and responsible for its transport across the plasma-membrane into the periplasm,
3	*pqqC*	Catalyzes final step of the PQQ biosynthesis,
4	*pqqD*	Interact with *PqqE* possesses reductively cleavage activity
5	*pqqE*	Catalyzes the first step of linking glutamate and tyrosine residues of *PqqA* peptide.
6	*pqqF*	Zn dependent peptidase,
7	*pqqH*	Transcriptional regulator, LysR family
8	*pqqI*	Aminotransferase
9	*pqqJ*	Putative cytoplasmic protein
10	*pqqK*	Probable DNA-binding protein
11	*pqqM*	Peptidase

Table 1.3: Functions of *pqq* genes

(Choi et al., 2008; Puehringer et al., 2008; Wecksler et al., 2010).

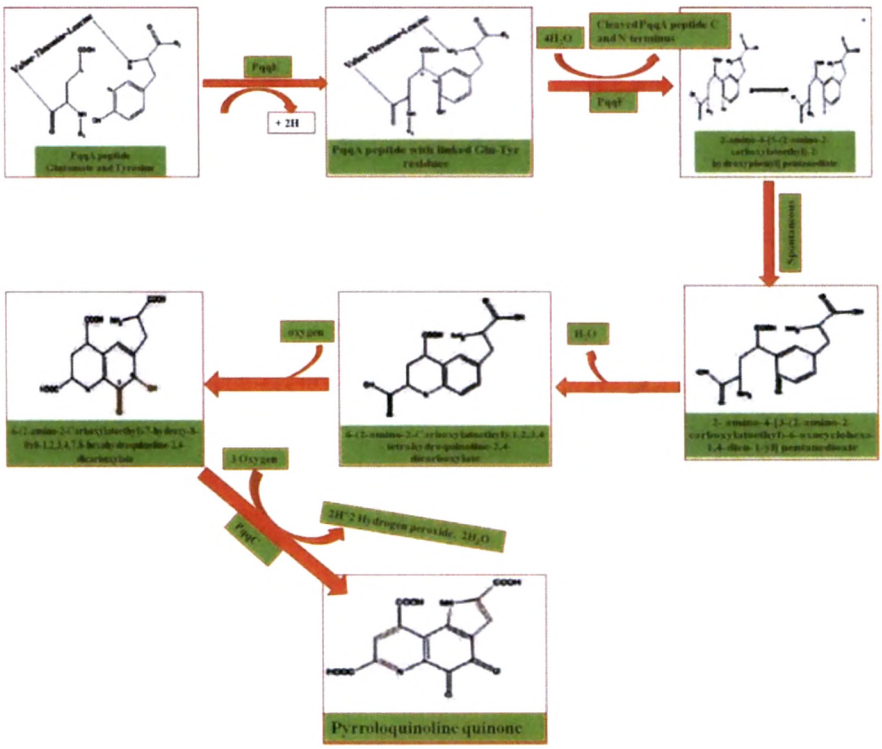


Fig. 1.14: PQQ biosynthesis pathway

(Schwarzenbacher et al., 2008; Caspi, 2010).

PGPR promote plant growth which is mediated by different triats, such as antibiotics or siderophores, phytohormones production, inhibition of pathogenic microorganisms and MPS in soil (Leong, 1986; Sivan and Chet, 1992; Xie et al., 1996; De Freitas et al., 1997). PQQ promote growth in mammals, biological function of PQQ is not fully understood (Smidt et al., 1991; Steinberg et al., 1994). PQQ has been found in various foods in nanogram range but plants and animals do not produce PQQ, and it has notable antioxidant properties (Kumazawa et al., 1992, 1995; Choi et al., 2008). PQQ directly neutralizes reactive oxygen species (ROS) (Misra et al., 2004). PQQ – GDH are involved in production of anti-pathogen compounds (James and Gutterson 1986; Schnider et al. 1995; Han et al. 2008; de Werra et al. 2009; Guo et al. 2009; Ahmed and Shahab, 2010).

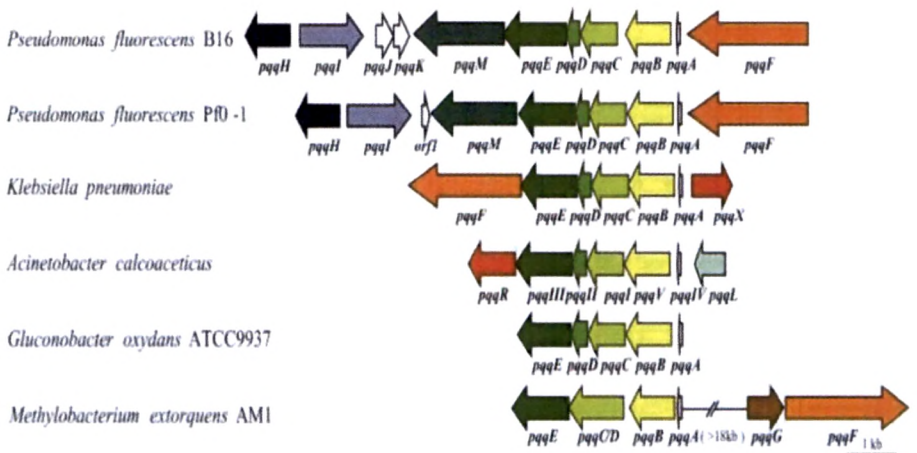


Fig. 1.15: Comparison of the *pqq* gene clusters of *P. fluorescens* B16, P. *fluorescens* Pf0-1, Klebsiella *pneumoniae*, *Acinetobacter calcoaceticus*, *Gluconobacter oxydans* ATCC9937 and *Methylobacterium extorquens* AM1 (Choi et al., 2008).

Species	Pathogenicity	Set of *Pqq* genes
Acinetobacter baumannii ATCC 19606	Opportunistic human pathogen	ABCDE
Acinetobacter hemolyticus ATCC 19194	Rare human pathogen	BCDE
Azoarcus sp. (strain BH72)	Plant symbiont	ABCDEF
Bradyrhizobium japonicum	Plant symbiont	ABCDEF
B*radyrhizobium* sp.(strainBTAi1/ATCC BAA- 1182)	Plant symbiont	ABCDEF
Burkholderia cenocepacia (strain HI2424)	Human cystic fibrosis pathogen	ABCDE
Burkholderia cepacia (strain J2315/LMG 16656)	Animal and plant pathogen	ABCDE
Burkholderia glumae (strain BGR1)	Plant pathogen	ABCDE
Burkholderia multivorans (strain ATCC 17616/ 249)	Animal pathogen in mammals	ABCDE
Burkholderia phymatum (strain DSM 17167/ STM815)	Plant symbiont	ABCDE
Colwellia psychrerythraea (strain 34H/ATCC BAA-681)	Plant pathogen	ABCDE
Cronobacter turicensis (strain DSM 18703/LMG 23827/z3032)	Neonatal pathogen	ABCDEF
Cupriavidus taiwanensis (strain R1/LMG 19424)	Plant pathogen	ABCDE
Dinoroseobacter shibae (strain DFL 12)	Animal symbiont	ABCDEF
Enterobacter intermedius	Opportunistic pathogen	ABCDEF
Enterobacter sakazakii (strain ATCC BAA-894)	Neonatal pathogen	ABCDEF
Erwinia amylovora (strain ATCC 49946/CCPPB 0273/Ea273/27-3)	Plant pathogen	ABCDEF
Erwinia pyrifoliae	Plant pathogen	ABCDEF
Erwinia tasmaniensis (strain DSM 17950/Et1/99)	Plant commensal	ABCDEF
Gluconacetobacter diazotrophicus (strain ATCC 49037/DSM 5601/PAl5)	Plant symbiont	ABCDE
Granulibacter bethesdensis (strain ATCC BAA-1260/CGDNIH1)	Human pathogen	ABCDE
Grimontia hollisae CIP 101886	Human pathogen	BCDE
K. pneumoniae	Opportunistic pathogen	ABCDEF

