Chapter 1

Review of Literature and Introduction

1.1 Rhizobia: The master microbe

Nitrogen fixing bacteria in legume nodules collectively designated as rhizobia have been known since 1888 (Quispel, A.1988). They were the first biofertilizers produced and allowed savings of millions of dollars in chemical fertilizers that may contaminate soil and water (Hungria et al 2006). In 1888, Beijerink obtained the first pure bacterial culture named by him *Bacillus radicicola* from nodule suspension. This isolate could nodulate *Pisum* and *Vicia* was later renamed as *Rhizobium leguminosarum* (Frank, 1889). The description of the first rhizobial species was mainly based on the legume, which acted as host. This fact led to the definition of the cross-nodulation groups, which was based on the nodulation specificity after infectivity experiments in several legumes (Baldwin and Fred, 1929).

1.1.1: General Taxonomy

The current taxonomy of rhizobia consists of several genera in the subclass Alpha- and Beta- Proteobacteria. *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Azorhizobium*, *Methylobacterium*, *Bradyrhizobium*, *Phyllobacterium*, *Devosia* and *Ochrobactrum* are genera that belong to rhizobial Alpha-Proteobacteria. Rhizobial Beta-Proteobacteria includes the following genera: *Burkholderia*, *Herbaspirillum* and *Cupriavidus* (Weir, 2012). This classification is based on taxonomically important strains that may not necessarily be important reference strains for legume growth improvement. Rhizobial strains commonly used in inoculants have good field performance and stability of symbiotic properties in culture, but are not necessarily well documented or used in taxonomy or molecular biology studies (Lindström et al., 2010).

1.1.2: Host Specificity and Nodulation

The legume-rhizobia association is specific (each rhizobial strain establishes a symbiosis with only a limited set of host plants and *vice versa*). Thus, a restricted number of inoculants fit with a leguminous plant and farmers need to be familiar with the

suitability of inoculants for application according to the characteristics of plant and soil (Mabrouk and Belhadj, 2010). Plants mutually compatible with the same species of rhizobia were listed in earlier years in so-called *cross inoculation groups* (**Table 1.1**).

Table 1.1: Cross-inoculation group and *Rhizobium*-legume association (Morel et al., 2012)

Rhizobia	Legume Cross-inoculation group
Ensifer meliloti	Alfalfa Group: alfalfa (*Medicago sativa*), sweet clover (*Melilotus* spp.) (yellow and white), fenugreek (*Trigonella* spp.)
R. leguminosarum bv *trifolii*	Clover Group (Clover I, II, III and IV): clovers (*Trifolium* spp.)
B. japonicum	Soybean Group: soybean (*Glycine max*)
Bradyrhizobium spp.	Cowpea Group: pigeon pea (*Cajanus cajan*); peanut (*Arachis hypogaea*); cowpea, mungbean, black gram, rice bean (*Vigna* spp.); lima bean (*Phaseolus lunatus*); *Acacia mearnsii; A. mangium; Albizia* spp.; *Enterlobium* spp., *Desmodium* spp., *Stylosanthes* spp., Kacang bogor (*Voandzeia subterranea*), *Centrosema* sp., winged bean (*Psophocarpus tetragonolobus*), hyacinth bean (*Lablab purpureus*), siratro (*Macroptilium atropurpureum*), guar bean (*Cyamopsis tetragonoloba*), calopo (*Calopogonium mucunoides*), puero (*Pueraria phaseoloides*)
R. leguminosarum bv *viciae*	Pea Group: peas (*Pisum* spp.), lentil (*Lens culinaris*), vetches (*Vicia* spp.), faba bean (*Vicia faba*)
R. leguminosarum bv *phaseoli*	Bean Group: beans (*Phaseolus vulgaris*), scarita runner bean (*Phaseolus coccineus*)
Mesorhizobium loti	Chickpea Group: chickpea (*Cicer* spp.), Birdsfoot trefoil (*Lotus corniculatus* L.)
Rhizobium lupini	Group Lupines
Rhizobium spp.	Crown vetch

The specificity of symbiotic interactions is achieved by exchange of molecular signals. In the early steps of symbiosis, a diverse array of compounds is exuded into the rhizosphere, including flavonoids, isoflavonoids, and non-flavonoid inducers (Peters et al. 1986; Caetano-Anolle´s et al., 1988; Dharmatilake and Bauer 1992; Spaink et al., 1991, 1995; Hungria and Stacey 1997).

Molecular determinants of host specificity during nitrogen-fixing symbiosis are depicted in **Fig. 1.1** (Wang et al., 2012) – (i) the plant produce flavonoid signals (such as the luteolin from *M. truncatula*) to free-living soil bacteria, activating the bacterial NodD proteins. NodD proteins, bind to the conserved *nod*-box in the promoters of bacterial nodulation genes to induce their expression.

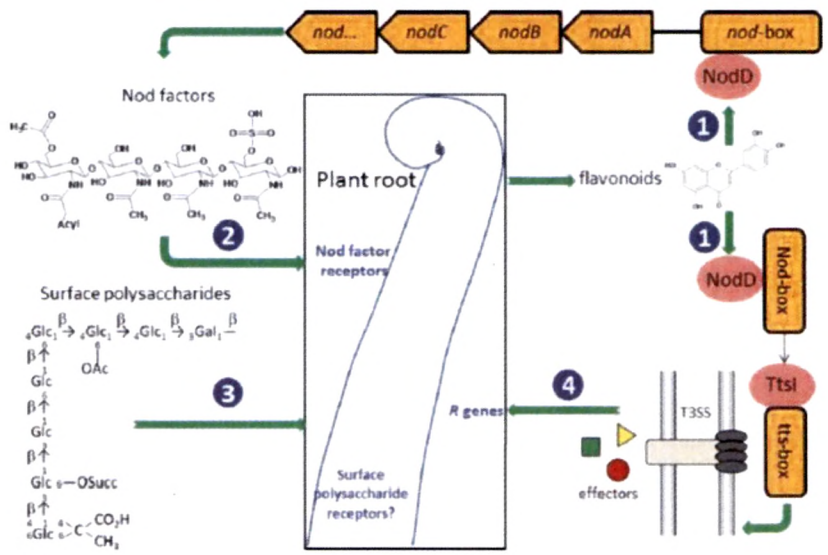


Fig. 1.1: Molecular determinants of host specificity during nitrogen-fixing symbiosis (Wang et al., 2012).

(ii) The *nod* genes code for enzymes for the synthesis of Nod factors, which are recognized on the plant surface by transmembrane Nod factor receptors in a strain- and ecotype-specific fashion. Modifications on the Nod factor such as the length and

saturation of the acyl group determine host specificity. Activation of Nod factor receptors triggers growth changes in the root hair to trap a small number of bacteria, which would give rise to the entire population colonizing the resulting nodule. (iii) Possibly downstream of Nod factors, rhizobia also use their surface polysaccharides (such as EPS from *S. meliloti*, depicted) to modulate host range. The plant receptor(s) are unknown, but may resemble animal receptors for surface polysaccharides from bacterial pathogens. (iv) In certain rhizobial strains, NodD also induces the expression of *TtsI*, which codes for a transcriptional regulator that binds to highly conserved promoter elements, called *tts* boxes, upstream of operons encoding the type III secretion machinery and effectors. Recognition of these effector proteins by *R* genes present in some varieties of plants limits host range.

The legume secretes flavonoids which induce the rhizobia to produce Nod factors and attract them to the plant root hair cells **(Fig. 1.2)** (Haag et al., 2012). Nod-factor signaling triggers a number of developmental changes, including root hair curling which traps the rhizobia in Shepherd's crooks. Inward growth of the root hair tip results in tubular structures called infection threads, which allow the rhizobia to enter the cortical cell layers of the plant root. The rhizobia escape the infection thread and are taken into the host cell *via* an endocytosis-like process **(Fig. 1.2, c-1),** forming vesicles composed of host-derived membrane and are known as symbiosomes. In legumes of the Inverted Repeat-Lacking Clade (IRLC) (consisting of legumes such as *Medicago, Pisum, Vicia, Trifolium, Galega* and *Astragalus*), the rhizobia are challenged with nodule-specific cysteine-rich peptides (NCR) peptides **(Fig. 1.2, c-2),** and differentiated into elongated bacteroids **(Fig. 1.2, c-3).** The bacterial BacA protein is essential for protecting the rhizobia against the antimicrobial activity of NCR peptides **(Fig. 1.2, c-2).** In contrast, BacA is dispensable for rhizobia infecting legumes of the phaseoloid clade that do not produce NCR peptides. In these host plants, rhizobia do not differentiate terminally and often multiple bacteroids can be found inside a single symbiosome membrane.

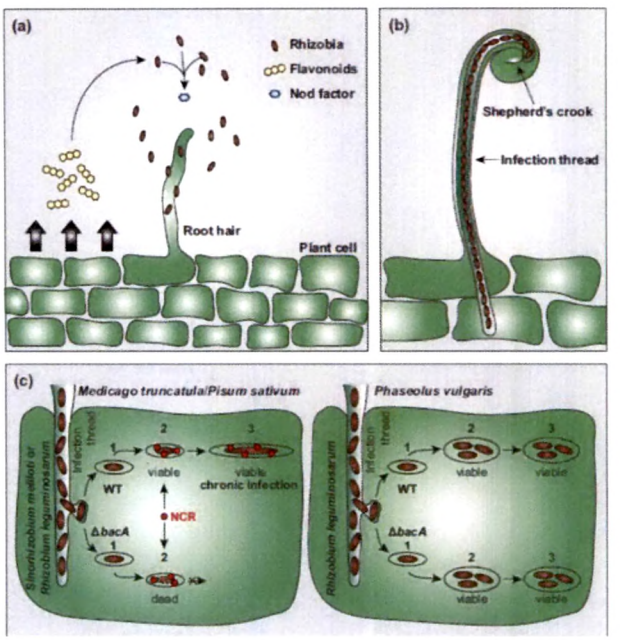


Fig. 1.2: Rhizobia interacting with legumes (Haag et al., 2012). (WT: wild-type).

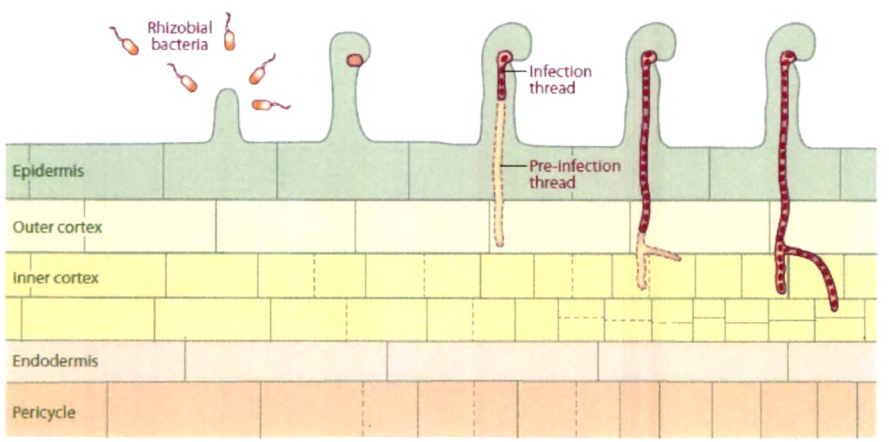


Fig. 1.3: Nodulation involves the coordinated development of bacterial infection and nodule organogenesis (Oldroyd et al., 2011). Cell division (*indicated with dotted lines*) in the inner or mid-cortex and pericycle is initiated early in the interaction between the root and rhizobial bacteria and precedes the initiation of infection events. Bacteria are entrapped in a curled root hair, and from this site infection threads (ITs) are initiated. The route of the IT is predicted by pre-infection threads that are densely cytoplasmic

subdomains with aligned cytoskeleton. Its progress into the inner cortex where the nodule primordium has formed is through a series of cell divisions. From these divided cells, the nodule meristem forms.

Depending on the host plant, changes in rhizobial metabolism are accompanied by changes in rhizobial cell size and shape and three distinct types of bacteroids (Vasse et al., 1990; Mergaert et al., 2006; Bonaldi et al., 2011; Haag et al., 2011b). The first type of bacteroids, develop in legumes of the IRLC. Infecting rhizobia undergo repeated rounds of genome amplification, increase their cell size by elongation and can even be branched **(Fig. 1.2 c)** (Bisseling et al., 1977; Paau et al., 1979; Kobayashi et al., 2001; Mergaert et al., 2006). Bacteroids that undergo such type of metamorphosis are terminally differentiated and are no longer viable once extracted from the nodule (Mergaert et al., 2006). The second type of bacteroids is found in legumes such as those of the Dalbergoid clade (*Aeschynomene* and *Arachis* species). Bacteroids are enlarged by either elongation as for example in *Aeschynomene afraspera* or by forming large spheres as in *Arachis, Aeschynomene indica* or *Aeschynomene evenia* (Sen and Weaver, 1984; Bonaldi et al., 2011). In contrast, the third type of bacteroids is found in rhizobia that infect phaseoloid legumes (i.e. *Phaseolus, Vignia,* Lotus and Glycine species). In this case, rhizobia do not undergo terminal differentiation and retain a cell shape and size and DNA content similar to free-living bacteria (**Fig. 1.2c**) (Bisseling et al., 1977; Paau et al., 1979; Mergaert et al., 2006). *Rhizobium* strains nodulate legumes of different clades and adopt a bacteroid morphotype according to the host, it was concluded that bacteroid metamorphosis was induced by host factors rather than being encoded in the bacterial genome (Sen and Weaver, 1984; Mergaert et al., 2006; Bonaldi et al., 2011). A class of nearly 600 genes encoding nodule-specific cysteine-rich peptides (NCRs) has been discovered and the expression of more than 300 NCRs confirmed (Mergaert et al., 2003; Graham et al., 2004; Young et al., 2011).

The list of metabolites produced by symbiotic enhancers is as follows: vitamins that may supplement the nutritional requirement of rhizobia (Marek-Kozaczuk and

Skorupska, 2001); hydrolytic enzymes that assist during rhizobial penetration in the root hair, or attack phytopathogenic fungi (Sindhu et al., 2002; Egamberdieva et al., 2010); or P-solubilizing acids that increase phosphorus availability (Elkoca et al., 2008). The use of Nitragina, as a donor of effective *Rhizobium,* for pea plants resulted in slightly higher Green Area Index values (Zając et al., 2013).

The growth of rhizobial infection threads (ITs) in root hairs may be further understood by the given model **(Fig. 1.3; Fig. 1.4)** (Oldroyd et al., 2011). Nod factors (NFs) produced by rhizobia within root hairs are perceived by an NF receptor complex that resides in signaling microdomains at the infection thread apex. One of the mechanisms may be that NFs induce the production of reactive oxygen species (ROS). Although their role is not clear, NADPH oxidases is required for both pollen tube and root-hair growth, so they may play an analogous role in IT growth. One of the main roles of ROS is the activation of calcium channels. The cytoskeleton is also essential for IT growth. Microtubules help to determine the direction of growth at the site of Ca^{2+} influx and actin provides the infrastructure for vesicle delivery at the site of growth root hairs For example, F-actin, which is found in the sub-apex of growing root hairs, along with microtubules participate in the recycling and endocytosis of PM sub domains, plays a key role for the cytoskeleton in signaling. Small G proteins also play crucial roles in support of tip growth. The proximity of the nucleus to the site of root-hair growth is significant, and its movement is mediated by actin. This invagination is associated with bacterial infection, and the promotion of cell division in the cortex leading to the nodule meristem. The nodules and associated symbiosomes are structured for efficient nitrogen fixation (Oldroyd et al., 2011). Infection thread is major key and check point for selection of competitive rhizobia, mutation in LPS and EPS which are cell surface components failed to be released IT, which fails to show symbiosis of rhizobia to plant (Gibson et al., 2008)

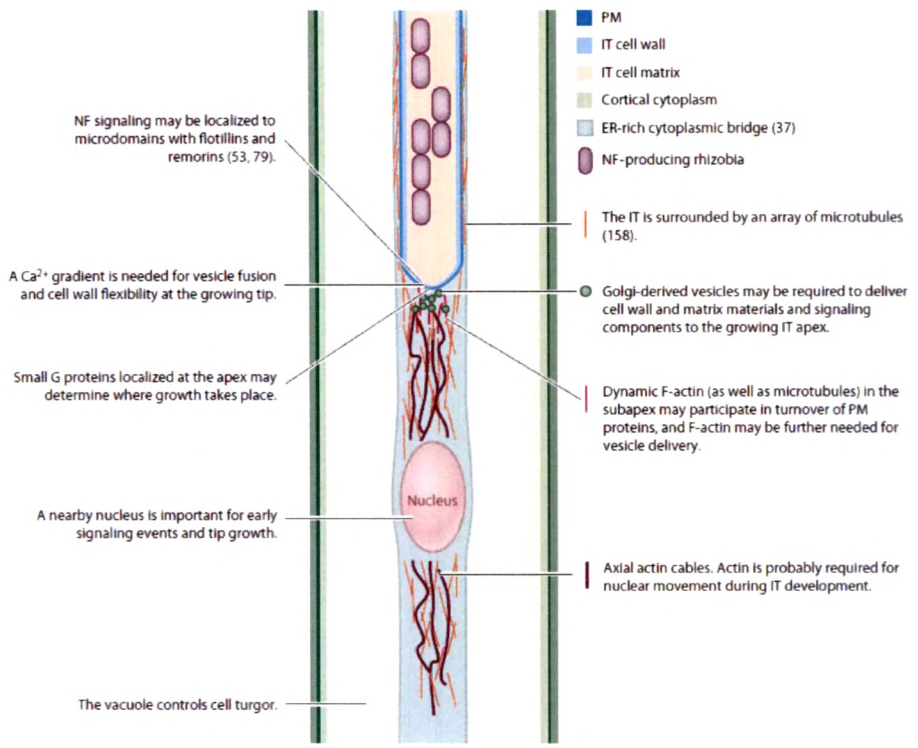


Fig. 1.4: A model for the growth of rhizobial infection threads (ITs) in root hairs (Oldroyd et al., 2011).