Klebsiella sp. 1_1_55	Opportunistic pathogen	BCDEF
Marinobacter algicola DG893	Plant symbiont	ABCDE
Methylobacterium nodulans (strain ORS2060/ LMG 21967)	Plant saprophyte and symbiont	ABCDEF
Methylobacterium populi (strain ATCC BAA-705/ NCIMB 13946/BJ001)	Plant endophyte	ABCDEF
Methylobacterium radiotolerans (strain ATCC 27329/DSM 1819/JCM 2831)	Plant symbiont	ABCDEF
Mycobacterium smegmatis [strain ATCC 700084/ mc(2)155]	Commensal in mammals	ABCDE
Pantoea ananatis	Plant pathogen	ABCDEF
Pseudomonas aeruginosa	Opportunistic human pathogen	ABCDEF
Pseudomonas entomophila (strain L48)	Insect pathogen	ABCDEF
Pseudomonas fluorescens	Potential pathogen to birds	ABCDEF
Pseudomonas mendocina (strain ymp)	Rare human pathogen	ABCDEF
Pseudomonas savastanoi pv savastanoi NCPPB 3335	Tumor-inducing pathogen	ABCDEF
Pseudomonas syringae pv phaseolicola (strain 1448A/Race 6)	Plant symbiont	ABCDEF
R. aquatilis	Opportunistic pathogen	ABCDEF
Ralstonia pickettii (strain 12D)	Opportunistic pathogen	ABCDEF
Rhizobium meliloti	Plant symbiont	ABCDE
Rhizobium sp. (strain NGR234)	Plant symbiont	ABCDEF
Rickettsiella grylli	Arthropod pathogen	BCDEF
Serratia marcescens	Opportunistic human pathogen	ABCDE
Serratia odorifera 4Rx13	Opportunistic pathogen	ABCDEF
Verminephrobacter eiseniae (strain EF01-2)	Animal endosymbiont	ABCDEF
Xanthomonas axonopodis pv citri (citrus canker)	Plant pathogen	ABCDE
Xanthomonas campestris pv Campestris	Plant pathogen	ABCDE
Xanthomonas oryzae pv Oryzae	Rice bacterial blight pathogen	ABCDE

Fig. 1.16: Organization of *pqq* genes in bacteria (Shen et al., 2012)

species	pathogenicity	set of Pqq gene
Acinetobacter baumannii ATCC 19606	opportunistic human pathogen	ABCDE
Acinetobacter haemolyticus ATCC 19194	rare human pathogen	BCDE
Azoarcus sp. (strain BH72)	plant symbiont	ABCDEF
Bradyrhizobium japonicum	plant symbiont	ABCDEF
Bradyrhizobium sp. (strain BTAi1/ATCC BAA-1182)	plant symbiont	ABCDEF
Burkholderia cenocepacia (strain HI2424)	human cystic fibrosis pathogen	ABCDE
Burkholderia cepacia (strain J2315/LMG 16656)	animal and plant pathogen	ABCDE
Burkholderia glumae (strain BGR1)	plant pathogen	ABCDE
Burkholderia multivorans (strain ATCC 17616/ 249)	animal pathogen in mammals	ABCDE
Burkholderia phymatum (strain DSM 17167/ STM815)	plant symbiont	ABCDE
Cobetia pacifica (strain 3-H/ATCC BAA-681)	plant pathogen	ABCDE
Cronobacter turicensis (strain DSM 18703/LMG 23827/z3032)	neonatal pathogen	ABCDEF
Cupriavidus taiwanensis (strain R1/LMG 19424)	plant pathogen	ABCDE
Dinoroseobacter shibae (strain DFL 12)	animal symbiont	ABCDEF
Enterobacter intermedius	opportunistic pathogen	ABCDEF
Enterobacter sakazakii (strain ATCC BAA-894)	neonatal pathogen	ABCDEF
Erwinia amylovora (strain ATCC 49946/CCPPB 0273/Ea273/17-3)	plant pathogen	ABCDEF
Erwinia pyrifoliae	plant pathogen	ABCDEF
Erwinia tasmaniensis (strain DSM 17950/Et1/99)	plant commensal	ABCDEF
Gluconacetobacter diazotrophicus (strain ATCC 49037/DSM 5601/PAL5)	plant symbiont	ABCDE
Granulibacter bethesdensis (strain ATCC BAA-1260/CGDNIH1)	human pathogen	ABCDE
Grimontia hollisae CIP 101886	human pathogen	BCDE
K. pneumoniae	opportunistic pathogen	ABCDEF
Klebsiella sp. 1_1_55	opportunistic pathogen	BCDEF

species	pathogenicity	set of Pqq gene
Mesorhizobium alhagi DSM9	plant symbiont	ABCDE
Methylobacterium nodulans (strain ORS2060/ LMG 21967)	plant saprophyte and symbiont	ABCDEF
Methylobacterium populi (strain ATCC BAA-705/ NCIMB 13946/BJ001)	plant endophyte	ABCDEF
Methylobacterium radiotolerans (strain ATCC 27329/DSM 1819/JCM 2831)	plant symbiont	ABCDEF
Mycobacterium smegmatis [strain ATCC 700084/ mc(2)155]	commensal to mammals	ABCDE
Paenibacillus mucilaginosus	plant pathogen	ABCDEF
Pseudomonas aeruginosa	opportunistic human pathogen	ABCDEF
Pseudomonas entomophila (strain L48)	insect pathogen	ABCDEF
Pseudomonas fluorescens	potential pathogen to birds	ABCDEF
Pseudomonas mendocina (strain ymp)	rare human pathogen	ABCDEF
Pseudomonas savastanoi pv savastanoi NCPPB 3335	tumor-inducing pathogen	ABCDEF
Pseudomonas syringae pv phaseolicola (strain 1448A/Race 6)	plant symbiont	ABCDEF
P. aquatilis	opportunistic pathogen	ABCDEF
Ralstonia pickettii (strain 12D)	opportunistic pathogen	ABCDEF
Rhizobium mellioti	plant symbiont	ABCDE
Rhizobium sp. (strain NGR234)	plant symbiont	ABCDEF
Rickettsia gylli	arthropod pathogen	BCDEF
Serratia marcescens	opportunistic human pathogen	ABCDE
Serratia odorifera 4Rx13	opportunistic pathogen	ABCDEF
Verminephrobacter eiseniae (strain EF01-2)	animal endosymbiont	ABCDEF
Xanthomonas axonopodis pv citri (citrus canker)	plant pathogen	ABCDE
Xanthomonas campestris pv Campestris	plant pathogen	ABCDE
Xanthomonas oryzae pv Oryzae	rice bacterial blight pathogen	ABCDE

Fig. 1.16: Organization of *pqq* genes in bacteria (Shen et al., 2012)

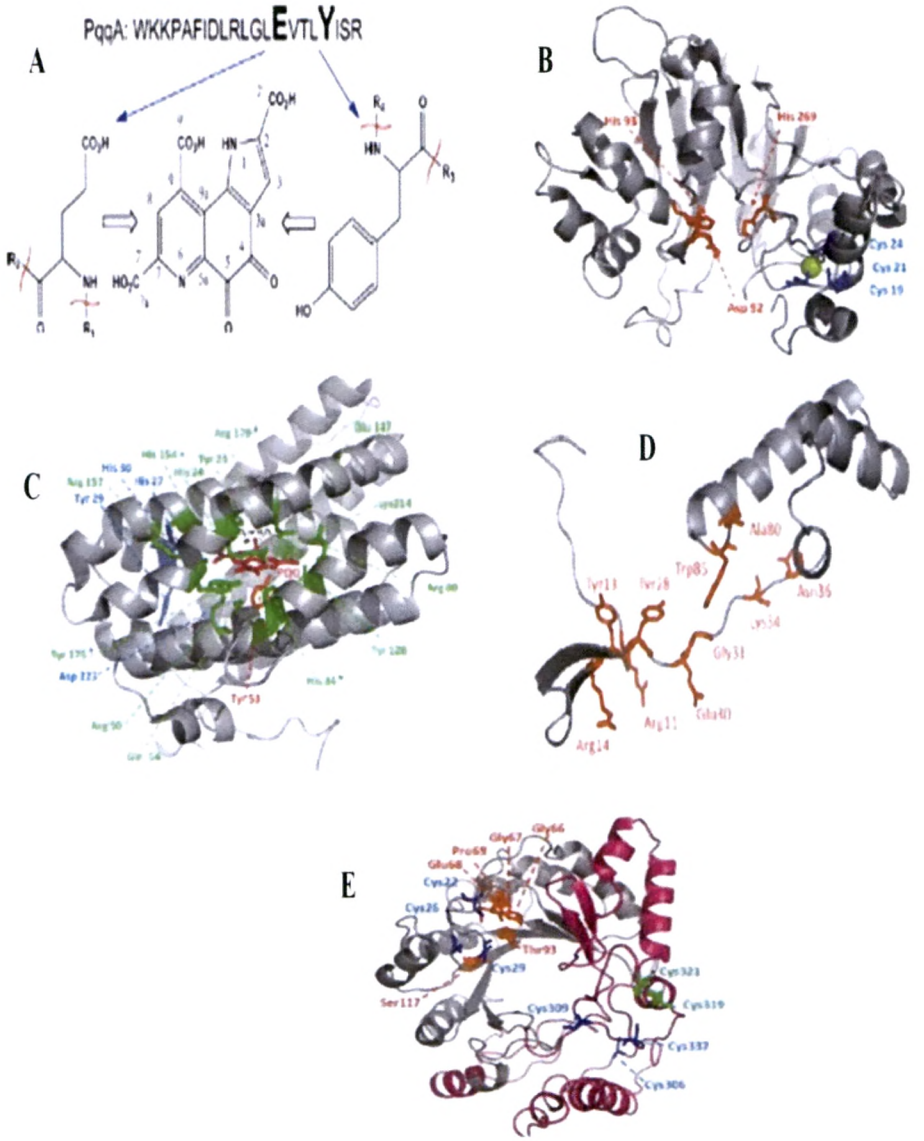


Fig. 1.17: Structures of Pqq genes . A- PqqA, B- PqqB, C- PqqC, D- PqqD and E- PqqE (Shen et al., 2012)

1.9: Citric acid secretion in microorganisms

Citric acid secretion is reported in large number of microorganisms including bacteria, fungus and yeast (Khan et al., 2006). Plants are also known to secrete citrate in root exudates. Bacteria secrete citrate in very low amounts (~ 1 mM) (Gyaneshwar et al., 1998). *A. niger* is known to produce citrate in molar amounts and thus the organism is used to produce citrate at large scale (Pappagiani, 2007).

1.9.1: Citrate synthase

Citrate synthase is a ubiquitous enzyme found in all eukaryotes and in most microorganisms. Its role as a catalyst of the entry point reaction for entry of two-carbon units into the citric acid cycle indicates its importance for organisms with a complete cycle, but even where the complete cycle does not function; it is an essential step in the biosynthesis of amino acids related to glutamate. Some organisms have a single CS, while a few, such as baker's yeast and *Bacillus subtilis*, have as many as three. *E. coli* citrate synthase contain 427 amino acid encoded by *gltA* gene. Most CS molecules are homodimers with a polypeptide chain between 380 and 440 residues in length. *E. coli* citrate synthase contain 427 amino acid encoded by *gltA* gene. Monomeric hexamer has molecular weight 48 KD. Type I CS is found in eukaryotes, archaea and Gram-positive bacteria. Type II CS, found in gram negative bacteria especially in members of *Enterobacteriaceae* family, was first recognized by Weitzman in 1966. Type II CS is a hexameric molecule, found only in Gram-negative bacteria. Its most striking functional property, which clearly distinguishes it from type I enzymes, is that type II CS is strongly and specifically inhibited by NADH via an allosteric mechanism (**Fig. 1.18**) (Weitzman, 1966).

E. coli CS is a mixture of dimers and hexamers of identical subunits, 426 amino acids in length. The dimer–hexamer equilibrium is affected by many factors as pH, ionic strength, and the presence of the allosteric inhibitor, NADH (Tong and Duckworth, 1975; Ayed et al., 1998). Citrate synthase, the ubiquitous enzyme which catalyzes entry

of acetyl-coA carbon into the Krebs cycle, is potentially a key control point for energy production. In eukaryotic and Gram-positive bacteria citrate synthase, is dimer of identical subunits. The more complex citrate synthases of Gram-negative bacteria are allosteric hexamers, displaying specific inhibition by NADH and a sigmoid saturation curve for substrate, acetyl-coA (Weitzman and Danson, 1976; Donald et al., 1991).

Acetyl-coA is enolized by simultaneous deprotonation of the methyl carbon by an aspartic acid and protonation of the carbonyl oxygen by a histidine. The neutral enol then attacks the carbonyl carbon of OAA to form the product; citryl-CoA which is subsequently hydrolyzed to citrate and coenzyme A. OAA is bound in the active site by 5 amino acids including 3 arginines and 2 histidines (Karpusas et al., 1990).

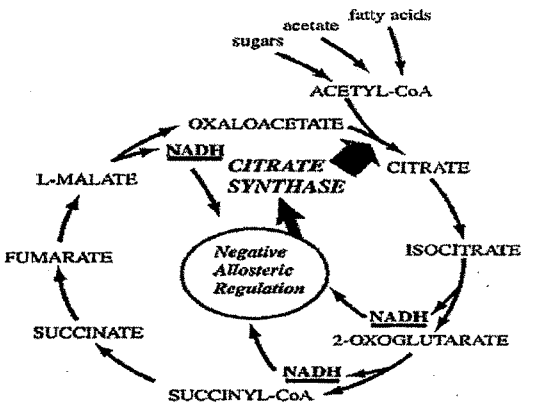


Fig. 1.18: Schematic representation of NADH inhibition of *E. coli* Type II CS (Nguyen et al., 2001).

E. coli citrate synthase loses most of its ability to discriminate between OAA and the inhibitor, α-KG, when the functional group of Arg^{407} was substituted to leucine by mutation (Pereira et al., 1993). *E. coli* citrate synthase is example of type II hexameric CS allosterically inhibited by NADH. Type I and type II subunit have overall similar fold. Type II hexamer is formed by three dimers each one folded in two domain arrangement containing 18 α helices. Presence of a 53 residue novel N-terminal

domain, rich in β sheet is the major structural difference which is absent in type I CS. Also, the length of interhelical loops is different in type I and type II CS.

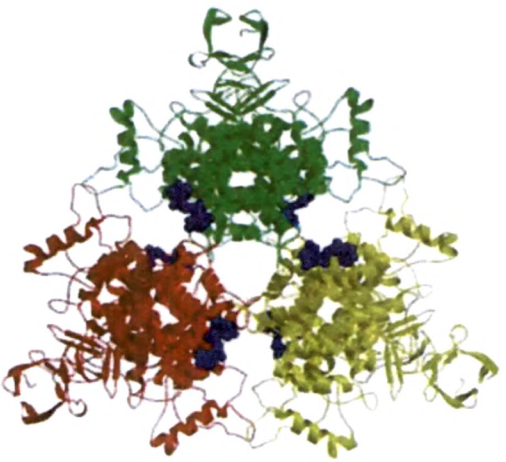


Fig. 1.19: Ribbon structure of NADH bound hexameric *E. coli* F383A type II CS. Green, red and yellow ribbons shows three equivalent dimers of this complex having central 3- fold axis while the location of the six bound NADH molecule (one per subunit) is shown in blue (Maurus et al., 2003).

CS variant in which active site phenylalanine (Phe 383) is replaced by alanine, kinetic properties suggest that the equilibrium between the R (active) and T (Inactive) confirmation strongly shifted to T state. NADH acts as allosteric inhibitor and selectively binds to T state. In CS dimer state NADH occupies approximately six sites making close contact (**Fig. 1.19**). One face of the hexamer having three identical sites arrange with 3-fold symmetry and three more identical site slightly different from first three, on the other face. Pocket of the binding site involved are cationic and bind NADH in an unusual, roughly horseshoe confirmation.

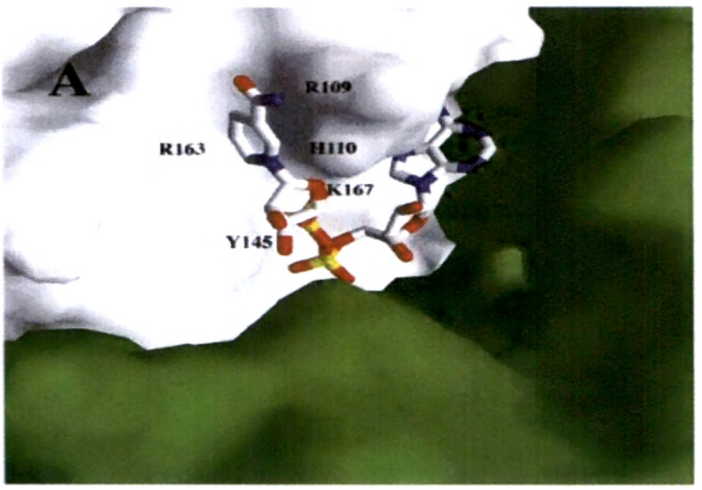


Fig. 1.20: Close-up view of the contour of the enzyme surface in the vicinity of one NADH binding site in hexameric *E. coli* CS (Maurus et al., 2003).