1.1.3: *Rhizobium* as nitrogen-fixer

Legume crops are not only used as human diet but also for improving soil fertility through biological nitrogen fixation (Anjum, 2006). Biological nitrogen (N) fixing microbes are free living in soil media that are also found in association with rhizosphere and the tissues (endophytes) of the healthy plant are beneficial for plants systems (**Fig. 1.5**) (Bashan and de-Bashan, 2005; Herridge et al., 2008). N fixing organisms having ability to enhance the N fixation performance, as well as they may also increase nutrient level in soil, which is due to the production of substances like hormones, siderophores, phosphate solubilization, improvement of nutrients, water uptake and also these microbes are also helps to enrich soil fertility and counteract agro environmental problems (Badawi

et al., 2011; Mader et al., 2011). The different N fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems are shown in **Fig. 1.5**.

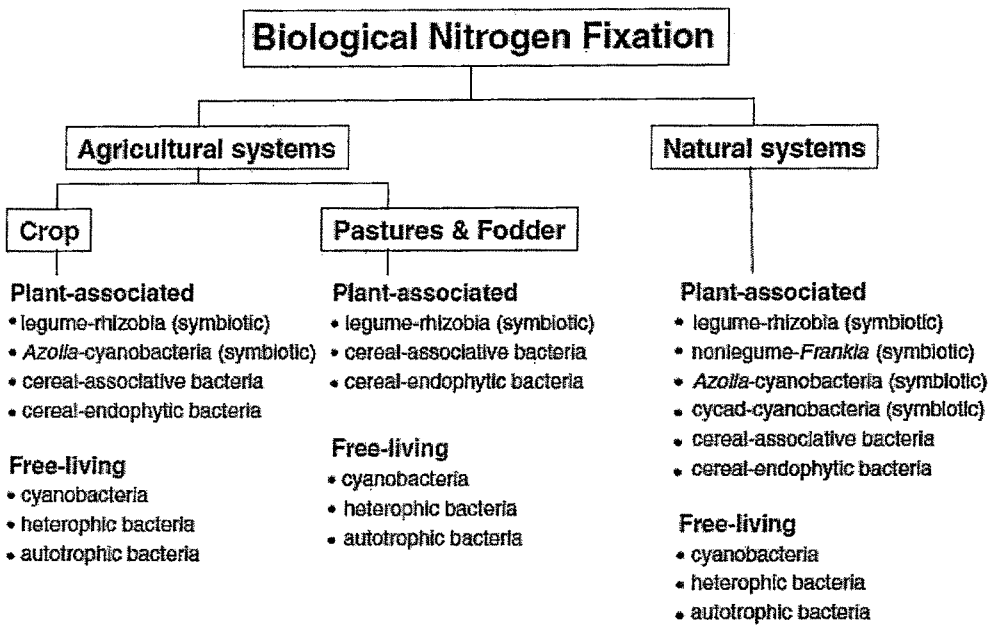


Fig. 1.5: The different N_2-fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems (Herridge et al., 2008).

In many tropical forests, N inputs via free-living N-fixers, which are ubiquitous and responsive across space and time, are critical for meeting high N demands and loss rates (Houlton et al., 2008; Hedin et al., 2009; Brookshire et al. 2012). Free-living N fixations are an important source of new N to ecosystems (Reed et al., 2011). Based on this nitrogen-fixing symbiosis, legume crops require 35–60% less fossil-based energy than conventional, N-fertilized crops (Jensen et al., 2012). On a global scale, biological nitrogen fixation in the legume–rhizobia symbiosis accounts for roughly 200 million tons of fixed nitrogen per year. Thus, legumes are agriculturally and ecologically very important and account for 25% of the world's primary crop production (Ferguson et al., 2010).

The soil borne, gram-negative *Rhizobia* bacteria have nitrogen reduction capacity catalyzed by nitrogenase, an enzyme not found in plants (Kessel and Hartley, 2000; Raven and Johnson, 2008; Taiz and Zeiger, 2010; Valentine *et al.*, 2011).

$$N_2 + 16ATP + 16H_2O + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi + 8H^+$$

This nitrogen fixation process is essential for life, because fixed nitrogen is required to biosynthesize the basic building blocks of life, e.g. nucleotides for DNA and RNA and amino acids for proteins (Raven and Johnson, 2008; Taiz and Zeiger, 2010). The family Fabaceae establishes symbiosis with diazatrophic bacteria (for example, the genus *Rhizobia,* which has the unique ability to establish N_2-fixing symbiosis on legume roots) in exchange for metabolites and reduced carbon (Kessel and Hartley, 2000; Valentine et al., 2011).

Nitrogen fixation is the most energetically expensive reaction known to occur in any plant cell (Raven and Johnson, 2008). Each symbiotically fixed ammonium molecule utilizes 8 ATPs for the reaction and the cost of symbiotic N_2 reduction in legumes is between 2 and 3 mg carbon (C) per mg fixed N, varying according to the species and specific genotypes (Valentine et al., 2011). Symbiotic nitrogen fixation required significantly more energy per N fixed than NO_3^- uptake and reduction. Abiotic stresses account for major reductions in nitrogen fixation, where more than 50% of legume crops are lost worldwide due to drought, salinity, aluminium toxicity and nutrient deficiencies.

1.1.4: Plant Growth Promoting Rhizobzcteria (PGPR)

Interactions between plants and micro-organisms in the rhizosphere can clearly affect crop yields. The most studied PGPR belong to gram-negative genera, and large number of strains belongs to fluorescent pseudomonads (Kloepper, 1993). Plant Growth Promoting Rhizobzcteria (PGPR) are divided into two groups according to the mode of action: PGPR that directly affect plant growth and biocontrol that indirectly benefit the plant growth (**Fig. 1.6; Fig. 1.7; Table 1.3**) (Glick, 1995; 1999). Direct mechanisms of plant growth by PGPR include (i) the provision of bioavailable phosphorus for plant

uptake, (ii) nitrogen fixation for plant use, (iii) sequestration of iron for plants by siderophores, (iv) production of plant hormones like auxins, cytokinins and gibberellins, and (v) lowering of plant ethylene levels (Podile and Kishore, 2006; Bhattacharyya and Jha, 2012). Indirect mechanisms used by PGPR include (i) antibiotic protection against pathogenic bacteria, (ii) reduction of iron available to phytopathogens in the rhizosphere, (iii) synthesis of fungal cell wall-lysing enzymes, and (iv) competition with detrimental microorganisms for sites on plant roots.

Phytohormones like indole-3-acetic acid (IAA), gibberellic acid and cytokinins PGPR increase root surface and length and promote plant development (Kloepper et al., 2007). Several PGPR as well as some pathogenic, symbiotic and free living rhizobacterial species produce IAA and gibberllic acid in the rhizospheric soil and plays a significant role in increasing the root surface area and number of root tips in many plants (Vessey, 2003; Bhattacharya and Jha, 2012). Ethylene, a gaseous phytohormone commonly induced by wounding in plants, causes root growth inhibition. Many PGPR have the capability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase which contributes to growth promotion even in stressful conditions such as flooded or soils contaminated with cadmium (Podile and Kishore, 2006).

Microorganisms and plants have evolved specific mechanisms to chelate insoluble iron through the release of siderophores and uptake of iron-siderophore complexes through specific outer membrane receptor proteins to meet their iron requirement (Sharma and Johri, 2003). These siderophores can be of different types: hydroxamates, phenol-catecholates, and carboxylates (Podile and Kishore, 2006). Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability all over world (Compant et al., 2005). Diverse PGPR antagonize the root pathogens through one or more different mechanisms, for example by production of bacterial allelochemicals such as volatile or non-volatile antibiotics, siderophores, detoxification enzymes, lytic enzymes and other secondary metabolites like HCN (Podile and Kishore, 2006).

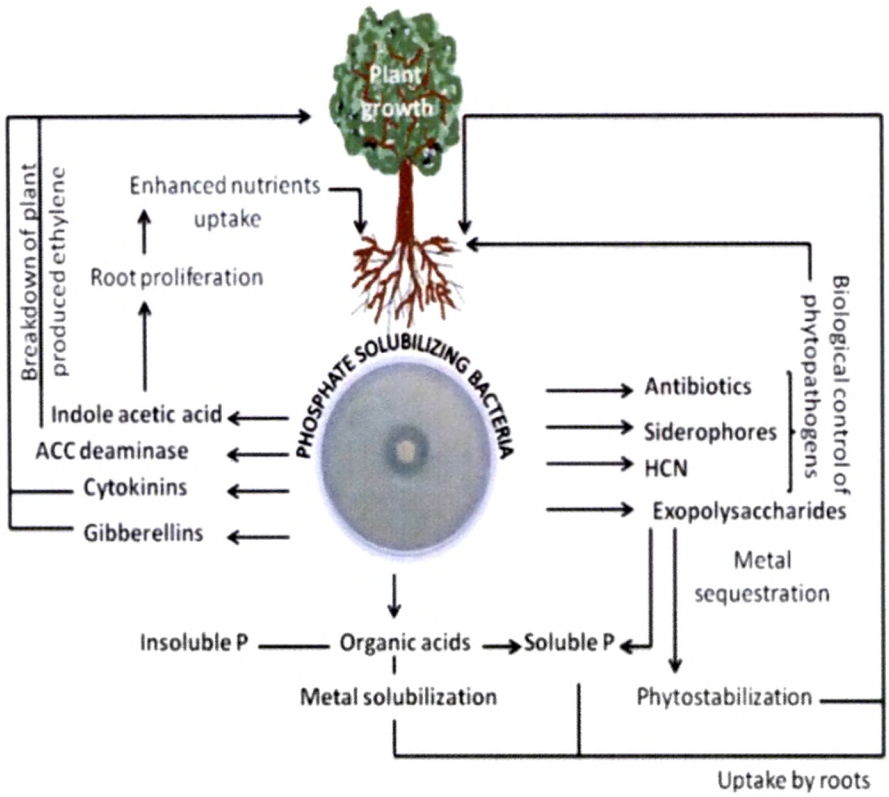


Fig. 1.6: Mechanism of growth promotion by phosphate solubilizing bacteria (Zaidi et al., 2009).

Production of these compounds is highly influenced by the qualitative and quantitative nutrient availability and is also subjected to quorum sensing (Haas and Keel, 2003). Many PGPR present the ability to produce peptide antibiotics, which are oligopeptides that inhibit synthesis of pathogens cell walls, influence membrane structures of cells, and inhibit the formation of initiation complex on small subunit of ribosomes system of the plant (Maksimov, et al., 2011). It was revealed that *B. subtilis* surfacine is able to stimulate induced systemic resistance by activation of components like lipoxygenases, lipid peroxidases and the formation of reactive oxygen species (Maksimov et al., 2011).

Table 1.2: Growth-promoting substances released by phosphate solubilizing bacteria (Zaidi et al., 2009).

Phosphate solubilizing bacteria	Plant growth-promoting traits	References
Pseudomonas sp., Bacillus sp.	IAA, siderophore	Rajkumar et al. (2006)
Bacillus spp.	IAA, siderophores, ammonia production, HCN, chromium reduction, metal solubilization	Wani et al. (2007a, 2007b, 2007c)
Mesorhizobium loti MP6	HCN, IAA	Chandra et al. (2007)
Dyella ginsengisoli, Burkholderia kururiensis, Pandoraea sp. ATSB30	Siderophore, IAA, salicylic acid, ACC Deaminase	Anandham et al. (2008)
Pseudomonas sp.	siderophore ACC deaminase, IAA,	Poonguzhali et al. (2008)
Bacillus subtilis	IAA, siderophore, antifungal activity	Singh et al. (2008)
Serratia marcescens	IAA, siderophore, HCN	Selvakumar et al. (2008)
P. fluorescens	ACC deaminase	Shaharoona et al. (2008)
Acinetobacter sp., Pseudomonas sp.	ACC deaminase, IAA, antifungal activity, N_2-fixation	Indiragandhi et al. (2008)
Enterobacter sp.	ACC deaminase, IAA, siderophore	Kumar et al. (2008)
Burkholderia	ACC deaminase, IAA, heavy metal solubilization siderophore,	Jiang et al. (2008)
P. jessenii	ACC deaminase, IAA, heavy metal solubilization siderophore,	Rajkumar and Freitas (2008)
P. aeruginosa	ACC deaminase, siderophore, IAA	Ganesan (2008)
Mesorhizobium sp., Bacillus sp., Azotobacter sp., Pseudomonas sp.	IAA, siderophore, antifungal activity, ammonia production, HCN	Ahmad et al. (2008)
P. aeruginosa, P. mosselii, P. plecoglossicida	Siderophore, IAA, protease, cellulose and HCN	Jha et al. (2008)

Some PGPR can induce plant growth promotion through a combination of modes of action and act synergistically to stimulate the growth of the host plant (Antoun et al., 1998; Belimov et al., 2001; Vessey, 2003; Dey et al., 2004; Khan et al., 2009). ACC deaminase activity along with expression of one or more of the traits such as suppression

of phytopathogens, solubilization of tricalcium phosphate, production of siderophore and/or nodulation promotion by the PGPR contributed to the enhancement of growth, yield, and nutrient uptake of peanut (Dey et al., 2004). Seed inoculation of rice (cv. Naveen) by the six individual PGPR isolates had a considerable impact on different growth parameters including root elongation that was positively correlated with ACC deaminase activity and IAA production and also showed other plant growth attributes including ammonia production and at least two isolates produced siderophores (Bal et al., 2013).

1.1.4.1: *Rhizobium* as PGPR for non-legumes.

Rhizobia also have an excellent potential to be used as PGPR and PSM with non-legume plants (Chabot, 1996). Beyond nitrogen fixation, rhizobia also colonize roots of non-legume species and promote their growth without forming any nodule-like structure (Mehboob et al., 2009; Mia and Shamsuddin, 2010). Inoculation with *Rhizobium* had significant effect on the plant height, number of branches, root and shoot dry weight, number of nodule, seed and biomass yields, number of pod, crude protein rate and phosphorus content of seed (Erman et al., 2009). Increased rice production was seen by inoculation with a *Rhizobium leguminosarum* bv. trifolii strain (Yanni and Dazzo, 2010). *R. leguminosarum* PETP01 and TPV08 are excellent biofertilizers for tomato and pepper in different production steps leading to increased yield and quality (Garcı´a-Fraile1 et al., 2012). Rhizobia strains establish endophytic relationships with rice plants to promote shoot growth and enhance grain production (Biswas et al., 2000a, b; Mia and Shamsuddin, 2010; Costa, et al., 2013). One native rhizobia, POA3 isolated from the Porto Alegre locality promoted growth of white clover (*Trifolium repens*) and rice plants (*Oryza sativa*) (Granada, et al., 2013).

The symbiotic rhizobia isolated from leguminous plants also promote plant growth *via* their inherent PGP capacities: siderophores and indolic compound production and nutrient solubilization (Ashraf et al., 2013; de Souza et al., 2013; Jida and Assefa, 2013). Indolic compounds production was the most common characteristic of the rhizobia

species isolated from *Cajanus cajan* (Dubey et al., 2010). Crop enhancement, plant nutrients like P, K, Ca, Mg and even Fe accumulation and biofertilizer attributes were observed in cereal crops due to rhizobial inoculation (Mia and Shamsuddin, 2010) Rhizobia also promotes plant growth by synthesis of vitamins, phytohormones and enzymes, producing siderophores, dissolving phosphates and other nutrients and prevention deleterious effects of phytopathogenic microorganisms besides biological nitrogen fixation (Boiero et al., 2007; Hayat et al., 2010; Ahemad and Khan, 2011).

The effects of *Rhizobium* inoculation, lime and molybdenum supply on yield and yield components of *Phaseolus vulgaris* L. significantly improved the number of pods per plant, number of seeds per plant, 100-seed weight and seed yield (Bambara and Ndakidemi, 2010). On average, an increase of 4-5% in crop yield has an important impact in agricultural production.

1.1.5: Effect of P on nodulation

Next to nitrogen, phosphorus is the most important element for adequate grain production. The evolution of science, particularly in the past century, has clearly demonstrated the significance of phosphorus for all animal and plant life on the earth (Ryan et al., 2012). Calcium increased root growth, number of nodule primordia, nodules, and growth of the soybean plant (Waluyo et al., 2013). Ca and P had a synergistic effect on BNF of soybean in acid soils. Ca is important for the establishment of nodules, whilst P is essential for the development and function of the formed nodules. P increased number of nodule primordia, thus it also has an important role in the initiation of nodule formation. This effect of P supply on nodule formation is because P supply affects the production of root-exudates including flavonoids that trigger nod-gene expression to form nodules, and also plays a role in nodule cell metabolism that affects nodule development (Raghothama et al., 1999; Abel et al., 2002). Improved soil P status enhanced the positive effect of elevated [CO_2] on grain yield, biomass and shoot total N and P contents of the legumes tested (Lam et al., 2012). Besides contributing to plant growth by making

soluble phosphorus more available, the legume-nodulating strains increased levels of soluble phosphate, thus improving the efficiency of biological nitrogen fixation (Silva et al., 2006). A positive correlation is observed between BNF and P availability in natural soils (Pearson and Vitousek, 2002; Labidi et al., 2003). Wherever soil P availability is low, elevated CO_2 does not increase BNF, and pasture quality decreased because of a reduction in above ground; at low P availability, there is a limited response of biomass production by grass community (Edwards et al., 2005; 2006).

Crop growth and yield reduce greatly due to low P availability especially for legumes, since legume nodules responsible for N_2 fixation have high P requirements (Sulieman and Tran, 2012). P is often the limiting element for biological productivity (Sato and Miura, 2011; Lopez-Arredondo and Herrera-Estrella, 2012). P deficiency is one of the critical limiting factors, adversely affecting nodulation and N_2 fixation, and thus legume growth and productivity, worldwide (Tesfaye et al., 2007). *M. truncatula* plants inoculated with either the *S. meliloti* 102F51 or 2011 strain but due to P deficiency severely inhibited plant growth and development of nodules as well as N and P assimilation (Sulieman and Schulze, 2010*a*). Plants engaged in symbiotic N_2 fixation have high P demand (Vance et al., 2003; Sulieman et al., 2013a). Low levels of phosphorus affected symbiosis by decreasing the supply of photosynthates to the nodule, which reduced the rate of bacterial growth and the total population of legume-nodulating microorganisms (Moreira et al., 2010). P availability dominantly controlled free-living N fixation in tropical rain forest (Reed et al., 2013). Thus, the efficiency of nitrogen fixation by the strains approved as inoculants may be related to a greater ability to solubilize low soluble phosphates.

1.1.6: Phosphorus deficiency and Nitrogen fixation

Phosphorus deficiency is commonly reported along with Al^{3+} toxicity as 40% of the world's arable soil is considered acidic. Phosphorus deficiency and Aluminium (Al^{3+}) toxicity are associated with each other in acid soils, and they both have major effects on legume plant growth and function and are collectively considered as inseparable factors

that limit crop productivity on such soils (Ward et al., 2008). Nodule biomass is strongly correlated to P availability to plants as about 3 times more P is required by nodules than the surrounding root tissues. An increase in P supplied to host legume plants led to a 4-fold increase in nodule mass (Olivera et al., 2004). P deficiency in soil severely limits plant growth productivity, in legumes, and this has a deleterious effect on nodule formation, development and function (Haque et al., 2005). Nodule construction cost and growth respiration of soybeans increased with P deficiency (Andrews et al., 2009). In the case of legumes, more P is required by symbiotic than non-symbiotic plants. Symbiotic nitrogen fixation (SNF) has a high demand for P, with up to 20% of total plant P being allocated to nodules during N_2 fixation. The process consumes large amounts of energy, such that the energy generating metabolism is depended upon the availability of P (Schulze et al., 1999; Schenk, 2012). The effects of P deficiency may be direct, as P is needed by nodules for their growth and metabolism, or indirect. The high requirement of P are linked to its role in nodule carbon and energy metabolism, therefore as the deficiency may affect the supply of carbon to the nodules, the bacteria will have greater respiratory demand on the host plant during nitrogen fixation (Sar and Israel, 1991; Valentine et al., 2011).

1.1.7: *Rhizobium*- PSM co-inoculation

Considering the main limitations to the biological N_2 fixation with soybeans and common beans inoculated with rhizobia and the benefits to crop growth attributed to *Azospirillum*, co-inoculation with both microorganisms might improve plant's performance. This approach is current with modern demands of agricultural, economic, social and environmental sustainability (Chaparro et al., 2012). Many evidences are there to show that co-inoculation with *Rhizobium* and PSM have additive or synergistic effect on plant growth and crop yield (**Table 1.4**) (Morel and Brana, 2012). The results confirm the feasibility of using rhizobia and PSM such as azospirilla as inoculants in a broad range of agricultural systems, replacing expensive and environmentally unfriendly N-fertilizers (Hungria et al., 2013).

Table 1.3: Ten years of studies on legume co-inoculation (2002-2012). Increase in legume symbiotic parameters and yield by co-inoculation compared to single-inoculation with rhizobia. Abbreviations are as follows: RDW: root dry weight; SDW: shoot dry weight; RL: root length; NN: nodule number; NFW: Nodule fresh weight; PDW: plant dry weight; PFW: plant fresh weight (Morel et al., 2012).

Rhizobium and PSM	Host plant	Observation (% increase)	Reference
Rhizobium and PSB	chickpea	enhanced nodulation, plant growth, yield and nutrient uptake	Rudresh et al., 2005.
R. leguminosarum D293 and AM fungi	Pea	increased plant biomass, nodulation parameters, N_2 fixation activity, increased significantly total P content in plant tissues and percentage of root colonization	Stancheva et al., 2006.
Bradyrhizobium sp. (*Vigna*) and *B. subtilis*	green gram plants	increased dry matter yield, chlorophyll content in foliage and N and P uptake	Zaidi and Khan, 2006
Bradyrhizobium spp./ *Rhizobium* and PGPR	legumes	increased root and shoot biomass, nodule dry matter, nitrogenase activity, N_2-fixation, and grain yield	Elkoca et al., 2008.
B. japonicum USDA110- P. putida	Mung bean (*Vigna radiata*	Increase in total Biomass and in Nodule Number	Shaharoona et al., 2006
Rhizobium sp. *-P.putida/ P. fluorescens/B. cereus*	Pigeon pea (*Cajanus cajan*)	Increase in Nodule Number	Tilak et al., 2006
Rhizobium sp.-*Bacillus* spp.	Pigeon pea (*Cajanus cajan*)	Increase in plant fresh weight and in Nodule Number	Rajendran et al., 2008
R. leguminosarum- B. thuringeinsis	Lentin (*Lens Culinaris L.)*	Increase in plant fresh weight and in Nodule Number	Mishra et al., 2009
R. leguminosarum- B. thuringeinsis	Pea (*Pisum sativum* L. cv. Capella	Increase in plant fresh weight and in Nodule Number	Mishra et al., 2009
R. leguminosarum bv viceae -P. fluorescens	Pea (*Pisum sativum* L. cv. Capella	Increase in plant dry weight	Kumar et al., 2001
R. leguminosarum bv. *viciae - A. brasilense* 30	Vetch (*Vicia sativa*)	Increase in SDW, nod gene induction and decrease in indoles content	Star et al., 2011

R. galegae bv. *orientalis* - *Pseudomonas* spp.	Galega (*Galega orientalis*)	Increase in SDW RDW and in Nodule Number	Egamberdieva et al., 2010
S. meliloti - *Delftia* sp.	Alfalfa (*Medicago sativa*)	increase in SDW and in nodulation rate	Morel et al., 2011
Bradyrhizobium sp. -*Pseudomonas* sp./ *Ochrobactrum cytisi*	Altramuz (*Lupinus luteus*)	66 in SDW and 20-40, 25, and 30-50 decrease in Cd, Cu and Zn – accumulation in roots, respectively	Dary et al., 2010
R. leguminosarum bv.trifolii - *P. fluorescens*	Clover (*Trifolium repens*)	20 in SDW; 100 in Nodule Number	Marek-Kozaczuk and Skorupska, 2001
R. leguminosarum bv. trifolii - *Delftia* sp	Clover (*Trifolium repens*)	50 in SDW and 80 in nodulation rate	Morel et al., 2011
Rhizobium sp. - *Thiobacillus* sp.	Peanut (*Arachis hypogaea*)	50 in PDW; 80 in Nodule Number	Anandham et al., 2007
M. ciceri - *Azotobacter chroococcum*	Chickpea (*Cicer arietinum*)	15 in Nodule Number; 25 in P-soil availability	Qureshi et al., 2009
M. ciceri - *Pseudomonas* sp/*Bacillus* sp	Chickpea (*Cicer arietinum*)	20 in PDW; 30 in Nodule Number; 100 in P-uptake	Wani et al., 2007
Mesorhizobium sp. *Cicer* - *Pseudomonas* spp.	Chickpea (*Cicer arietinum*)	70 in Nodule Number; 30 in SDW, 30 in Nuptake	Goel et al., 2002
Mesorhizobium sp. *Cicer* -*Pseudomonas* spp.	Chickpea (*Cicer arietinum*)	1,2-1,86 in Nodule Number; 1,3-2,11 NFW; 1-2,93 in PDW	Malik and Sindhu, 2011
Rhizobium -*B. subtilis/ megaterium*	Chickpea (*Cicer arietinum*)	18 in SDW; 16-30 in RDW; 14 in total biomass yield	Elkoca et al., 2008
Rhizobium spp. -*A. brasilense*	Common bean (*Phaseolus vulgaris*)	30 total yield	Remans et al., 2008b
R. tropici - *Paenibacillus Polymyxa*	Common bean (*Phaseolus vulgaris*)	50 in Nodule Number; 40 in N uptake in non-drought stress	Figuereido et al., 2008
R. tropici/etli - *A. brasilense*	Common bean (*Phaseolus vulgaris*)	18-35 and 20-70 in RDW; 29 and 28 in SDW under non saline and saline conditions, respectively.	Dardanelli et al., 2008
R. etli - *C. balustinum*	Common bean	35 in SDW; 35 in Nodule Number under non-saline	Estevez et al., 2009

	(*Phaseolus vulgaris*)	conditions; and 39 in SDW; 63 in RDW under saline conditions	
Rhizobium spp. - *P. putida /B. subtilis/A. brasilense*	Common bean (*Phaseolus vulgaris*)	30 in Nodule Number; 20 in SDW; 30-45 in RDW	Remans et al., 2007
Rhizobium spp. - *A. brasilense*	Common bean (*Phaseolus vulgaris*)	70 in Nodule Number	Remans et al., 2008a
R. tropici - Paenibacillus polymyxa	Common bean (*Phaseolus vulgaris*)	50 in Nodule Number; 40 in N uptake in non-drought stress	Figuereido et al., 2008
Rhizobium spp. - *P. fluorescens /A. lipoferum*	Common bean (*Phaseolus vulgaris*)	25 in Nodule Number; 13 in SDW; 74 in seed yield	Yadegari et al., 2010
S. meliloti B399 and the *Bacillus* sp. M7c	alfalfa plants	increase in root and shoot dry weight, length, and surface area of roots, number, and symbiotic properties	Guiñazú et al., 2010; Lorena et al 2010.
E. fredii - Chryseobacterium balustinum	Soybean (*Glycine max*)	56 and 44 in SDW; 100 and 200 in RDW; 155 and 286 in Nodule Number	Estevez et al., 2009
B. japonicum USDA110- P. putida	Soybean (*Glycine max*)	40 in SDW; 80 in Nodule Number; 45 in RDW	Rosas et al., 2006
B. japonicum USDA110- B. subtilis/ S. proteamaculans	Soybean (*Glycine max*)	12 in SDW; 10 in P-uptake	Han and Lee, 2005
B. japonicum USDA110- A. brasilense	Soybean (*Glycine max*)	47 in Nodule Number	Cassán et al., 2009
Rhizobium and PSB	grass	enhanced nodulation and increased the number and weight of nodules	Abusuwar and Omer, 2011.
Pseudomonas fluorescens P-93 and *Azospirillum lipoferum S-21* *Rhizobium* strains Rb-133 and Rb-136	Common bean seeds	increased nodule number and dry weight, shoot dry weight, amount of nitrogen fixed as well as seed yield and protein content.	Yadegari et al., 2010

*B. japonicum USDA110*RCR 3407 strain *B. subtilis*	Common bean seeds	influence plant growth, vitality, and the ability of the plant to cope with pathogens	Elkoca et al., 2010; Tsigie et al., 2012
Rhizobium and phosphate solubilizing bacteria	faba bean plants	increased yield and seed quality decreased seeds carbohydrate content	Rugheim and Abdelgani, 2012
Pseudomonas and *Rhizobium* isolates	common bean	improved growth and yield production	Samavat et al., 2012
Rhizobium and AM fungi	Chick pea plant	significantly increased fresh and dry weights of shoot and root	Moradi et al., 2013
P. chlororaphis and A. pascens amendment with RP	Walnut	highest plant height, shoot and root dry weight, P and nitrogen (N) uptake of walnut seedlings, and the maximum amounts of available P and N in soils	Xuan Yu et al., 2012
*B. japonicum USDA110*with *A. brasilense*	Soybean and common bean	increased seed yield, improved nodulation	Hungria et al., 2013
tetra inoculants *R. leguminosarum* + *A. chroococcum* + *P. aeruginosa* + *T. Harzianum*, tri inoculants of *R. leguminosarum* + *A. chroococcum* + *P. aeruginosa* and *R. leguminosarum* + *A. chroococcum* + *T. harzianum*	Common bean seeds	significant nodulation, grain yield and nutrient uptake	Varma and Yadav, 2012

1.1.8: Phosphate solubilization by *Rhizobium* spp.

The phosphate-solubilizing activity of *Rhizobium* is associated with the production of 2-ketogluconic acid, indicating that phosphate-solubilizing activity of the organism is entirely due to its ability to reduce pH of the medium (Halder and Chakrabarty, 1993). Since 1950s it is reported that P-solubilizing bacteria release phosphorus from organic and inorganic soil phosphorus pools through mineralization and solubilization **(Fig. 1.7)** (Khan et al., 2009).

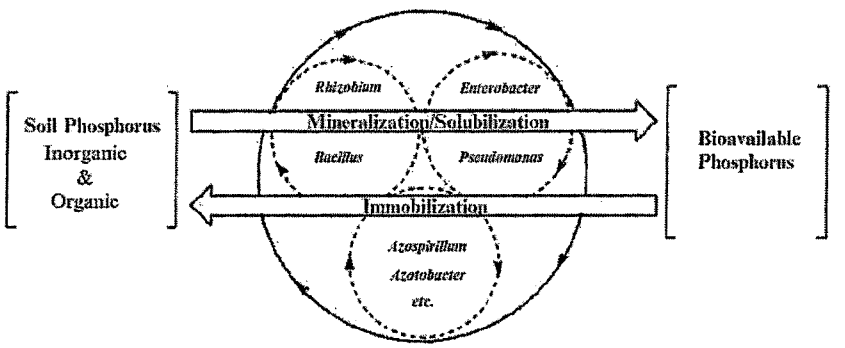


Fig.1.7: Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (Khan et al. 2009)

General sketch of P solubilization in soil is shown in **(Fig. 1.8).** A wide range of microbial P solubilization mechanisms exist in nature and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi (Banik and Dey, 1982). Phosphatic rocks are solubilized by acid producing microorganisms to release more P for plant uptake (Gyaneshwar et al., 2002). A few strains or species of *Rhizobium* are involved in phosphate solubilization also along with symbiotic nitrogen fixation (Deshwal et al., 2003). Lowering of soil pH by microbial production of organic acids such as acid phosphatases, lactate, citrate, and succinate, gluconic and 2-ketogluconic acids etc. and proton extrusion is the main principal mechanism of mineralization of organic form of phosphorus (Goldstein, 1995; Deubel et al., 2000). Phosphate availability in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes (Alikhani et al., 2006). Many phosphate-solubilizing bacteria are

found in soil and in plant rhizospheres and potentially represent 40% of the culturable population (De Freitas, et al., 1997; Richardson, 2000; Chen et al., 2006). PBS produces a range of organic acids such as citrate, lactate, and succinate that solubilize mineral phosphates. To make P available for plant nutrition *Bacillus*, *Pseudomonas*, *Klebsiella* and *Enterobacter* spp. are involved in the stepwise degradation of phytate to lower phosphate esters of myo-inositol and phosphorous by means of acid and alkaline phosphatase enzymes (Podile and Kishore, 2006). Bacteria also enhance phosphorus availability to crops by solubilizing precipitated forms of phosphorus (Chen et al., 2006). Single and dual inoculation with *Rhizobium* along with P fertilizer is 30-40% better than only P fertilizer for improving grain yield of wheat, where *Rhizobium* with non-legumes could act as phosphate solubilizer, hormone producer and to some extent as N-fixer (Afzal and Bano, 2008). Bacteria assimilate soluble phosphorus, and make it available by preventing it from adsorption (Khan and Joergensen, 2009). Phosphate solubilization activity of rhizobia is related with the production of 2-ketogluconic acid, due to its ability to reduce pH of the medium (Hayat et al., 2010). During phosphate solubilization, the nature of organic acid produced by rhizobia is more important than the quantity (Hayat et al., 2010).