This is significantly different from the Rossman fold where NADH binds to the most common nucleotide binding protein motif and showed extended confirmation. Thus, NADH sites can only formed in hexameric form, by interaction of two different dimers subunits. Most of the binding pocket, including positive charge of pyrophosphate-binding is provided by one subunit and residue from adjacent dimer required to complete formation of the adenine subsite. Arg 163, Arg 109, Tyr 145, Lys 167, His 110, Tyr 204 and Gln 182 are identified as key residues that interact with the NADH. The binding site is made up of structural component having two different subunit which are part of two different enzyme dimers in overall hexameric confirmation of *E. coli* CS (**Fig. 1.20**) (Maurus et al., 2003). Binding site of NADH is distinct from active sites. Thus, NADH inhibition weakened by different condition like salt or alkaline pH, which favors enzyme activity of CS. Treatment with sulfydryl reagent and Ellman's reagent, affects the activity but abolish the NADH inhibition. *E. coli* CS exits in two conformational state i.e. R state which binds acetyl-CoA selectively whereas T state binds with NADH.

R109L variant protein of *E. coli* CS, residue 262-298 refold into a confirmation towards functional acetyl CoA binding site. In wild type and R109L variant CS structure, residue 316-342 form helix turns helix which acts as helical linker. Hexameric CS shows helical linker region (residue 316-341) structurally links refolded active site polypeptide chain segment region (262-298) to an NADH binding site. However, the NADH binding site involved is not from the same subunit, instead being located across the dimer-dimer interface on an adjacent subunit (**Fig. 1.21**).

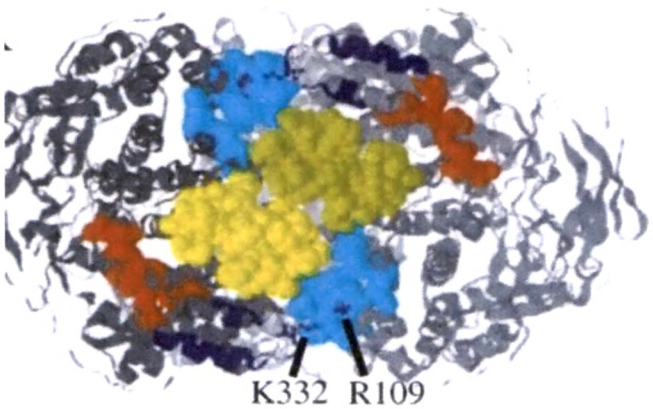


Fig. 1.21: RasMol model of *E. coli* citrate synthase (hexamer). Figure shows perpendicular 3 fold axis that relate dimer subunit and face onto one of the three relatively flat sides. Running through the middle of each side face is a 2-fold axis that relates two NADH binding sites and two active sites (Stokell et al., 2003).

In T (inactive) state in wild type CS, Arg-109 positioned so as to cause high thermal motion in helical linker region. This leads to refolding of polypeptide chain in active site so enzyme unable to bind substrate acetyl CoA. In this condition NADH stabiles hexameric structure of enzyme in this T state confirmation. This indicates the presence of NADH has negligible effect on the segmental mobility and overall folding of the R109L CS protein (**Fig. 1.22**).

In case of wild type CS, switching of R (active) state involves the release of NADH and movement of Arg to alleviate the charge- charge conflict with adjacent residue present in helical linker region. Rigidity of this linker region promote refolding of residue 262-298 form acetyl CoA binding site to catalytic reaction to be happen. This way helical linker region play a role in cross talk between NADH and active site which in close proximity across the dimer-dimer interface in hexameric form of enzyme.

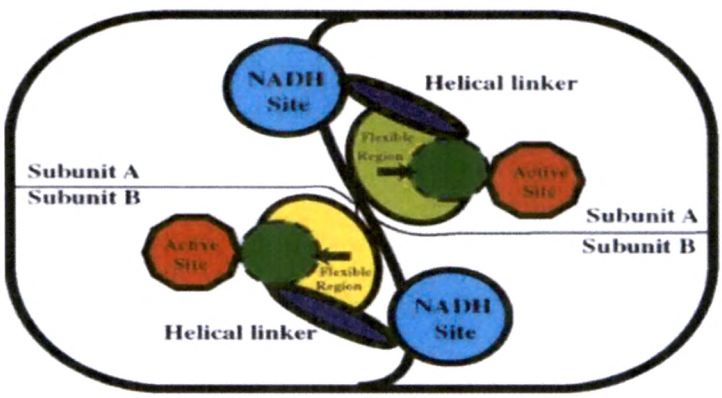


Fig. 1.22: Diagram showing proposed route of communication between NADH binding sites and active sites in the Type II CS of *E. coli* (Stokell et al., 2003).

Replacement of Arg 109 (in the NADH binding site) by Leu is accompanied by substantial changes in position and mobility of two other regions: the "helical linker" (residues 316–342, dark blue) and the "flexible region" (residues 262–298, bright yellow for one dimer, and greenish yellow for the other). A regulatory signal, originating at the NADH binding sites (cyan circles), is transmitted to the 316–342 linker of the other dimer and hence via the flexible 262–298 region to the active site (red decagons; the entry points for the two visible active sites are from the lower left and upper right, respectively). The initial step in this process may be modulated by a charge interaction between Arg and Lys 332. Changes in position and mobility of the 316–342 linkers provide a more suitable surface upon which the flexible 262–298 region can pack. This packing occurs partially in R109L structure leading to refolding a

part of the flexible region to form a binding site (green circle) for the substrate, acetyl-CoA. This mechanism suggests how the six NADH binding sites and six active sites in the Type II CS hexamer might communicate so as to induce either the T (inactive) or R (catalytically active) states that are required for the control exerted by NADH on this system.

Nine amino acids whose side chains were proposed to make hydrogen bonds with the NADH. Functional properties of nine sequence variants, in which these amino acids have been replaced by nonbonding residues, were checked. All of the variants show some changes in NADH binding and inhibition and small but significant changes in kinetic parameters for catalysis. NADH inhibition in three mutants (Y145A, R163L, and K167A) has become extremely weak (Maurus et al., 2003). Rep*lac*ement of Arg163 by Leu is accompanied by substantial changes in position and mobility of two other regions: the "helical linker" (residues 316–342, dark blue) and the "flexible region" (residues 262–298, bright yellow for one dimer, greenish yellow for the other) they propose that a regulatory signal, originating at the NADH binding sites (cyan circles), is transmitted to the 316–342 linker of them other dimer and thence via the flexible 262–298 region to the active site (red decagons; the entry points for the two visible active sites are from the lower left and upper right, respectively). Changes in position and mobility of the 316–342 linker provide a more suitable surface upon which the flexible 262–298 region can pack which induces refolding of part of the flexible region, to form a binding site (green circle) for the substrate acetyl-CoA (**Fig. 1.22**). This mechanism suggests how the six NADH binding sites and six active sites in the Type II *CS* hexamer might communicate so as to induce either the T (inactive) or R (catalytically active) states that are required for the control exerted by NADH on CS activity.

1.9.2: Citrate transporter

Citrate is very abundant in nature and most bacteria have transport proteins in the cytoplasmic membrane for uptake of citrate. The carriers belong to the class of secondary transporters which use the free energy stored in transmembrane electrochemical gradients of ions to drive the uptake of the substrates (Poolman and Konings, 1993). The citrate transporter CitH of *Klebsiella pneumoniae* is driven by the proton motive force and the transporters CitS and CitC of *K. pneumoniae* and *Salmonella* serovars are driven by both the proton motive force and sodium ion motive force (Lolkema, 2006; 2007). Mechanistically these transporters catalyze coupled translocation of citrate and H^+ and/or Na^+ (symport). However, citrate carriers of lactic acid bacteria take up citrate by an electrogenic uniport mechanism or by exchange with lactate (Lolkema, 2006). These citrate transporters are involved in generation of metabolic energy. A number of structural genes coding for citrate transporters have been cloned, and the primary sequences have been deduced from the base sequences (**Table 1.3**).