Rhizobium ciceri inoculation and phosphorus application in combination increased growth rate and P utilization of chickpea cultivars as compared to the control, greatly affected the P Efficiency Index (EI) and P utilization performance of chickpea cultivars.(Karaman et al., 2013). Certain strains of *R. leguminosarum* (bv. viciae, bv. phaseoli, bv. trifolii), *R. leguminosarum* sp, *B. japonicum, Mesorhizobium ciceri, Mesorhizobium mediterraneum* and *S. meliloti* are good P-solubilizers (Antoun et al., 1998; Peix et al., 2001; Alikhani et al., 2006; Daimon et al., 2006; Rivas, 2006; Boiero et al., 2007). *B. japonicum USDA110*518 strain showed the ability to solubilize insoluble tricalcium phosphate (Marinkovic et al., 2013).

1.1.9: Phosphorus in agriculture

Phosphorous is going to be plant nutrient that will limit the agricultural production in the next millennium. It is a major growth-limiting nutrient, and unlike the

case of nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa et al., 2002). As regards the role of P, it stimulates root development and growth, gives plant rapid and vigorous start leading to better tillering, essential for many metabolic processes in plant life and for seed formation and organization of cells, encourages earlier maturity. In most soils, its content is about 0.05% of which only 0.1% is plant available (Achal et al., 2007). About 20-25% of total phosphorous in arid soils of India is organic in nature and 68% organic phosphorous in the soil is present as phytin (Yadav and Tarafdar, 2007), which are not directly available to plants. Phosphorous is taken up from soil in the form of soluble orthophosphate ions; $H_2PO_4^{-1}$, HPO_4^{-2} and PO_4^{-3} and generally the availability of these ions to the plants is in the order of $H_2PO_4^{-1} > HPO_4^{-2} > PO_4^{-3}$. Only about 20% of the phosphorus used in agriculture reaches the food we consumed, most of the rest is lost in inefficient steps along the phosphorus cycle (Cordell et al, 2011) (**Fig. 1.8**).

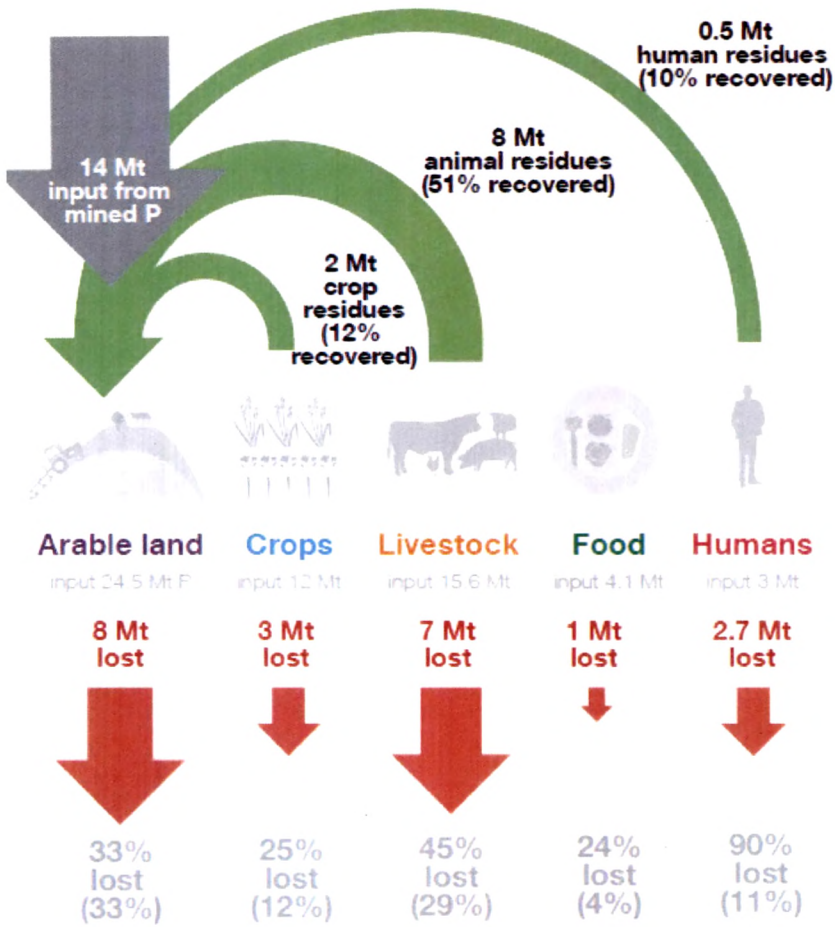


Fig. 1.8: Simplified cycle of phosphorus in agriculture (based on data from Cordell et al., 2009 and 2011). Red arrows represent losses into water systems ultimately, and green arrows represent current recoveries into arable land from the different subsystems. The percentages under the red arrows represent the percentage losses from each subsystem, and shown in brackets are the percentage losses relative to the total input into agriculture land. For example, the livestock system loses about 45% of the phosphorus entering the livestock system itself, and this represents about a 29% loss of the phosphorus entering the agriculture system overall.

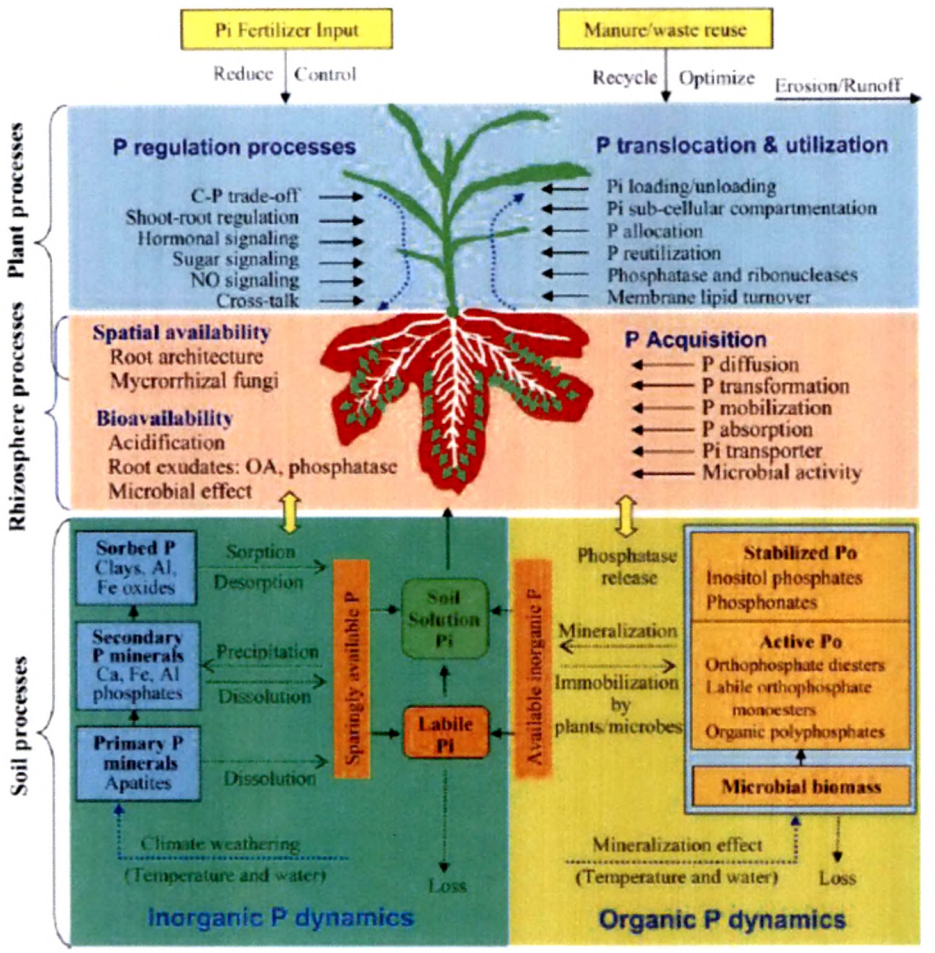


Fig. 1.9: P dynamics in the soil/rhizosphere-plant continuum. C-P, Carbon- P; NO, nitric oxide; OA, organic acids (Shen,et al 2011).

Rhizobacteria secrete organic acids as end products or by-products of primary metabolism. In most cases, sugars are catabolized by glycolytic or Entner-Doudroff pathway. The amount of the organic acid secretion differs between members of the same genus and sometimes between strains of the same species due to presence or absence of enzymes (Vyas and Gulati, 2009; Buch et al., 2010). Organic acids of aerobic or anaerobic respiration such as gluconic acid, 2-ketogluconic acid are directly formed extracellularly or in the in the periplasm by the membrane bound enzymes (**Fig. 1.10**) (Archana et al., 2012). However, organic acids formed by intracellular enzymes require

specific transport proteins that aid in their extracellular secretion. Mono-, di- and tri-carboxylate transporters are located in the plasma membrane mediate their secretion.

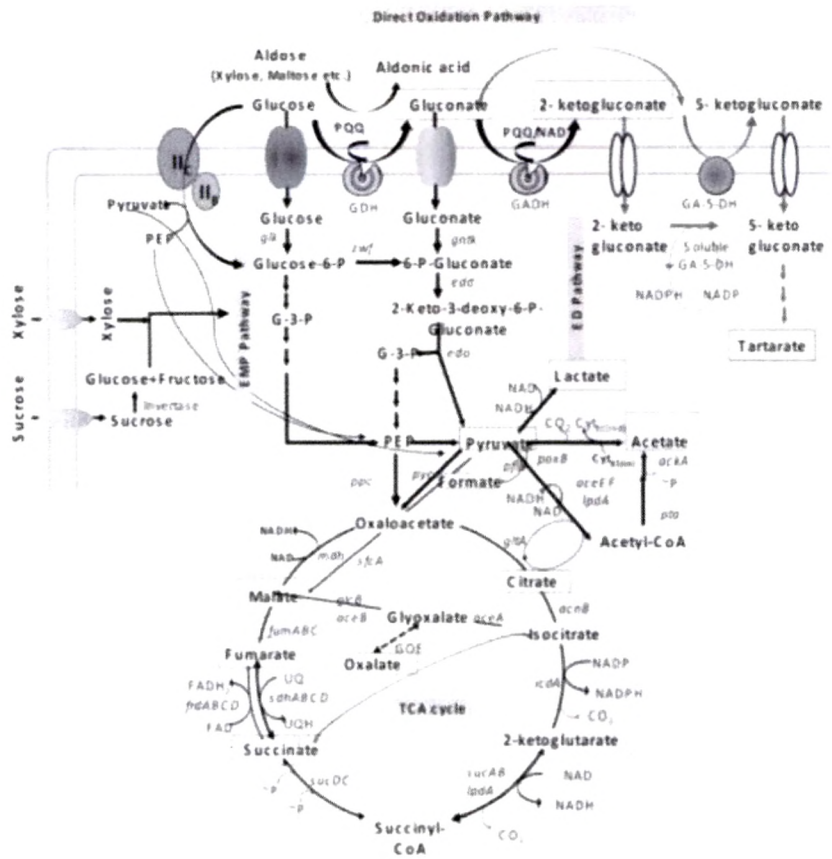


Fig.1.10: Pathways and enzymes involved in organic acid biosynthesis by rhizobacteria (Archana et al., 2012). The organic acids secreted are depicted in boxes. The diagram depicts a comprehensive set of pathways – all may not be present in any given organism. Abbreviations: GDH glucose dehydrogeanse, GADH gluconate dehydrogenase, GA-5-DH gluconate-5-dehydrogenase, glk Glucokinase, zwf Glucose-6-phosphate dehydrogenase, gntk Gluconate kinase, edd 6-phosphogluconate dehydratase, eda 2-keto-3-deoxy-6-phosphogluconate aldolase, ppc phosphoenolpyruvate carboxylase, pyc pyruvate carboxylase, gltA citrate synthase, acnB Aconitase, icdA Isocitrate dehydrogenase, icl Isocitrate lyase, sucABa ketoglutarate dehydrogenase, sucDC succinyl-CoA synthetase, sdhABCD succinate dehydrogenase, fumABC Fumarase, frdABCD fumarate reductase, mdh Malate dehydrogenase, sfcA malic enzyme, aceA Isocitrate lyase, aceB/glcB Malate synthase, GOE Glyoxalate oxidizing enzyme, ldh Lactate dehydrogenase, aceEF-lpdA pyruvate dehydrogenase, pta phosphotransacetylase, ackA acetate kinase A, poxB pyruvate oxidase, pfl pyruvate formate lyase.

Table 1.4: Organic acids involved in P-solubilization and produced by PS bacteria (Zaidi et al., 2009).

Bacterial communities	Organic acids produced	References
Burkholderia cepacia DA23	Gluconic	Song et al. (2008)
Pseudomonas corrugata (NRRL B-30409)	Gluconic, 2-ketogluconic	Trivedi and Sa (2008)
Citrobacter sp. DHRSS	Acetic, gluconic	Patel et al. (2008)
Burkholderia, Serratia, Ralstonia and Pantoea	Gluconic	Elizabeth et al. (2007)
Bacillus, Rhodococcus, Arthrobacter, Serratia and one Chryseobacterium, Delftia, Gordonia, Phyllobacterium, Arthrobacter ureafaciens, Phyllobacterium myrsinacearum, Rhodococcus erythropolis and Delftia sp.	Citric, gluconic, lactic, succinic, propionic	Chen et al. (2006)
Enterobacter intermedium	2-ketogluconic	Hwangbo et al. (2003)
B. amyloliquefaciens, B. licheniformis, B. atrophaeus, Penibacillus macerans, Vibrio proteolyticus, xanthobacter agilis, E. aerogenes, E. taylorae, E. asburiae, Kluyvera cryocrescens, P. aerogenes, Chryseomonas luteola	Lactic, itaconic, isovaleric, acetic isobutyric	Vazquez et al. (2000)
Pseudomonas cepacia	Gluconic, 2-ketgluconic	Bar-Yosef et al. (1999)
Bacillus polymyxa, B. licheniformis, Bacillus spp.	Oxalic, citric	Gupta et al. (1994)

Table 1.5 Organic acids produced by phosphate solubilizing fungi (Zaidi et al 2009)

Organism	Organic acids 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	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. tamari, A. terricola,A. amstelodemi,	Oxalic, citric	Gupta et al. (1994)
A. japonicus, A. foetidus	Oxalic, citric gluconic succinic, tartaric	Singal et al. (1994)

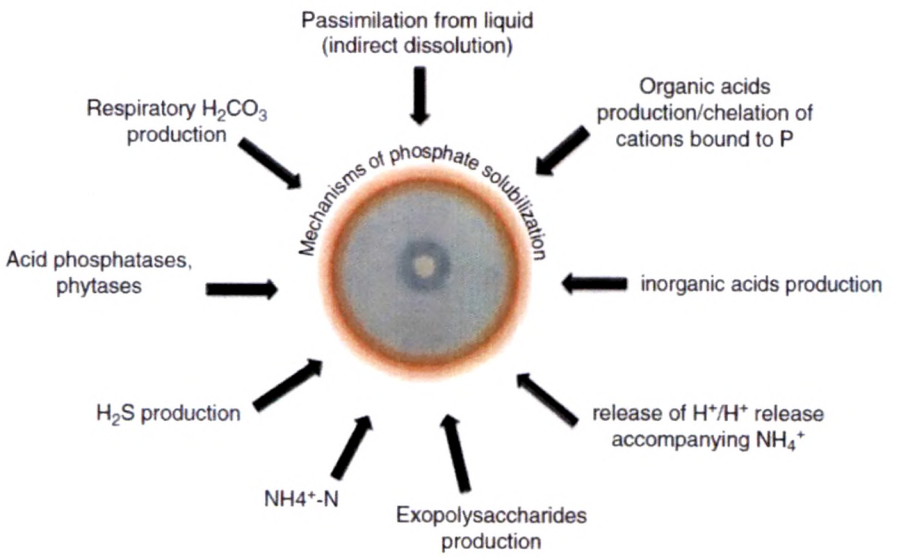


Fig. 1.11: Mechanisms of P-solubilization by phosphate solubilizing bacteria (Zaidi et al., 2009).

PS microbes are well known for making soluble P accessible for uptake by plants. They can also facilitate growth and development of plants by producing essential nutrients (Thomas et al., 2005) or by changing the concentration of plant growth promoting substances including phytohormones such as indoleacetic acid (Wani et al., 2007a, b), through asymbiotic or symbiotic N_2 fixation (Zaidi, 2003; Zaidi and Khan, 2007), soil conditioning, exhibiting bio-control activity (Pandey et al., 2006), by synthesizing siderophores (Vassilev et al. 2006), antibiotics, and cyanide (Lipping et al., 2008), by synthesizing an ACC deaminase that can modulate plant ethylene levels (Anandham et al., 2008; Poonguzhali et al., 2008), and by solubilizing or reducing the toxicity of metals (bioremediation) (Khan et al., 2009).