The 2-hydroxycarboxylate transporter (2HCT) family is a family of bacterial secondary transporters for substrates like citrate, malate and lactate. The 2HCT family of secondary transporters contains 54 unique members that are all found in the bacterial kingdom. The characterized members of the family are transporters for citrate, malate and lactate which are substrates that contain the 2-hydroxycarboxylate motif. The members of the 2HCT family represent different modes of energy coupling like other families of secondary transporters. The transporters are H^+ or Na^+ symporters or they catalyze exchange between two substrates. Mostly under anaerobic conditions, the transporters of the 2HCT family function in citrate and malate breakdown pathways. Na^+ -coupled citrate transporters like CitS of *Klebsiella pneumonia* and CitC of *Salmonella enterica* found in the γ subdivision of the phylum Proteobacteria which are involved in the fermentative degradation of citrate to acetate and carbon dioxide yielding ATP (Lolkema, 2006; 2010).

Table 1.4: Characterized members of 2HCT family (Lolkema, 2006).

Transporter	Bacteria	Substrates	Transport mode	Reference
CitS	*K. pneumoniae*	citrate	Na^+ symport	Dimorth and Thomer, 1986
CitC	*Salmonella typhimurium*	citrate	Na^+ symport	Ishiguro et al., 1992
CitW	*K. pneumoniae*	citrate, acetate	exchange	Kastner et al., 2002
MleP	*Lactococcus lactis*	malate, lactate	exchange	Molenaar et al., 1993
CitP	*Leuconostoc mesenteroides*	Citrate, lactate	exchange	Marty-Teysset et al., 1995
CimH	*Bacillus subtilis*	Citrate, malate	H^+ symport	Krom et al., 2001
MalP	*Streptococcus bovis*	malate	H^+ symport	Kawai et al., 1997
MaeN	*Bacillus subtilis*	malate	Na^+ symport	Wei et al., 2000

The pathway that consists of only exchanger and the decarboxylase results in generatation of proton motive force in the form of both a membrane potential and a pH gradient across the cytoplasmic membrane. Similarly, CitP generates membrane potential by exchange of divalent citrate for monovalent lactate. Secondary transporters are widely distributed in nature and they come in a great genetic and structural diversity which probably reflects many different translocation mechanisms (Sobczak and Lolkema, 2005). The transporters in the 2HCT family are integral membrane proteins consisting of about 440 amino acid residues. The core of the structure is formed by two homologous domains that are connected by a large hydrophilic loop which resides in the cytoplasm. The domains contain 5 transmembrane segments (TMSs) each and they have opposite orientations in the membrane (Lolkema, 2006; 2010).

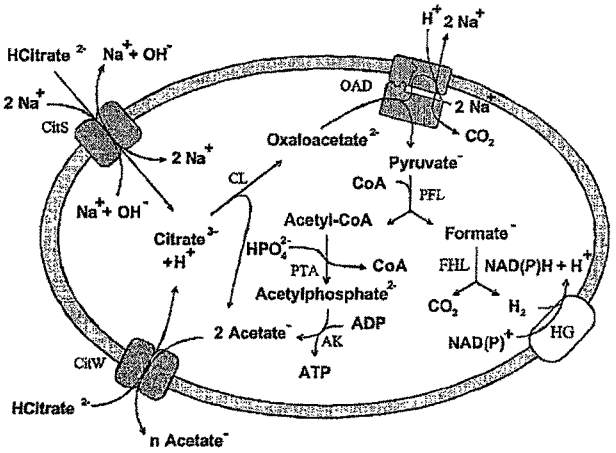


Fig. 1.23: Citrate fermentation pathway in *Klebsiella pneumonia*
(Kustner et al., 2002).

In *K. pneumoniae* citrate is transported into the cell by the Na^+-dependent citrate carrier CitS and the citrate/acetate exchanger CitW. Internal citrate is cleaved by the citrate lyase (CL) to oxaloacetate and acetate. Oxaloacetate is decarboxylated to pyruvate by the oxaloacetate decarboxylase Na^+ pump (OAD). Pyruvate formate lyase (PFL) converts pyruvate to formate and acetyl-CoA. Acetyl-CoA is converted to acetyl-phosphate by phosphotransacetylase (PTA) and further to acetate by the acetate kinase (AK) with ATP generation. Formate is converted to CO_2 and H_2 by formate hydrogen lyase (FHL) and the H is utilized for NAD (P) reduction by membrane-bound hydrogenase (HG) (Steuber et al. 1999). The end product acetate is expected to leave the cell via the citrate/acetate antiporter CitW in exchange for citrate (**Fig. 1.23**) (Kustner et al., 2002).

1.10: Potassium solubilization.

After phosphate, nitrogen potassium (K) is important macronutrient, deficiency of K in soil limits the plant growth and development as it reduces crop yield (Sparks and Huang, 1985; Richardson et al., 1994). Potassium is tightly bound with metals and the non-exchangeable potassium which is unavailable for plant uptake. Majority of three different forms of potassium are present in soil but they are unavailable for plants (Goldstein, 1994). Feldspar are group of rock forming tectosilicates minerals ($KAlSi_3O_8$ - $NaAlSi_3O_8$ - $CaAl_2Si_2O_8$). These tectosilicates contains three end members. Potassium-Feldspar (K-spar) $KAlSi_3O_8$, Albite $NaAlSi_3O_8$ and Anorthite $CaAl_2Si_2O_8$. However solid three types of feldspar, are characterized on the basis of their forms between solid solutions. Solid solution between K-feldspar and albite are alkali feldspar, solid solution between albite and anorthite are plagioclase, and Barium feldspar. Alkali feldspars-Orthoclase (monoclinic) - $KAlSi_3O_8$, Sanidine (monoclinic) - (K,Na) $AlSi_3O_8$, Microcline (triclinic)- $KAlSi_3O_8$, Anorthoclase (triclinic) - (Na,K)$AlSi_3O_8$. Plagioclase feldspars- Albite - $NaAlSi_3O_8$, Oligoclase - (Na,Ca)(Al,Si)$AlSi_2O_8$, Andesine - $NaAlSi_3O_8$ -$CaAl_2Si_2O_8$, Labradorite - (Ca,Na)Al(Al,Si)Si_2O_8, Bytownite - (NaSi,CaAl) $AlSi_2O_8$, Anorthite - $CaAl_2Si_2O_8$. Barium feldspars are monoclinic ; Celsian $BaAl_2Si_2O_8$, Hyalophane (K,Na,Ba) $(Al,Si)_4O_8$ (Lesure, 1968; Anderson and Burnham 1983; Potter, 1991; Bourne, 1994; Kauffman and Van Dyk, 1994; Potter, 1996; 1997; Drever and Stillings 1997; Walther 1997). The K is in various forms of non-exchangeable and exchangeable K in soil solution (Sparks and Huang, 1985; Mutscher, 1995) (**Fig. 1.24**).

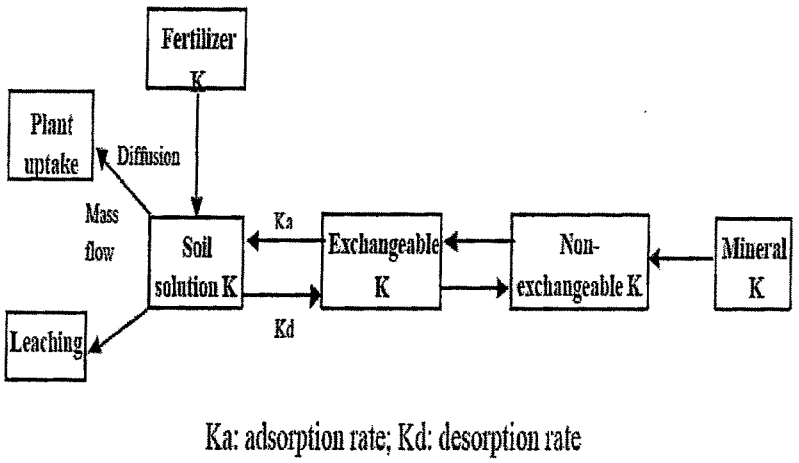


Fig. 1.24: Diagramatic representation of the potassium cycle in soils. Sparks and Huang (1985); Mutscher (1995).