1.2: Central Carbon Metabolism

The term "central carbon metabolism" (CCM) describes the integration of pathways of transport and oxidation of main carbon sources inside the cell. In most bacteria, the main pathways of the CCM are those of the phosphotransferase system (PTS), glycolysis, ED pathway gluconeogenesis, pentose phosphate (PP) pathway, and the tricarboxylic acid cycle (TCA) with the glyoxylate bypass. As a whole, the system has a complex structure and it is regulated by complex networks of reactions. The knowledge about regulation in CCM has great industrial relevance as it allows the engineering of selected metabolic steps to reroute carbon fluxes toward precursors for industrially important metabolites (Nielsen, 2011).

Carbon Metabolic networks of active reactions were identified using ^{13}C constrained metabolic-flux analysis that relies on the detection of mass isotopomer pattern in proteinogenic amino acids (Fischer and Sauer, 2003; Fischer et al., 2004). Based on the established amino acid biosynthesis schemes, intracellular-flux ratios was calculated from the labeling patterns of the amino acids by using the algebraic equations of METAFoR analysis (Fischer et al., 2003). These flux ratios represent direct, local evidence for the in vivo activity of particular pathways and reactions. Thus from the elucidated network topology of active reactions and from literature data, organism-specific metabolic-reaction models were deduced using the master network model **(Fig. 1.12).**

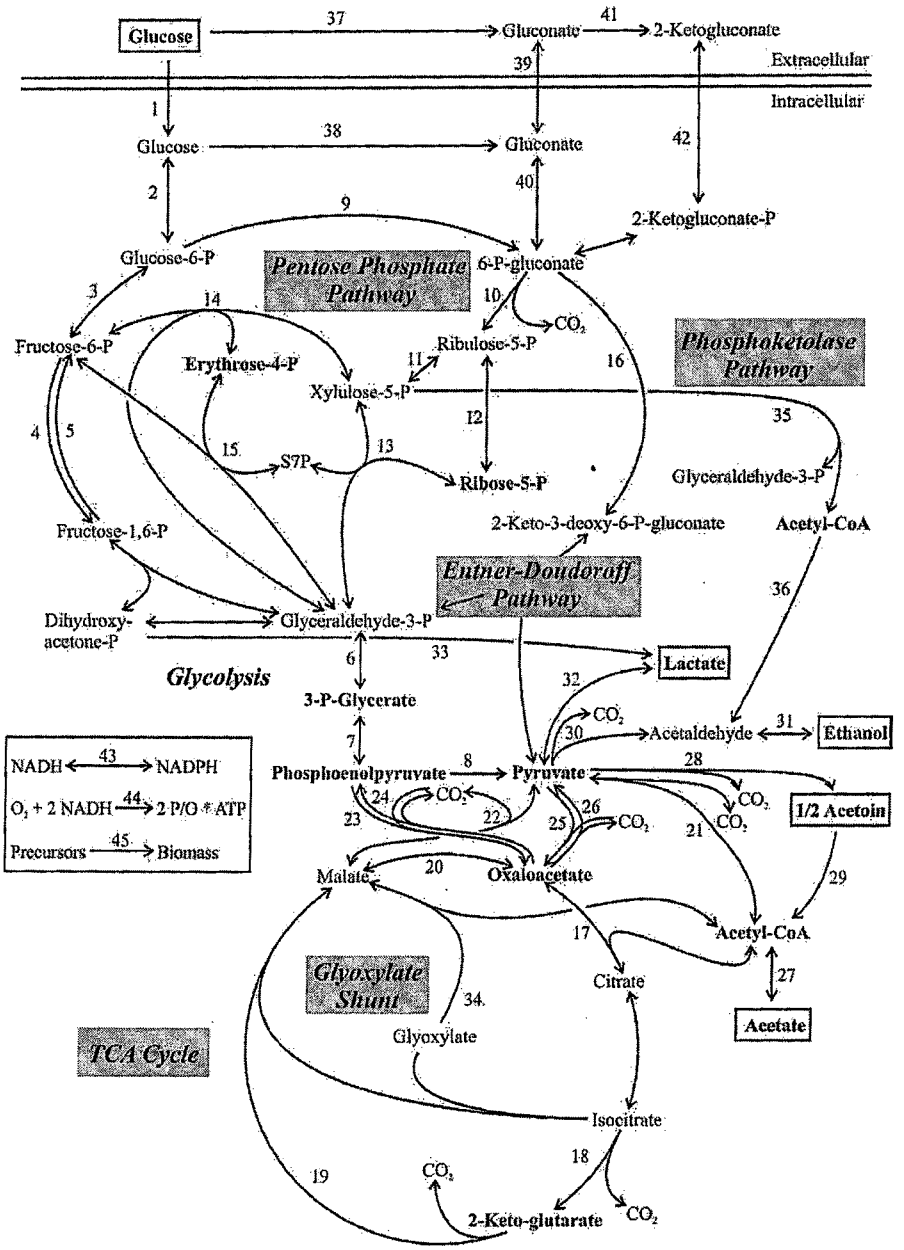


Fig. 1.12: Master network reaction that was used as the basis for net-flux analysis (Fuhrer et al., 2005). Metabolites in bold were precursors for amino acid biosynthesis, and metabolites in boxes were extracellular substrates or products. Doubled-headed arrows indicate reactions assumed to be reversible. Abbreviations: S7P, sedoheptulose-7-P; Acetyl-CoA, acetyl coenzyme A.

1.2.1: *Escherichia coli* –as the Model Organism

The CCM of *E. coli* and specifically the metabolism of glucose are intensively studied and well known topics (Raab et al., 2010; Blankschien, et al., 2010). Glucose metabolism starts with its uptake *via* the PTS and proceeds with several interconnected pathways with the major being: glycolysis, gluconeogenesis, the pentose- monophosphate bypass with the Entner-Dudoroff pathway, the TCA cycle with the glyoxylate bypass, anaplerotic reactions and acetate production and assimilation **(Fig. 1.13).**

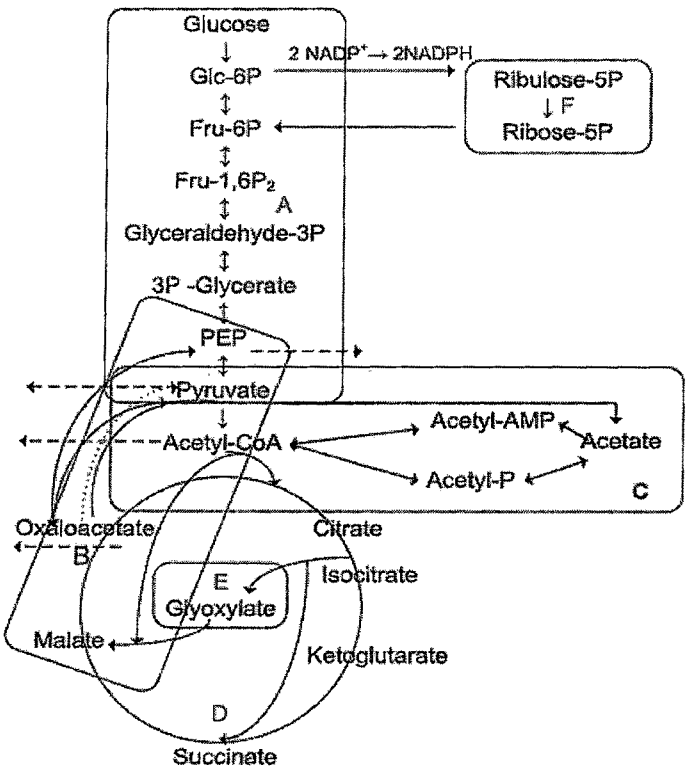


Fig. 1.13: Simplified representation of the central carbon metabolism of *E. coli.* (A) glycolysis and gluconeogenesis, (B) anaplerotic reactions, (C) acetate formation and assimilation, (D) TCA cycle, (E) Glyoxylate shunt, (F) PP pathway. The dotted line arrow from oxaloacetate to pyruvate indicates the anaplerotic reaction catalysed by pyruvate carboxylase. The broken line arrows indicate the removal of metabolites.

The extensive knowledge gained on *E. coli* CCM (Sauer and Eikmanns, 2005) offers key advantages in metabolic engineering efforts to achieve increased metabolite production. Such efforts have been focused intensively rather on the upper part of the carbon assimilation network, consisting of glycolysis and glyconeogenesis and their genetic and metabolic regulation.

The terminal stages of glycolysis in *E. coli* involve complex interplays. PEP conversion is coupled to two metabolic processes: PEP forms pyruvate by pyruvate kinase (PK) in the PTS or it forms oxaloacetate by the PEP carboxylase (ppc)-catalyzed anaplerotic reaction. The final products of glycolysis PEP and pyruvate enter the TCA cycle via acetyl-CoA and via the formation of oxaloacetate by carboxylation. This route, referred to as anaplerosis, replenish the intermediates of the TCA cycle that were used for anabolic purposes. Under gluconeogenic conditions, the TCA cycle intermediates oxaloacetate or malate are converted to pyruvate and PEP by decarboxylation and this way the PEP-pyruvate-axaloacetate node provides the precursors for gluconeogenesis. Therefore, the metabolic link between glycolysis, gluconeogenesis and the TCA cycle is represented by the PEP-pyruvate-oxaloacetate node (Sauer and Eikmanns, 2005)

1.2.2: Glucose catabolic pathways in pseudomonads

In pseudomonads although organic acids are the preferred carbon sources presence of glucose as the sole carbon source does induce the glucose metabolizing pathways. Pseudomonads do not catabolize glucose to triose phosphate via the traditional EMP pathway as they lack the key glycolytic enzyme PFK (Lessie and Phibbs, 1984). Unlike *E. coli*, pseudomonads generally lack PEP-PTS system for glucose uptake (Romano et al., 1970). Instead, pseudomonads catabolize glucose by two different routes: the direct oxidative pathway which acts on glucose extracellularly and the simultaneously operating intracellular phosphorylative pathway. 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 (Lessie and Phibbs, 1984; Fuhrer et al., 2005) **(Fig.1.14).**

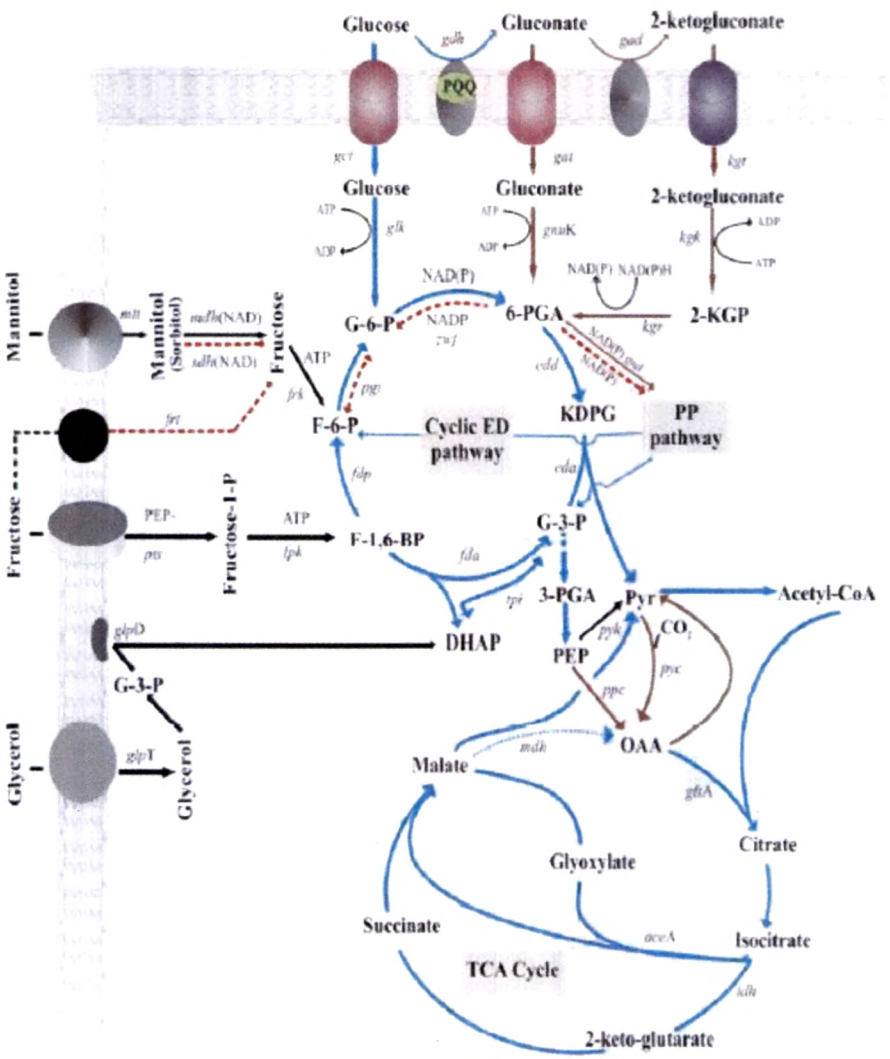


Fig.1.14: Carbohydrate metabolism in pseudomonads. Key to the pathway: Blue lines/arrows=Pathway operating in presence of glucose; **Bold Black arrows**=Reactions occurring when carbon source is other than glucose; Blue dashed arrow=Flux through that reaction is very low; Brown arrows=Occurrence of those genes is highly variable from strain to strain; Red dashed arrows=Reactions specific to *Pseudomonas cepacia*

1.2.3: Glucose Metabolism in *Bacillus subtilis*

B. subtilis is a gram positive spore forming bacterian and is the second most intensively studied bacteria after *E. coli.* Glucose is internalized via PTS *and* metabolizes

a large proportion of it to pyruvate and acetyl CoA, and subsequently converts these compounds to lactate, acetate and acetoin as by-products of metabolism which are excreted into the extracellular environment. The overall flux distribution done by ^{13}C metabolic flux analysis suggested glycolysis as the main catabolic pathway for glucose, acetate secretion, significant anaplerosis, and absent gluconeogenesis (**Fig. 1.15**) (Martin et al., 2011).

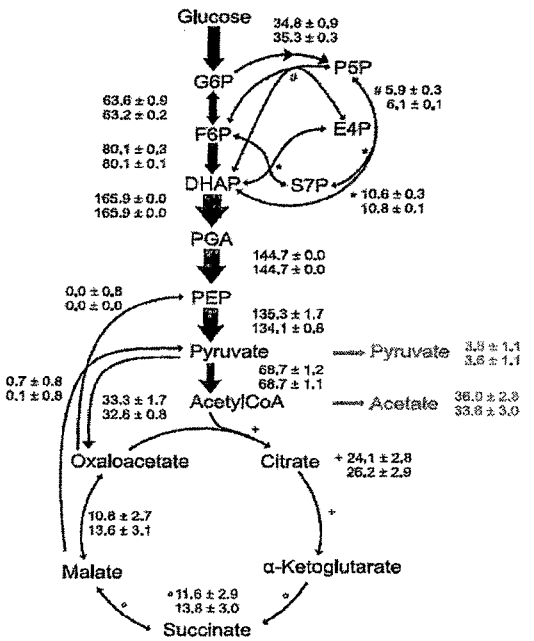


Fig. 1.15: Glucose Metabolism in *Bacillus subtilis* (Martin et al., 2011). Shown are relative flux values normalized to the glucose uptake rate of 8.2mmol g-1 h-1. Black arrows depict maximum and inner white arrows the minimum estimated flux value based on the Monte Carlo bootstrap error estimates with a confidence interval of 95%.

1.2.4: Glucose Metabolism in *Rhizobium*

The mechanism of glucose transport is established in both fast and slow-growing rhizobia, neither fast nor slow-growing rhizobia possessed a phosphoenolpyruvate phosphotransferase system and the uptake of glucose proceeded via an active process requiring an energized membrane state (Stowers et al., 1977; Mulongoy et al., 1978; DeVries et al., 1982). Carbohydrate supply is a major factor limiting nitrogen fixation by

the *Rhizobium*-legume symbiosis (Bethlenfalvay and Phillips, 1977; Hardy, 1977; Pate, 1977). Both fast- and slow-growing species possess the Entner-Doudoroff pathway (Katznelson and Zagallo, 1957; Keele et al., 1969; Martinez-de Drets and Arias, 1972; Mulongoy and Elkan, 1977 a). Fast-growing rhizobia also possess $NADP^+$-dependent 6-phosphogluconate dehydrogenase the key enzyme of the pentose phosphate pathway, but it was not found in slow-growing rhizobia (Katznelson and Zagallo, 1957; Keele et al., 1969; Martinez-de Drets and Arias, 1972; Mulongoy and Elkan, 1977a, b). The tricarboxylic acid cycle also operated in hexose catabolism in *B. japonicum USDA110*(Keele et al., 1969; Mulongoy and Elkan, 1977a). ED pathway was established as the presence of 6PG dehytratase (EC 4. 2. 1. 12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) activities were observed in glucose-grown cells (Keele et al.,1970; Stowers et al., 1985)(**Fig. 1.16.**)

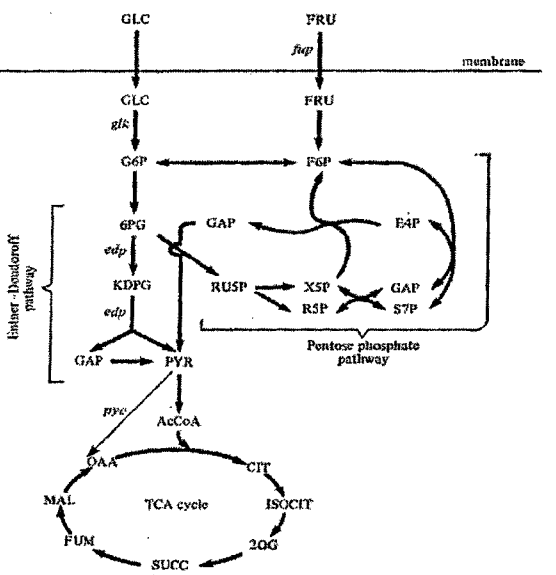


Fig. 1.16. Pathways of glucose and fructose catabolism available to *R. trifolii* strain 7000 (Ronson and Primrose, 1979). The mutants are blocked at the steps indicated: *glk,* strains 7009, 7013 and 7039; *fup,* strain 7039; pyc, strain 7049. Strain 7028 is blocked at one of the two steps labelled *edp*. Abbreviations: GLC, glucose; FRU, fructose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 6PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconatGe;A P, glyceraldehydes 3-phosphate; RUSP, ribulose 5-phosphate; X5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose

4-phosphate; PYR, pyruvate; AcCoA, acetyl-CoA; OAA, oxaloacetate; CIT, citrate; ISOCIT, isocitrate; 20G, 2-oxoglutarate; SUCC, succinate; FUM, fumarate; MAL, malate; TCA, tricarboxylic acid.

Rhizobium trifolii strain 7000 contained key enzyme activities of the ED and PP pathways (**Fig. 1.16**). The lack of phosphofructokinase indicated that the EMP pathway was absent (Ronson and Primrose, 1979).

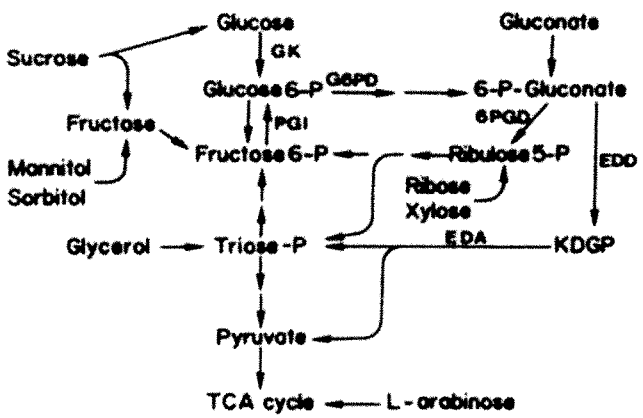


Fig.1.17 Outline of Possible pathways of Carbohydrate metabolism in *R. meliloti* (Arias et al., 1979)

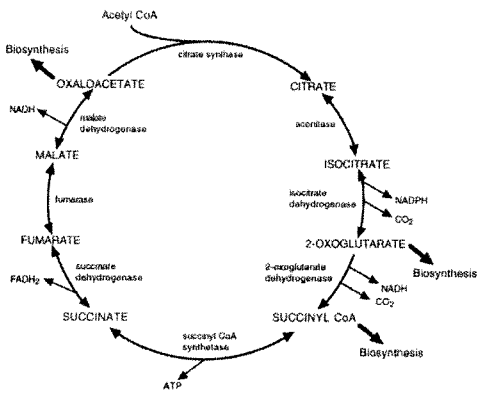


Fig.1.18: Reactions of the TCA cycle. (Dunn, 1998) Cycle intermediates are in capital letters. Additional products shown are for the forward (clockwise) reactions. Metabolites which are commonly used in biosynthetic reactions are also indicated.

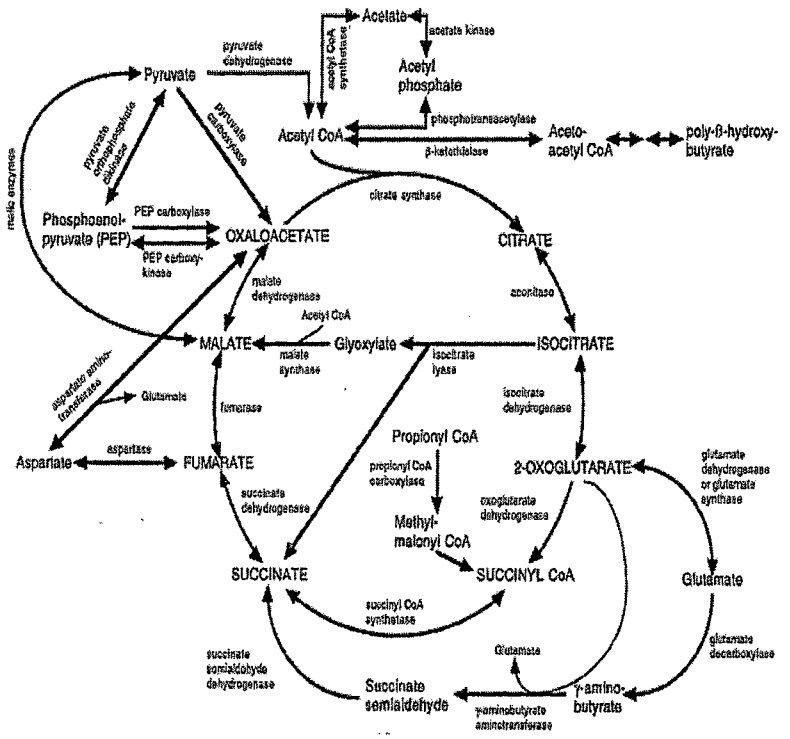


Fig. 1.19: Possible integration of anaplerotic and bypass pathways with the TCA cycle. (Dunn, 1998) Intermediates of the TCA cycle are in capital letters. Not all reaction products are shown.

The sources of acetyl-CoA are pyruvate and carbohydrate metabolism product in *S.meliloti* **(Fig. 1.20)** (Imperlini et al., 2009).. The main fates of acetyl-CoA are (i) its oxidation by the TCA cycle,(ii) its incorporation into PHB, and(iii) its utilization via the glyoxylate bypass. TCA cycle intermediates are commonly used in biosynthetic reactions and some reactions are inhibited by NADH.

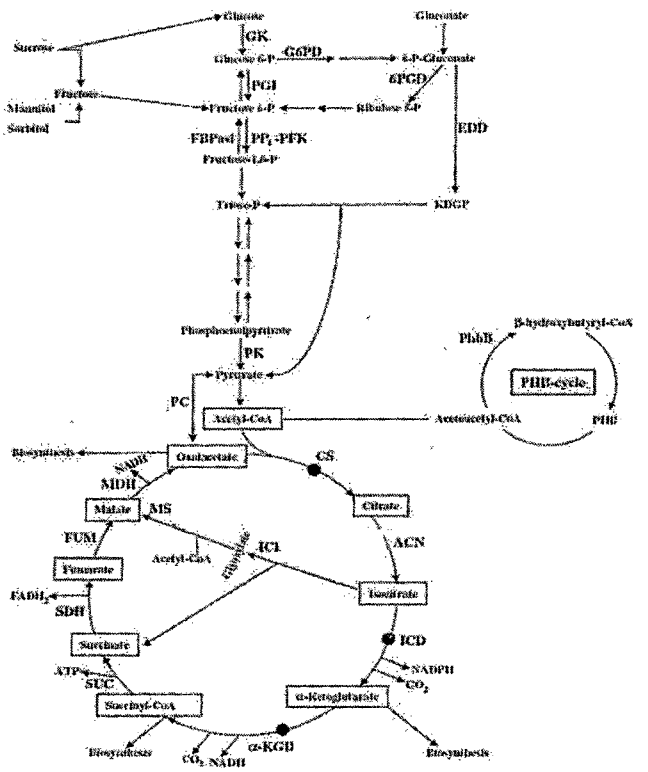


Fig.1.20: Carbohydrate metabolism in *S. meliloti* (Imperlini et al., 2009). MS malate synthase; PhbA β-ketothiolase; PhbB acetoacetyl-CoA reductase.

S. meliloti converted glucose to gluconate, which then entered metabolism (Stowers, 1985; Portais et al., 1999). Pyruvate carboxylase was assumed to be the anaplerotic reaction by analogy to *A. tumefaciens* (Dunn et al., 2001). Both the PP and ED pathways were present, and glycolysis was absent, in both rhizobia (Arthur et al., 1979; Stowers, 1985) In rhizobia, the ED pathway was basically the exclusive pathway of glucose degradation, while the pentose-5-P precursors for biomass were generated through the oxidative and nonoxidative branches of the PP pathway **(Fig. 1.21).** With a flux well above 100% relative to the glucose uptake rate high TCA cycle flux is seen. ^{13}C- and ^{31}P-NMR profiles of bacteroids presented quantitative and qualitative differences from that of their vegetative state, which reflect some physiological adaptations of rhizobia to the plant host environment.

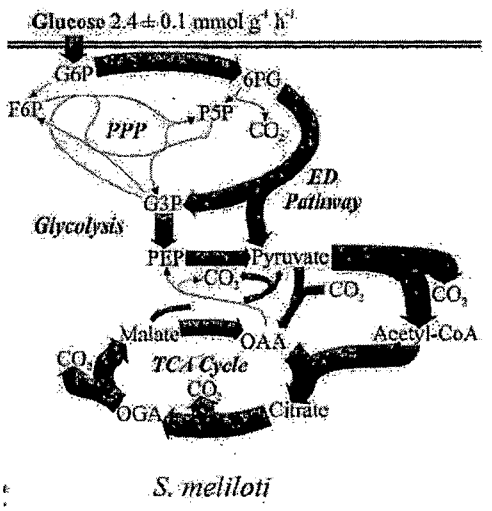


Fig. 1.21: *In vivo* carbon flux distribution in *S. meliloti* (Fuhrer et al., 2005). All fluxes normalized to the glucose uptake rate that is given at the top of each panel, and the widths of the arrows are scaled to the relative percentages of flux. Fluxes below 2.6% of the glucose uptake rate are represented by nonscaled hairlines.

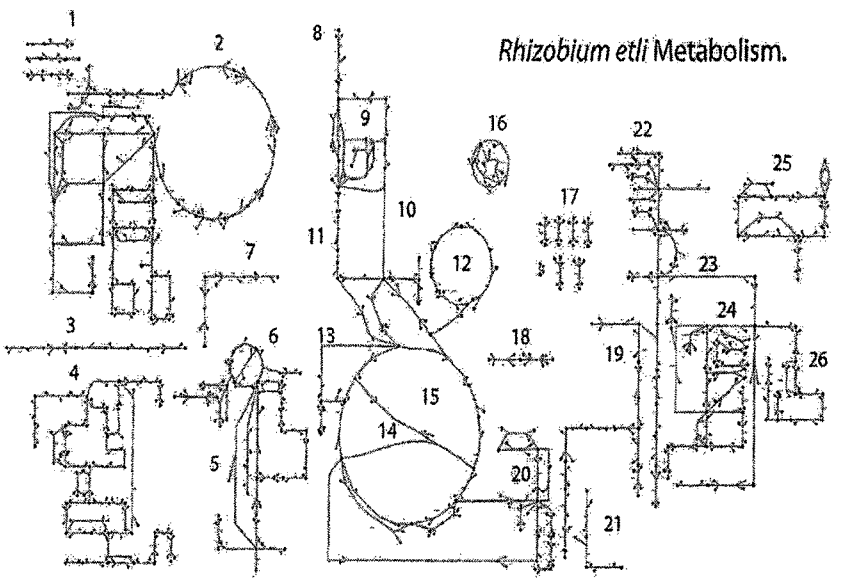


Fig. 1.22: Metabolic pathways for *Rhizobium etli* (Resendis -Antonio et al., 2007).
The metabolic reconstruction for *R. etli* includes 26 metabolic pathways involving 363 genes and 383 metabolic reactions. **(Fig. 1.22)**There are some major differences between free living *B. japonicum USDA110*and bacteroids **as depicted in Fig. 1.23.** (Vauclare et al., 2013).

1. Pentose and glucuronate.
2. Purine metabolism.
3. Histidine metabolism.
4. Pyrimidine metabolism.
5. Arginine and proline metabolism.
6. Amino group/ Urea.
7. Inositol metabolism.
8. Glycogen metabolism.
9. Penthose phosphate.
10. Entner-Doudoroff.
11. Glycolysis.
12. PHB cycle.
13. Aspartate metabolism
14. Glyoxylate shunt.
15. TCA cycle.
16. Oxidative phosphorylation.
17. Aminoacids tRNA.
18. Nitrogen fixation.
19. Valine, leucine and isoleucine.
20. Glutamate-glutamine metabolism.
21. Glutathione metabolism.
22. Glycine, serine and threonine metabolism.
23. Methionine metabolism.
24. Sulfur assimilation and cysteine metabolism.
25. Nicotine/nicotinamide.
26. Lysine.

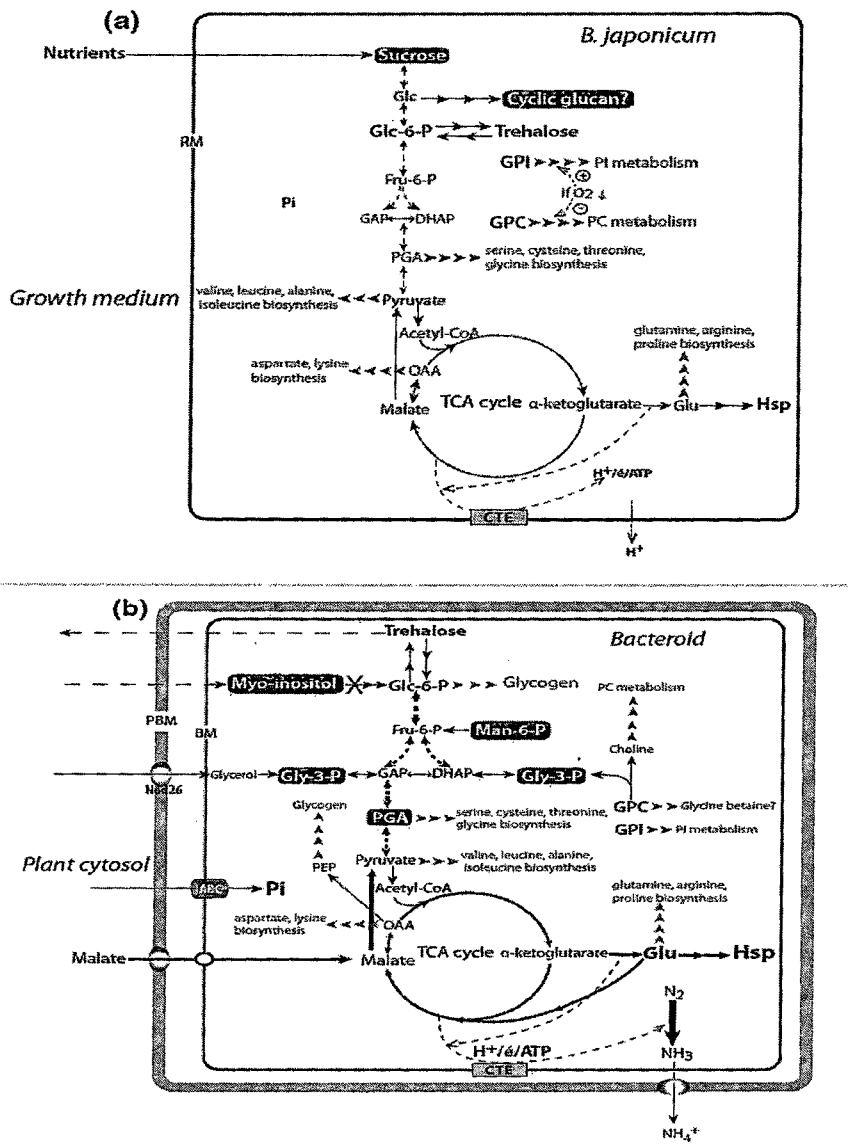


Fig. 1.23: Schematic representation of major differences between(a) free living *B. japonicum USDA110*and (b) bacteroids (Vauclare et al., 2013).