Potassium solubilization in soil is an important aspect for plants. In K cycle, microorganisms play a major key role (Sperberg, 1958). Rhizobacteria solubilizes potassium such as micas, illite, Silicate silicon and aluminum from rock K mineral powder, and make it available for plants. Potassium solubilizing microorganisms (KSB) secrete organic acids which account for K solubilization (Aleksandrov *et al.*, 1967; Sparks and Huang, 1985; Friedrich, 1991; Glick, 1995; Ullman, 1996; Barker *et al.*, 1998; Sheng *et al.*, 2003).). Silicon, aluminium and potassium are solubilized by silicate bacteria (Aleksandrov *et al.*, 1967).

1.11: *Herbaspirillum seropedicae Z67*

Herbaspirillum seropedicae is a gram negative belonging to β-group of proteobacteria, vibrioid, has one to three polar flagellae, endophytic nitrogen-fixer. It grows on organic acids under both N-fixing and non-N-fixing conditions. and is associated with many economically important cereals such as rice, sorghum, sugarcane and maize (Baldani et al., 1986; 1996). This endophyte colonizes efficiently the

intercellular space and vascular tissues of plants, fixes biological nitrogen and promotes growth (Urquiaga et al., 1992). The endophytic bacteria contributes ~40% of total nitrogen fixation from air (Shrestha and Ladha, 1996; Malarvizhi and Ladha, 1999). The genome sequence of *H. seropedicae* strain SmR1 is available (Pedrosa et al. 2011). This bacterium interacts efficiently with host plants, particularly with members of the family Poaceae, and colonizes in intercellular regions of plants. (**Fig. 1.25**).

Isolation *Herbaspirillum* species has been reported form C4-grasses, *Miscanthus sinensis* and *Pennisetum purpureum* (Kirchhof et al. 2001). *Herbaspirillum seropedicae* is found in banana (Musa spp.) and pineapple (*Ananas comosus* (L.) Merril) plants (Cruz et al. 2001; Weber et al. 2001). *Herbaspirillum* colonization in the intracellular tissue of rice plants was confirmed by immunogold labeling, and *gus* infection assay, microscopical analyses of root surfaces showed the presence of bacterium after 10 days of inoculation (Gyaneshwar et al., 2002) (**Fig. 1.26** and **Fig. 1.27**). This endophytic bacterium is associated with dicotyledonous plants, such as *Phaseolus vulgaris* nodules and soybean (Valverde et al. 2003; Kuklinsky-Sobral et al. 2005).

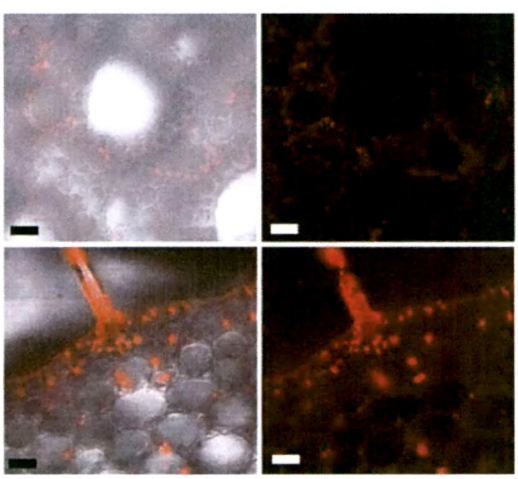


Fig. 1.25: *H. seropedicae* colonization in *Phaseolus vulgaris* (Fabaceae). Transmitted light **(A)** confocal fluorescence microscopy **(B)** cross-section of maize roots (Monteiro et al., 2012).

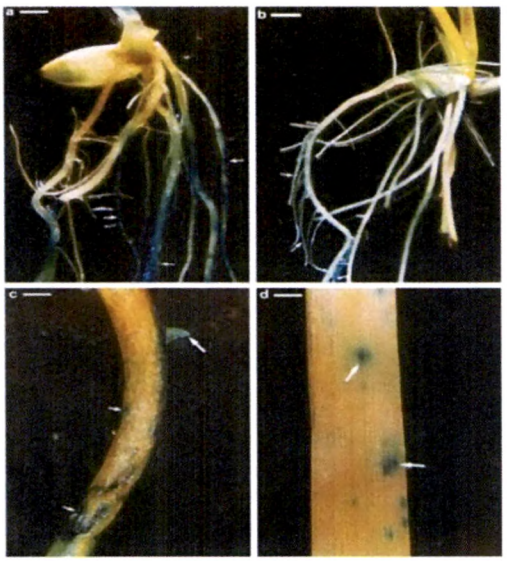


Fig. 1.26: β-Glucuronidase (GUS) staining of rice seedlings with *H. seropedicae* Z67-*gusA* (Gyaneshwar et al., 2002).

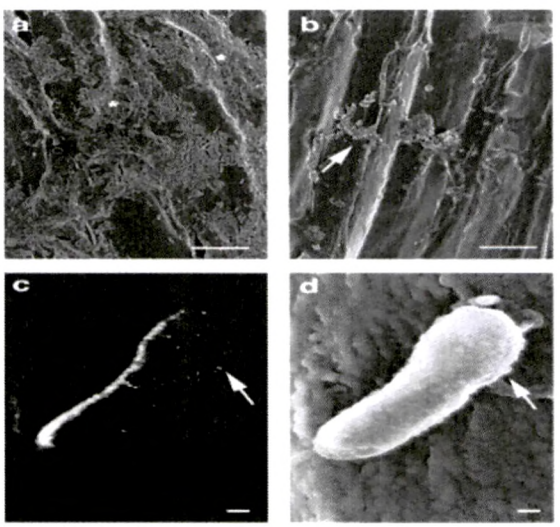


Fig. 1.27: Scanning electron microscope (SEM) images of *H. seropedicae* Z67-*gusA* (Gyaneshwar et al., 2002).

1.11.1: General metabolism

H. seropedicae proficiently grows on monosaccharides such as D-glucose, D-fructose, D-galactose and L-arabinose, but not on oligo- or poly-saccharides (Baldani et al., 1986; 1996). The genome of *H. seropedicae* shows complete set of genes for the Entner-Doudoroff and pentose phosphate pathways but it lacks the Embden-Meyerh off-Parnas (EMP) pathway. It does not possess usual 6-phosphofructokinase (PFK, E.C. 2.7.1.11). To metabolize D-glucose, D-fructose *H. seropedicae* possibly requires the contribution of the Entner-Doudoroff and the pentose phosphate pathways or D-mannose to pyruvate via the EMP pathway. Complete set of genes required for citric acid cycle are present in *H. seropedicae* cycle. The glyoxylate cycle (isocitrate lyase and malate synthase) is intermediates of the pathway and has all genes required for the synthesis of NADH dehydrogenase, succinate dehydrogenase, cytochrome c reductase and for ATP synthase (Pedrosa et al., 2011). *H. seropedicae* genome also encodes genes for utilization of aromatic hydrocarbons and colonization (**Fig. 1.28 and Fig. 1.29**).

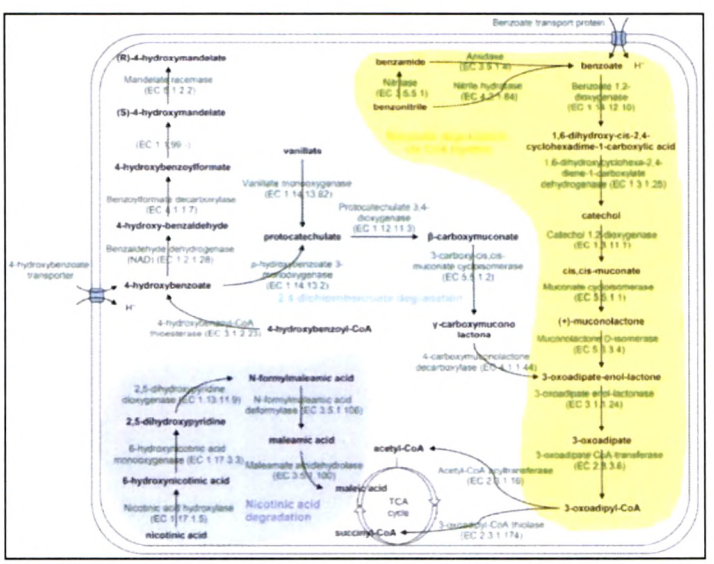


Fig. 1.28: Proposed pathways for aromatic compounds metabolism in *H. seropedicae* SmR1 (Pedrosa et al., 2011).