1.2.5: Importance of PEP-Pyruvate-OAA branch point in the cellular metabolism

In most chemotrophic, aerobic and facultatively anaerobic bacteria, the Embden–Meyerhof–Parnas pathway (glycolysis) or the Entner–Doudoroff pathway and the tricarboxylic acid (TCA) cycle are the main pathways of central metabolism. The former two are the primary routes for carbohydrate breakdown to phosphoenolpyruvate (PEP), pyruvate and acetyl-CoA,there by providing energy and building blocks for the synthesis of cellular components. The TCA cycle also serves a dual role in catabolism and anabolism by catalyzing complete oxidation of acetyl-CoA to CO_2 for respiratory ATP formation and by providing carbon precursor metabolites and NADPH for biosynthetic processes. Upon growth on TCA cycle intermediates or on substrates that enter central metabolism via acetyl- CoA (e.g. acetate, fatty acids and ethanol), the cycle intermediates malate or oxaloacetate must be converted to pyruvate and PEP for the synthesis of glycolytic intermediates.

This gluconeogenic formation of sugar phosphates from PEP is accomplished by the reversible reactions of glycolysis and one further enzyme, fructose-1,6-bisphosphatase.The metabolic link between glycolysis/gluconeogenesis and the TCA cycle is represented by the PEP–pyruvate–oxaloacetate node, also referred to as the anaplerotic node **(Fig. 1.24).**

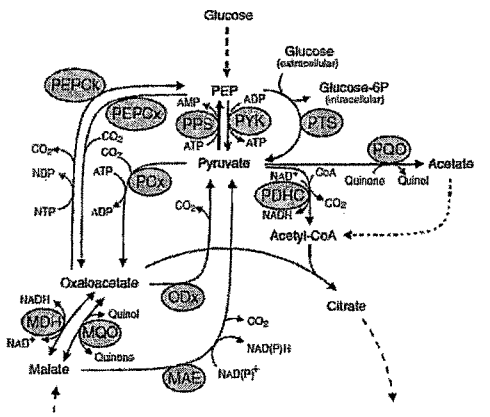


Fig. 1.24: The enzymes at the PEP–pyruvate–oxaloacetate node in aerobic bacteria.

The carbon flux control at the PEP–pyruvate–oxaloacetate node is often more complex than simple on/off regulation under a given condition and in some bacteria, two C3-carboxylating and up to three C4-decarboxylating enzymes are simultaneously active, even during growth on glucose as sole carbon and energy source (Dauner et al., 2001; Fischer et al., 2003) One enzyme, although operating in the same direction, fulfills a different function under certain conditions (Petersen et al., 2000).In other organisms or under different conditions, the same enzyme can operate in the reverse direction and thus contribute to a third function. These examples show that there is a complex interplay of the enzymes at the PEP–pyruvate–oxaloacetate node and it is obvious that sophisticated control is realized to ensure an optimal carbon and energy flow within central metabolism.

In most aerobic and facultatively anaerobic bacteria the chief metabolic pathways are the EMP pathway (glycolysis), ED pathway and the TCA cycle. The former two are involved in breakdown of carbohydrate to PEP and pyruvate which serve as precursors for biosynthesis of several cellular components. The pyruvate dehydrogenase (PDH) complex links the glycolytic/ED pathway and TCA cycle by further breakdown of pyruvate to acetyl-CoA which enters directly into TCA cycle. TCA cycle also performs dual functions of complete catabolism of acetyl-CoA for respiratory ATP formation as well as supplying the precursors for anabolism. On the other hand, when grown on TCA cycle intermediates or substrates that enter the central metabolism via acetyl-CoA, the cell diverts the metabolism towards gluconeogenic pathways for synthesis of the PEP and pyruvate to synthesize the essential sugar phosphates. Hence, the balance in the cellular physiology is highly dependent on the interactions between the catabolic and anabolic pathways.

The crucial metabolic link between the glycolytic / gluconeogenic / ED pathway and TCA cycle is the PEP-Pyruvate-OAA node often referred to as the anaplerotic node (Sauer and Eikmanns, 2005). The set of reactions operating at this node decide the carbon flux in a particular direction depending on the growth condition, thus acting as a key

switch governing the overall cellular metabolism. Under glycolytic conditions, PEP and pyruvate enter the TCA cycle by two routes, one by oxidative decarboxylation forming acetyl-CoA and second by C_3 carboxylation to form OAA which together energize the first reaction of TCA cycle. The formation of OAA by carboxylation of PEP or pyruvate is defined as anaplerosis, a process that replenishes the TCA intermediates utilized for anabolic purposes. Commonly, enzymes involved in C_3 carboxylation are PEP carboxylase (PPC) and pyruvate carboxylase (PYC). C_4 decarboxylation is irreversibly catalyzed by OAA decarboxylase (ODx). PEP carboxykinase (PEPCk), PEP carboxytransphosphorylase and malic enzyme perform the same function in a reversible manner.The differential occurrence of these enzymes in some of the commonly known bacteria is as listed in **Table 1.6.**

Table 1.6: Distribution of enzymes acting at PEP-pyruvate-OAA node in different bacteria. The numbers indicate the number of isozymes present in a given organism. Zero means that the organism has been tested for the enzyme or the respective gene however no activity is found so far. Empty space means that there is lack of evidence for the enzyme or the functional gene (Sauer and Eikmanns, 2005).

Organism	PEPCK	PPC	PYC	ODx	MAE	PPS	PDHC
E. coli	1(ATP)	1	0	0	1(NAD),1(NADP) 1(NADP)	1	1
C. glutamicum	1(ATP)	1	1	1	1 (NADP)	0	1
B. subtilis	1(ATP)	0	1	0	2(NAD) 1(NADP)	0	1
Rhizobium etli	1(ATP)	1	1		1(NAD) 1(NADP)	1	1
S. meliloti	1(ATP)	0	1		1(NAD) 1(NADP)	1	1
Rhododpseudomonas palustris	1(ATP)	1			1 (NAD)	1	1
P. citronellolis	0	1	1	1		1	1
P.fluorescens	1	1	1		1 (NADP)	1	1
Zymomonas mobilis		1			1		1

PEP–pyruvate–oxaloacetate node is very critical, still this node is quite flexible with respect to the metabolite pool and the prevailing biomolecular regulatory circuits. In certain organisms like *E. coli*, the regulation at this node is very simple, mediated by catabolite repression that does not allow gluconeogenic enzymes to express in presence of sugars. But in certain organisms like *Bacillus, Corynebacterium* and specific strains of *Pseudomonas*, more than one enzyme is responsible for C_3 carboxylation and C_4 decarboxylation, As a result, much more complex regulation is required at the anaplerotic node (Sauer and Eikmanns, 2005).

1.2.5.1: PEP-Pyruvate-OAA node in *E. coli*

The carbon flux at this node in *E. coli* is much more stringently regulated because PEP is involved in three major metabolic processes (i) PTS mediated sugar transport, (ii) PPC mediated anaplerotic reaction and (iii) as a precursor in the biosynthesis of amino acids (Clark, 1989; Gokarn et al., 2001). The enzymes participating in the PEP-Pyruvate-OAA interconversion in *E. coli* are as shown in **(Fig. 1.25).** PPC is the exclusive C_3 carboxylating enzyme while ATP-dependent PEPCk is primarily involved in C_4 decarboxylation and gluconeogenesis (Yang et al., 2003). Other options for C_4 decarboxylation are NADP dependent malic enzyme (ME) *mae*A and *mae*B whereas *sfc*A encodes NAD dependent ME which convert malate to pyruvate under physiological conditions but upon pyruvate accumulation can also act in reverse but thermodynamically favorable direction (Stols and Donnelly, 1997). However, malic enzymes are dispensable because PEP formation can be mediated by malate dehydrogenase and PEPCk. Interconversion of PEP and pyruvate is mediated by PYK and PEP synthetase (*pps*A, especially during growth on C3 acids like lactate and pyruvate). When grown on acetate, glyoxylate shunt also contributes to anaplerosis replenishing the essential C_4 intermediates of TCA cycle. Pyruvate apart from being converted to acetyl-CoA by PDH complex is acted upon by pyruvate oxidase to form acetate in the stationary phase (Dittrich et al., 2005). Hence, more than one metabolic reactions or enzymes are competing for the same metabolite to regulate this node.

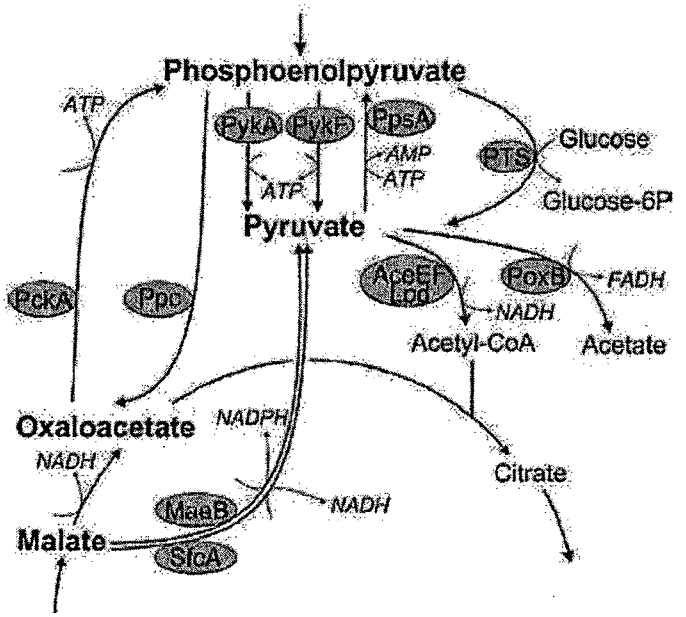
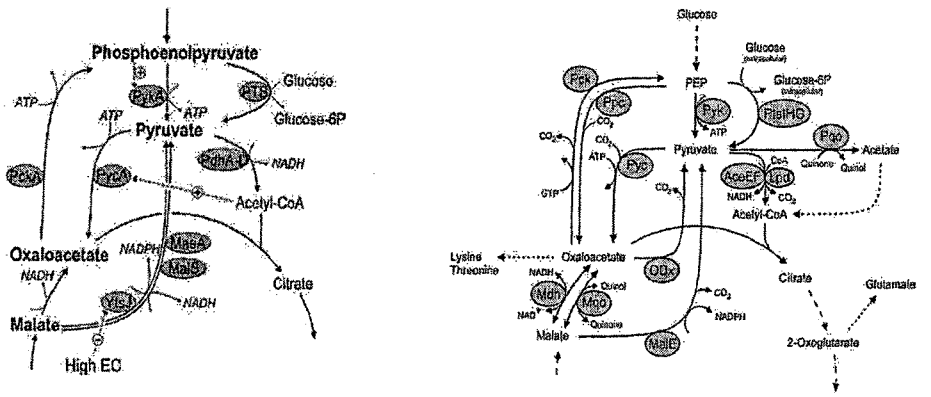


Fig. 1.25: The PEP-Pyruvate-OAA node in aerobic *E. coli.*

1.2.5.2: PEP-Pyruvate-OAA node in *B. subtilis and Corynebacterium glutamicum*

Bacillus is different from *E. coli* in being aerobic Gram-positive organism. The variations in the enzymes catalyzing the metabolic activity at this node in *B. subtilis* are as evident in **Fig. 1.26a**.The major difference is that instead of PPC, PYC acts as the sole anaplerotic enzyme to synthesize OAA. Due to absence of glyoxylate shunt, the organism fails to utilize carbon source that are metabolized via acetyl-CoA. PEPCk serves dual functions, primarily being involved in gluconeogenesis while performs a minor catabolic role in PYK and certain other mutants, by acting in reverse direction, despite being thermodynamically unfavorable (Sauer and Eikmanns, 2005). *B. subtilis* contains four paralogues *mle*A, *yts*J, *mal*S and *mae*A encoding putative ME, of which *yts*J encodes the major NADP-ME which is expressed constitutively on either glucose or malate (**Fig. 1.26a**). PYK and ME(s) constitute *pyruvate shunt* that substantially contributes to glucose uptake rate on carbon-limited conditions. Under these conditions, PEPCk flux is high which along with PYC and PYK constitutes an ATP dissipating futile cycle. Under gluconeogenic conditions PEPCk and MEs play a major role in redirecting the flux

through PEP Pyruvate-OAA node. Major regulation at this point is brought about by allosteric mechanisms and not by transcriptional control, unlike *E. coli*.



(a) ***B. subtilis*** (b) ***C. glutamicum***

Fig. 1.26: The PEP–pyruvate–OAA node in (a) *B. subtilis* and (b) *C. glutamicum*. Abbreviations denote the gene products that catalyze a given reaction: AceEF, subunits E1 and E2 of the pyruvate dehydrogenase complex; Lpd, subunit E3 of the pyruvate dehydrogenase complex; MalE, malic enzyme; Mdh, malate dehydrogenase; Mqo, malate: quinone oxidoreductase; ODx, oxaloacetate decarboxylase (gene not annotated); Pck, PEP carboxykinase; PtsIHG, phosphotransferase system; Pqo, pyruvate: quinone oxidoreductase; Pyc, pyruvate carboxylase; Pyk, pyruvate kinase

C. glutamicum shows some of the major differences in the type of enzyme occurring at this node as compared to *E. coli* and *B. subtilis* (**Fig. 1.26b**). It has both PPC and PYC as anaplerotic enzymes for C3 carboxylation, which are regulated by different allosteric effectors. Additionally, PEPCk, ME or ODx function for C4 decarboxylation converting OAA or malate to PEP or pyruvate (**Fig. 1.26b; Table 1.6**) of which PEPCk (GTP dependent) is the main enzyme with no anaplerotic functions. Unlike *E. coli* and *B. subtilis*, there is only one NADP dependent ME which acts for malate decarboxylation rather than for the reverse pyruvate carboxylation. In glucose grown cultures of *C. glutamicum*, the major anaplerotic role is played by PPC while PYC is the main enzyme for glutamate and lysine production (Shirai et al., 2007). Under glycolytic conditions, PYC, PEPCk and PYK are responsible for an energy (ATP/GTP) consuming (futile) cycle (**Fig. 1.26b**) but its physiological significance is unclear. The PDH complex is

exclusively involved in oxidative decarboxylation of pyruvate to acetyl-CoA . The reaction of the PDH complex may be bypassed by the combined activities of pyruvate:quinone oxidoreductase (catalyzing the oxidative decarboxylation of pyruvate with a naphtoquinone as electron acceptor), acetate kinase and phosphotransacetylase (both constitutively expressed and function to form acetyl-CoA from acetate), but the bypass will be thermodynamically unfavorable due to ATP requirement of acetate kinase. On acetate, the glyoxylate cycle was found to be the essential anaplerotic pathway (Reinscheid et al., 1994; Gerstmeir, R., 2003).

Several other check points are also functional in the cellular metabolism, apart from the PEP-Pyruvate-OAA branch point, which might be influenced by altered flux distribution at the anaplerotic node. The direct involvement of the anaplerotic node in the TCA cycle is evident because the interplay of enzymatic reactions utilizing PEP and pyruvate at this node supplies the substrates OAA and acetyl-CoA for citrate synthase.CS is non-redundant enzyme for catalyzing the first step of TCA cycle to form citrate and it catalyzes a crucial step at the branch-point of oxidative, lipogenic, and anaplerotic pathways (Walsh and Koshland, 1985a; 1985b). CS activity is regulated in *E. coli* at the transcriptional and allosteric level depending on the nature of the available carbon source (Park et al., 1994). Because of its key position as the first enzyme of the TCA cycle, CS had been assumed to be an important control point for determining the metabolic rate of the cell and the carbon flux at the anaplerotic node (PEP levels) which could directly determine the flux through TCA cycle (Peng et al., 2004).

Another branch point in the central metabolism occurs between TCA cycle and the glyoxylate shunt which is mainly governed by the two enzymes NADP dependent ICDH and ICL competing for the common metabolite isocitrate. ICL in *E. coli* is mainly regulated at the level of expression depending on the growth conditions (acetate or glucose as carbon source) while ICDH is regulated by phosphorylation/ dephosphorylation (Walsh and Koshland, 1985b). Low PEP levels increase flux via glyoxylate cycle (Yang et al., 2003; Peng et al., 2004) and PEP *in vitro* inhibits ICDH as well as ICL; however its physiological significance is yet unclear (Ogawa et al., 2007).

1.2.6: Comparison of Central Carbon metabolism

The ED pathway and the TCA cycle were the almost exclusive pathways in *P. fluorescens* and the overall TCA flux was higher in pseudomonads along with very low acetate overflow metabolism as compared to *E. coli,* (Fuhrer et al., 2005). The ICL and ICDH activities at TCA-glyoxylate branch-point in *P. fluorescens* as well the activities of malate synthase and CS alter significantly in response to aluminum stress (Hamel and Appanna, 2001). The gram-positive and gram-negative model bacteria *E. coli* and *B. subtilis* relied primarily on the EMP pathway for glucose catabolism and the relative TCA cycle flux was much lower than that in the other species because secretion of the incompletely oxidized overflow product acetate was extensive. **(Fig. 1.27).** At 38%, the PP pathway flux in *B. subtilis* was the highest observed in all species (Fuhrer et al., 2005).