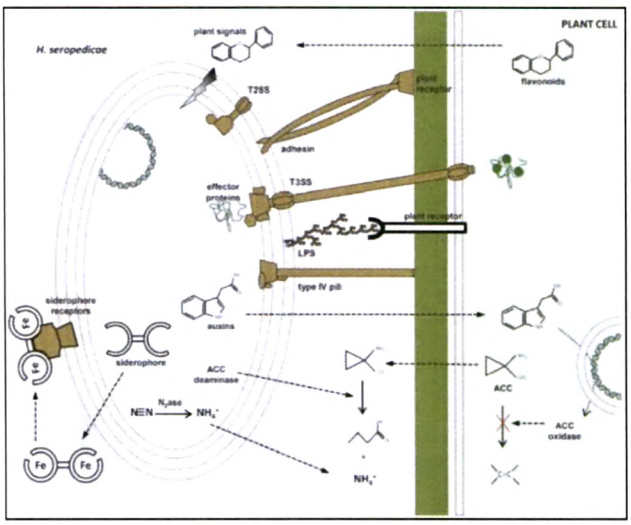


Fig. 1.29: Molecular mechanisms probably involved in plant colonization and plant growth promotion identified in the *H. seropedicae* SmR1 genome

(Pedrosa et al., 2011).

1.11.2: Nitrogen metabolism of *H. seropedicae* Z67.

1.11.3: Nif gene regulation.

The transcription of *nif* genes in *H. seropedicae* is operated by four promoters, which gets activated by the NifA protein, a bacterial enhancer binding protein (bEBP) dependent on the RNA polymerase sigma factor, σ54. Common mechanism is followed to activate transcription by this family of proteins: the RNA polymerase- σ54 holoenzyme binds to promoter DNA which forms closed complex which is incapable of proceeding for transcription (**Fig. 1.30**). The *nif* genes expression requires NifA as transcriptional activator, in *H. seropedicae* which is depend on the levels of fixed nitrogen and oxygen. Moreover, Ntr system regulates nitrogen metabolism and controls *nif*A expression (Chubatsu et al., 2012).

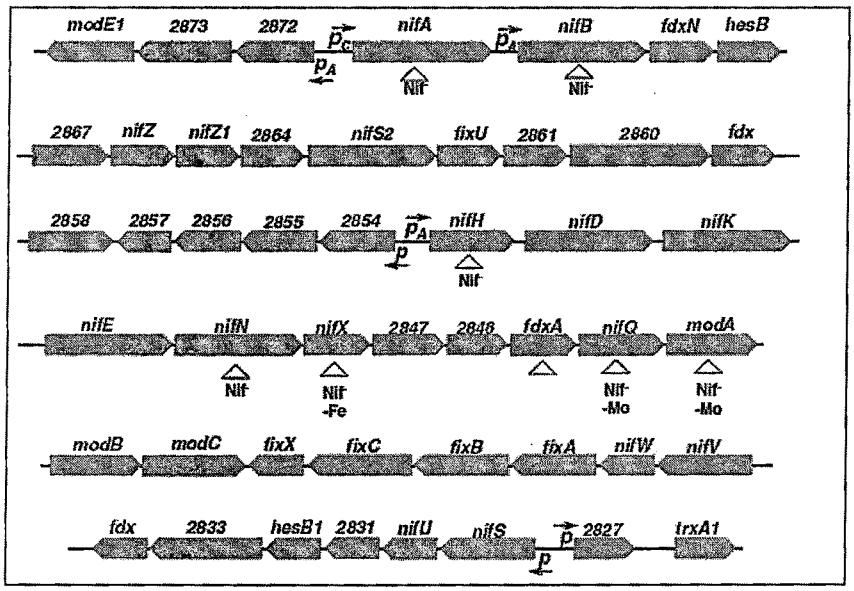


Fig. 1.30: Structural organization of the *nif* and *fix* gene cluster in the *H. seropedicae* genome (Chubatsu et al., 2012).

1.11.4: Post-transcriptional control of nitrogenase in *H. seropedicae*

Constitutive expression of *glnB* is regulated by σ70-dependent promoter. During excess levels of fixed nitrogen condition, the deuridylylation of GlnB is done by GlnD which induced by glutamine. In such status, NtrB is known for dephosphorylation of NtrC and accordingly inactivates NtrC. Even NtrB is involved in catalyzing phosphorylation of NtrC, during nitrogen-fixing conditions; it helps in activation of transcription of *nif*A, *nlm*A, *gln*K, *amt*B operon. Under this situation, GlnD uridylylates proteins GlnB and GlnK. Further, GlnK in uridylylated form activates NifA which activates other *nif* genes required for active nitrogenase (Chubatsu et al., 2012) (**Fig. 1.31**).

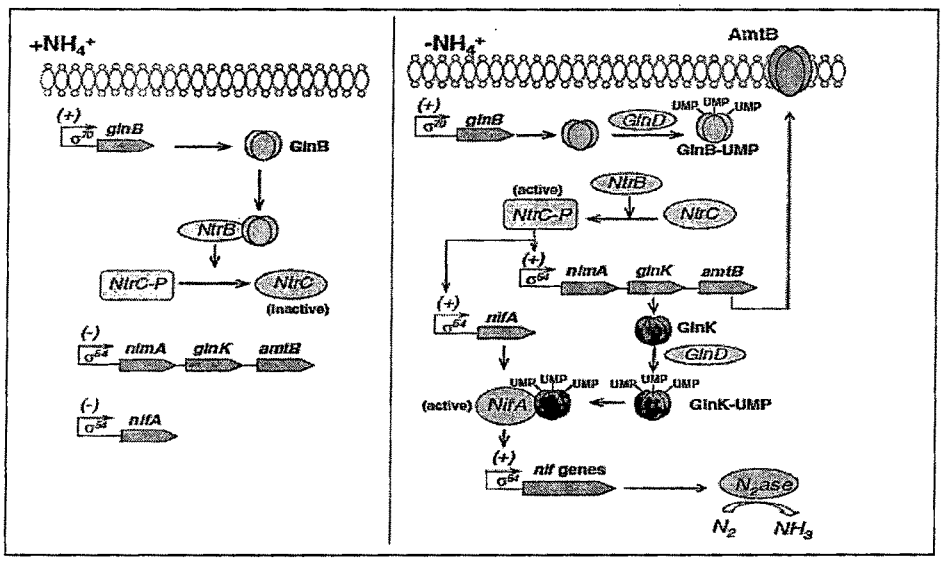


Fig. 1.31: Model of transcriptional regulation of nitrogen fixation in *H. seropedicae* (Chubatsu et al., 2012).

1.11.5: Post-translational control of nitrogenase in *H. seropedicae.*

Nitrogen fixation is an energy-consuming process; it controls the level of *nif* transcription and by operating the activity of nitrogenase. This post-translational regulation is depending on fixed nitrogen and the utilization of energy which leads to inactivation of nitrogenase activity. Nitrogenase activity gets inhibited, as the extracellular concentration of ammonium increases. Upon utilization of supplemented ammonium, the activity of nitrogenase is restored. Uridylylated GlnK maintains its cytosolic form under nitrogen-fixing conditions. Under excess ammonium concentration, GlnK gets deuridylylated by GlnD and binds with AmtB in the membrane and nitrogenase is inactivated (Chubatsu et al., 2012) (**Fig. 1.32**).

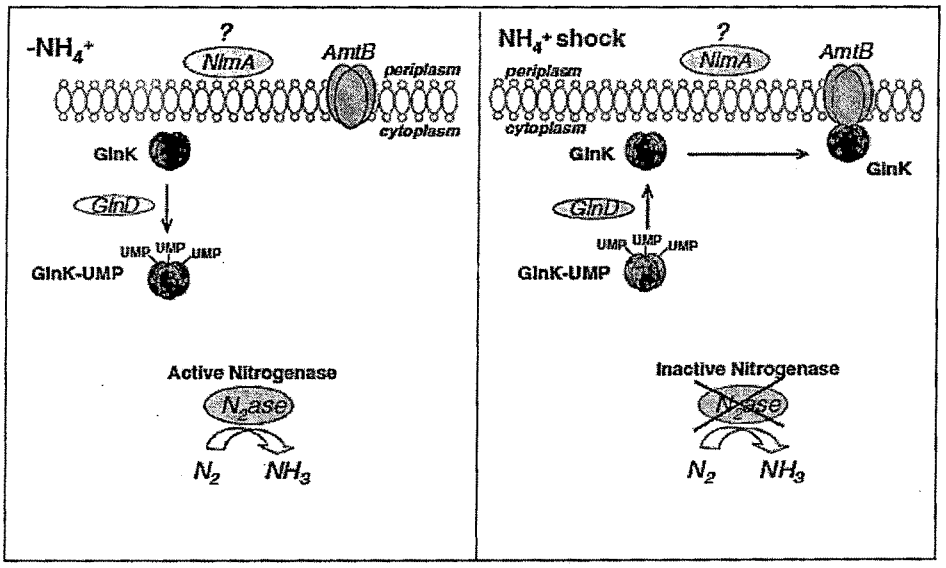


Fig. 1.32: Model of posttranslational control of nitrogen fixation in *H. seropedicae.* (Chubatsu et al., 2012).

1.12: Rational of study.

Biological nitrogen fixation is of tremendous importance to the environment and to world agriculture. Nitrogen fixation is an important part of the nitrogen cycle as it replenishes the overall nitrogen content of the biosphere and compensates for the losses that are incurred owing to denitrification. The availability of fixed nitrogen is frequently the limiting factor for crop productivity, making demands on global agriculture to provide food security as the world's population increases in the twenty-first century. The increased use of chemical fertilizers, which constitutes the largest human interference in the nitrogen cycle, has prompted concerns regarding the increased emissions of nitrogen oxides, soil acidification, and water eutrophication. The fixed nitrogen that is provided by biological nitrogen fixation is less prone to leaching and volatilization as it is utilized *in situ* and therefore the biological process contributes an important and sustainable input into agriculture. **Diazotrophs** are found in a wide variety of habitats including free-living in soils and water, associative symbioses with grasses, symbiotic associations in termite guts, cyanobacterial symbioses with various plants and root–nodule symbioses with legumes. Endophytes are the most efficient microorganisms which benefit the plant by colonizing whole plant and fixes nitrogen in micro aerobic condition. The nitrogen fixation is energy dependent process which requires upto 40 mol of ATP to fix 1 mol of nitrogen, also it is oxygen sensitive. Energy production is depend on the availability of major nutrients and phosphorous is a key nutrient.

Next to nitrogen, phosphorus (P) is the second major nutrient required for plant development and its availability determines the plant growth and crop yields. On an average, most mineral nutrients in soil solution are present in millimolar amounts; however, P is present only in micromolar quantities. This P is present in the soil as organic and inorganic phosphates. Inorganic P in acidic soils is associated with Al and Fe compounds whereas Ca phosphates are the predominant form of inorganic phosphates in calcareous soils. Thus, majority of the P is complexed with metal ions rendering it insoluble and thus unavailable for the plant uptake. Moreover, fixation reactions in soil may allow only a small fraction (10–15%) of the P in fertilizers to be

taken up by plants in the year of their application. Consequently, farmers are advised to apply several times more phosphorus than the plant needs. Excessive application of P causes environmental and economic problems.

Plant growth-promoting rhizobacteria (PGPR) are soil and rhizosphere bacteria that can benefit plant growth by different mechanisms. Negative environmental impact of chemical fertilizers and their increasing costs necessitated the use of PGPR as natural fertilizers for the development of sustainable agriculture. PGPR are known to exhibit phosphate solubilizing ability and provide soluble phosphates to plants. Organic acids secretion, such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, 2-ketogluconate, etc., is the major mechanism responsible for contributing to this P-solubilizing ability of PGPR. Solubilization of mineral phosphates by rhizobacteria has been extensively studied using glucose as the carbon source. Gluconic acid is one of the prominent organic acids responsible for P solubilization. Citric acid is strong acid and most effective for phosphate solubilisation (Gyaneshwar et al., 1998; Srivastava et al., 2006). High concentration of oxaloacetate (OAA) and acetyl-CoA are required for citric acid biosynthesis by the citrate synthase (CS). CS controls the carbon flux into the tricarboxylic acid (TCA) cycle which produces the cellular energy and biosynthetic precursors (Park et al., 1994). Previously our laboratory study showed that overexpression of *Escherichia coli* citrate synthase gene in *Pseudomonas fluorescens* ATCC 13525 enhanced citric acid production and phosphate solubilisation (Buch et al., 2009). However, citric acid levels could be regulated by NADH dependent inhibition of CS activity and citrate transport for secretion across the plasm membrane.

Gluconic acid is produced by direct oxidation of glucose *via* membrane bound quinoprotein glucose dehydrogenase (GDH) enzyme. The GDH enzyme is known to exhibit broad substrate specificity in some organisms in that it can also convert other aldose sugars such as xylose, galactose, maltose, etc. apart from glucose to their corresponding aldonic acids which can also bring about efficient P solubilization. Gluconic acid can further be oxidized to 2-ketogluconic acid by another periplasmic enzyme gluconate dehydrogenase (GAD).

Phosphate solubilizing microorganisms (PSM) have been reported to overcome the problem of phosphate deficiency of plant growth. Many microorganisms found to solubulise phosphate from soil by organic acid secretion, mainly by gluconic acid. 2-ketogluconic acid is a stronger acid than gluconic acid and thus more efficient in solubilizing mineral phosphates. *Herbaspirillum seropideace* Z67 can utilize both mono and di-saccharides but does not possess the ability to solubilize mineral phosphates. Glucose dehydrogenase (GDH) requires the pyrollo quinolinequinone (PQQ) as a cofactor. *H. seropideace* Z67 genome encodes for GDH apoprotein but not for *pqq* biosynthesis genes. Thus, overexpression of *pqq* synthase and gluconate dehydrogenase (*gad*) genes in *H seropideaceZ67* could produce 2-ketogluconic acid.

Metal toxicity on plant is mainly discussed topic by scientist (Brown and Jones 1975). As nutrient function of cadmium is not yet clearly understood, but it is highly toxic metal due to its high mobility even at low concentration it shows adverse effect on plants and microorganisms (Niess 1999; Barceli'o and Poschenriender 1990). Cd affects the chlorophyll biosynthesis and reduces the enzyme efficiency for CO_2 fixation (Stobart et al., 1985; De Fillips and Ziegler 1993). Cd effect on plant results directly decrease in roots leaves and yield production (Blum 1997). Recently rhizobacteria are used for the control of plant pathogens (Bloemberg and Lugtenberg, 2001). Some rhizobacteria have the ability for producing antimicrobial products and metabolites, including hydrogen cyanide (HCN) (Haas and Keel, 2003). Overexpression of *Serratia pqq* biosynthesis genes in *E. coli* showed antifungal activities against *M. grisea* (Yong et al., 2006). Similarly, an antibacterial compound which requires *pqq* and *gdh* genes has been identified in *Rahnella aquatilis*.

Solubilization of mineral phosphates by microbes has been well established under laboratory conditions; however, their efficacy in the field studies has been highly variable. This variability has hampered the large-scale use of PSB in agriculture. Many reasons have been suggested for this variability, but none of them have been extensively

investigated. Root exudates are known to serve as a source of reduced carbon compounds which are released in the rhizosphere. Microbes in the rhizosphere utilize root exudates as nutrients and this forms the major basis for rhizosphere colonization

1.13: OBJECTIVES OF THE PRESENT STUDY

The objectives of the present study were:

1. Monitoring the MPS ability of *H. seropidecae* Z67 containing *E. coli* NADH insensitive citrate synthase (Y145F) - *Salmonella typhimurium* Na^+ dependent citrate transporter (*citC*) operon.

2. Monitoring the MPS ability of *H. seropedicae* Z67 harboring *pqq* gene clusters.

3. Determining the MPS ability of *H. seropidecae* Z67 containing *pqq* cluster and *gad* genes.

4. Determining the alleviation of Cd toxicity and biocontrol abilities of *H. seropedicae* Z67 harboring *pqq* gene clusters in rice plants.