As a fast-growing rhizobium with a generation time of less than 6 h on complex medium, *S. meliloti* converted glucose to gluconate, which then entered metabolism. Pyruvate carboxylase was assumed to be the anaplerotic reaction by analogy to *A. tumefaciens* (Fischer and Sauer, 2003) Both the PP and ED pathways were present, and glycolysis was absent, in both rhizobia (Arthur et al., 1979; Stowers, 1985), which was confirmed by METAFoR analysis. In both rhizobia, the ED pathway was basically the exclusive pathway of glucose degradation, while the pentose-5-P precursors for biomass were generated through the oxidative and nonoxidative branches of the PP pathway **(Fig. 1.27)**. With a flux well above 100% relative to the glucose uptake rate, *Rhizobium* species exhibited higher TCA cycle fluxes than those of *Agrobacterium tumefaciens*, two pseudomonads, *Sinorhizobium meliloti*, *Rhodobacter sphaeroides*, *Zymomonas mobilis*, and *Paracoccus versutus* investigated here. The *E. coli* and *B. subtilis* PP pathway flux contributed substantially to catabolism (12 and 27%, respectively), Second, the *E. coli* and *B. subtilis* TCA cycle flux was relatively low, metabolism was not fully respiratory, and there was extensive overflow metabolism that is also referred to as aerobic fermentation. In addition to the linear ED pathway, which may be inducible in *E. coli*

during growth on gluconate or constitutive, as it is in *Z. mobilis*, cyclic operation of the ED pathway was reported for organisms that lack phosphofructokinase, e.g., pseudomonads (Lessie et al., 1984) and *S. meliloti* (Irigoyen et al., 1990).

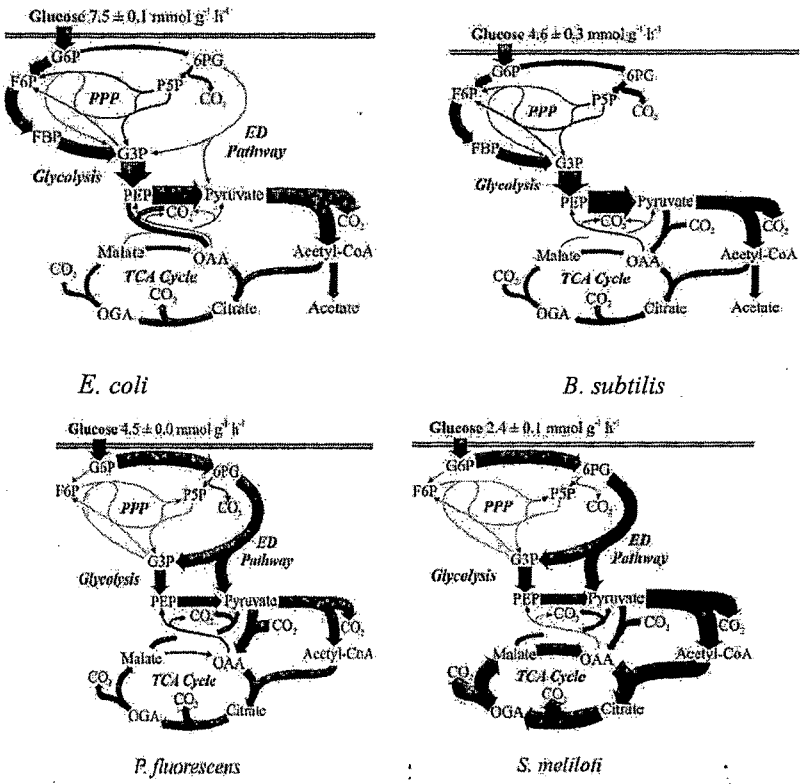


Fig. 1.27: In vivo carbon flux distribution in *E. coli B. subtilis P. fluorescens* and *S. meliloti*. All fluxes were normalized to the glucose uptake rate that is given at the top of each panel, and the widths of the arrows are scaled to the relative percentages of flux. Fluxes below 2.6% of the glucose uptake rate are represented by non-scaled hairlines.

Under any given condition, the metabolic network and its regulatory circuits synchronize to balance the catabolic and anabolic reactions to meet the requirements of energy and biomass. For smooth functioning of the metabolism, there are several check-points existing in the system at the junction of any two metabolic pathways. These branch points are sufficiently flexible so as to maintain a tight control over the carbon flux through a particular path under a particular condition. The following few sections discuss the importance of these check-points in the metabolism of several organisms and their role as potential new targets for metabolic engineering.

1.3: Metabolic engineering

Metabolic engineering is referred to as the "directed improvement of cellular properties through the modification of specific biochemical reaction(s) or the introduction of new ones, with the use of recombinant DNA technology" (Stephanopoulos, 1999). It is explained as the targeted and purposeful alteration of metabolic pathways found in an organism in order to understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly (Lessard, 1996). This multidisciplinary field involves implementation of principles from chemical engineering, biochemistry, mathematical and computational sciences, and owes its existence to fast developing molecular biology techniques.

Earlier strategies for genetic modifications were based on random chemical mutagenesis and selection of strains exhibiting desired phenotypes but its success relied heavily on mutagens and creative selection techniques (Koffas et al., 1999). Studies by Bailey (1991) and Stephanopoulos and Vallino (1991) pioneered the major transition of this classical approach to a more systematic and rational approach called *Genetic Engineering* involving the use of recombinant DNA technology. The technical manifestation of genetic engineering involving manipulation of enzymatic, transport and regulatory functions of the cell by using recombinant DNA technology was better referred to as "**Metabolic Engineering**" (Stephanopoulos and Vallino, 1991; Koffas et al., 1999).

An integrated approach towards engineering of microorganisms into 'microbial chemical factories' (MCFs) that can be used in a 'biorefinery' for the conversion of biomass into both fuels and value added biochemicals. Biosynthesis of high value chemical compounds from biomass using natural or engineered pathways in microorganisms also serves as a promising alternative to chemical synthesis processes that employ expensive, hazardous and non-renewable raw materials and reagents as well as harsh processing conditions. The evolution of the field of metabolic engineering has

developed principles and tools that enable construction and optimization of MCFs by tapping into naturally occurring pathways in specific host organisms, heterologous expression of non-native pathways in well characterized hosts, or engineering de novo biosynthetic pathways for synthesis of various natural and non-natural products. De novo pathway engineering refers to the design and construction of novel pathways (hitherto unknown in nature in any single organism) by assembling multiple existing partial pathways from different organisms or using promiscuous or engineered enzymes as biocatalysts to catalyze a series of biotransformations with non-natural substrates (Prather and Martin 2008).

The steps involved in the design of novel pathways for MCFs and various tools and approaches used for their optimization are outlined in **Fig. 1.28**. Once a pathway has been designed and selected for experimental exploration (Martin et al., 2009), suitable pathway enzymes are selected to catalyze the reaction steps. Pathway enzymes are the tireless machines of the MCFs that sequentially process raw materials into desired value added products and govern the pathway rates, selectivity, yield and overall productivity. Whenever possible, enzymes known to specifically catalyze reactions with the respective pathway intermediates are selected. The selected enzymes are then expressed in a suitable organism grown in a culture medium supplemented with the required starting materials for the microbial synthesis of the desired value added product.

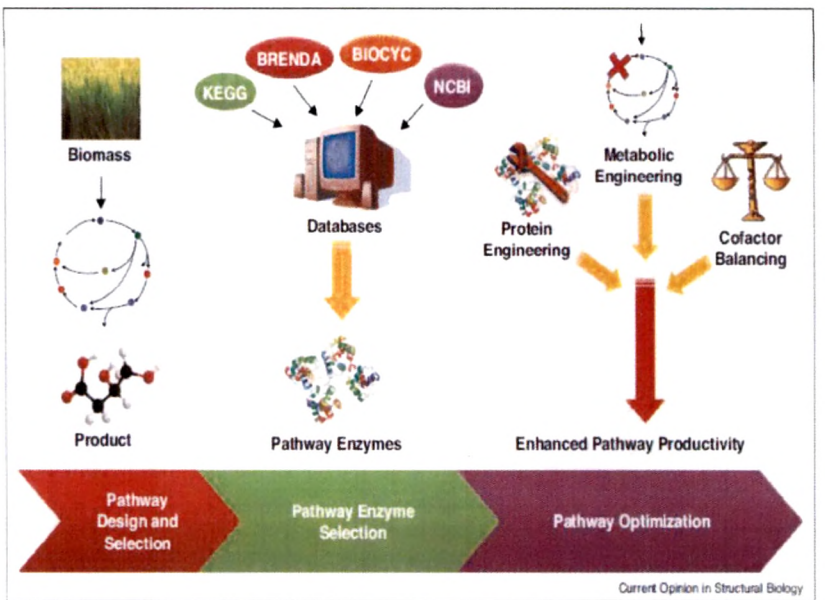


Fig. 1.28: Design and engineering of pathways for microbial chemical factories (MCFs) (Dhamankar et al., 2011).

The first step in engineering novel or natural pathways for MCFs is to identify potential natural cell metabolites or biomass derived feedstocks that can serve as starting materials and the series of biochemical reactions required to convert these into the desired product. Some of the computational tools available for identifying and selecting from the multiple possible pathways connecting different starting materials to a product of interest are studied (Martin et al., 2009). Once a pathway is selected, appropriate natural enzymes expected to catalyze pathway reactions need to be selected using enzyme information from various databases. *In silico* approaches such as protein BLAST searches and molecular docking may help in such enzyme selection. Further pathway optimization to enhance product titers relies on an integrated approach composed of metabolic engineering to enhance precursor metabolite availability using gene knockouts and enzyme expression level manipulation, Protein engineering to enhance pathway enzyme specificity and activity and cofactor balancing via effective cofactor recycling(Prather and Martin 2008; Martin et al., 2009).Protein engineering can be used for improving the selectivity and activity of the pathway enzymes and can effectively complement

conventional metabolic engineering approaches such as increasing the precursor supply by varying pathway enzyme expression levels or knocking out competing pathways to enhance productivity.

This has resulted in the metabolic engineering 'toolbox' greatly expanding from conventional approaches such as rationally deleting and/or over-expressing endogenous genes and introducing heterologous genes to now include tools capable of controlling gene expression and modulating regulatory networks throughout the cell (**Fig. 1.29**) (Klein-Marcuschamer et al., 2007; Tyo et al., 2007 and 2010; Blazeck and Alper, 2010).

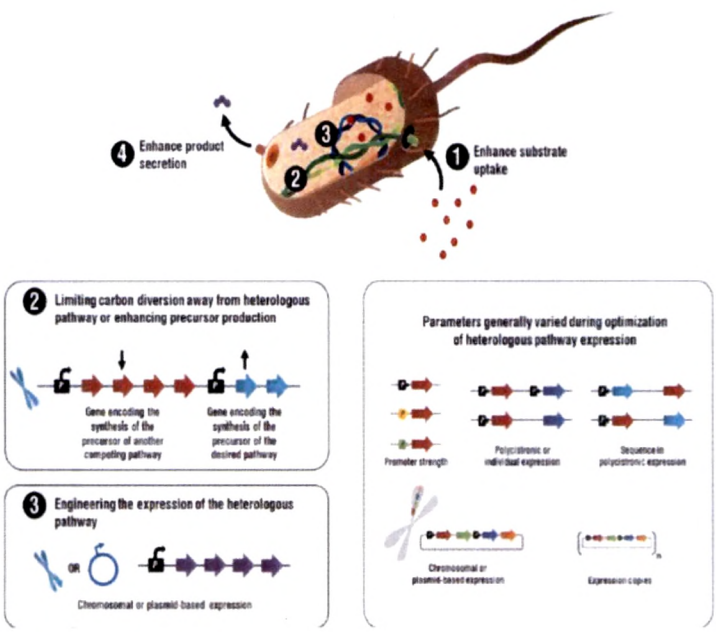


Fig. 1.29: A holistic view of metabolic and cellular engineering (Yadav et al., 2012) Methods for manipulating the flux from a substrate towards the product can be grouped into four categories: (1) enhancement in the rate of substrate uptake, (2)reduction of flux to undesirable by-products and enhancement of precursor and cofactor flux,(3)introduction of the heterologous pathway and optimization of the activity of its constituent enzymes and (4)export of the product to the extracellular medium in order to shift equilibrium towards product formation.

1.3.1 Metabolic engineering of organic acids for P solubilization.

Genetic manipulation of PS bacteria can be done to improve their ability to improve plant growth may include cloning genes involved in both mps and organic P-solubilization (ops),followed by their expression in selected rhizobacterial strains. For example, have shown that Mps activity is genetically coded in a gene cluster on plasmids of microbes endowed with PS activity (Goldstein and Liu, 1987 Rodriguez et al., 2006).They further transferred this gene cluster to an *E. coli* strain that did not previously possess PS activity but could demonstrate that the transferred gene was expressed in the transgenic *E. coli* strain. They have also found that the gene expression and mps activity of bacteria is affected by the presence of soluble P in the medium (feed-back regulation).Chromosomal insertion of these genes under appropriate promoters is another interesting approach (Rodriguez and Fraga 1999) (**Table 1.7**).

Table 1.7 Cloning of genes involved in mineral phosphate solubilization (Zaidi et al., 2009)

Microorganisms	Gene or plasmid	Features	Reference
Serratia Marcescens	pKG3791	Produces gluconic acid and solubilizes P	Krishnaraj and Goldstein, 2001
Rahnella aquatilis	pKIM10	produces gluconic acid and Solubilizes P in E. coli DH5a	Kim et al., 1998
Enterobacter agglomerans	pKKY	Solubilizes P in E. coli 109; Does not lower pH	Kim et al., 1997
Pseudomonas Cepacia	Gab Y	Produces gluconic acid and solubilizes mineral P in E. coli JM109; No homology with PQQ genes	Babu-Khan et al., 1995
Erwinia herbicola	Mps	Produces gluconic acid and solubilizes mineral P in E. coli HB101; Probably involved in PQQ synthesis	Goldstein and Liu, 1987
			Buch et al., 2009
			Buch et al., 2010
E. asburae PSI3	*gad*	Produces gluconic acid and 2-KGA solubilizes RP	Kumar et al., 2012
	Yf citC	Produces gluconic acid and citric	Adhikary, 2012

		solubilizes RP	
Citrobacter DHRSS	Yf citC and *vgb*	Produces citric acid and solubilizes RP	Yadav 2013
Citrobacter DHRSS	////	Produces oxalic acid and solubilizes P	Yadav 2013
***Herbaspirillum seropedicae* Z67**	Yf citC	Produces citric acid and solubilizes P	Wagh, 2013
***Herbaspirillum seropedicae* Z67**	*Pqq,gad*	Produces gluconic and 2-KGA and solubilizes P	Wagh, 2013

Gene technologies can be used to enhance specific traits that may increase an organism's capacity to mobilize soil P directly, enhance its ability to colonize the rhizosphere (ie, rhizosphere competence, Lugtenberg *et al.*, 2001), or even to form specific associations with plant roots (Bowen and Rovia, 1999). Alternatively, microorganisms may provide a novel source of genes for directly modifying plants. For example, it has been reported that, when expressed in roots, a bacterial citrate synthase gene increases the exudation of organic acids and significantly improves plant access to soil P (López-Bucio *et al.*, 2000).

1.3.2: Genetic manipulations at the anaplerotic node in *E. coli, B. subtilis* and *C. glutamicum*

Anaplerotic node has been manipulated not only for understanding the regulatory network but also for altering molecular fluxes for improving the bioprocesses. One of the most successful examples is the case of fermentative and aerobic succinate overproduction in *E. coli* (detailed in Section 1.3). Pyruvate overproduction was achieved in various *E. coli* mutants having block in conversion of PEP to OAA and pyruvate to acetyl-CoA, PEP, acetate, lactate and ethanol by deletion of the genes coding for the PDH complex (*ace*EF), pyruvate formate lyase (*pfl*B), PEP synthetase (*pps*), pyruvate: quinone oxidoreductase (*pox*B), acetate kinase, lactate dehydrogenase (*ldh*A), PPC and alcohol dehydrogenase (Wendisch et al., 2006). Anaplerotic node in *E. coli* has been successfully engineered for optimizing the amino acid production as PEP forms a key precursor molecule (Bongaerts et al., 2001; Kramer et al., 2003). These strategies include avoiding the drain of PEP to pyruvate by mutation in gene encoding PYK; a non-PTS

sugar uptake and increasing the gluconeogenic fluxes to PEP (e.g. overexpression of PEP synthetase), coupled with overexpression of transketolase which increases erythrose-4P level (Patnaik and Liao, 1994; Flores et al., 1996).

Several mutations in the enzymes acting at this node affect the physiological state of other relevant enzymes and *in vivo* pathway fluxes. Deletion of PYK gene developed a local catabolic loop involving PPC and ME which jointly function for both anapleriosis and catabolism thereby highlighting a newer function of the two enzymes (Emmerling et al., 2002) in *E. coli* but not in *B. subtilis*. *E. coli ppc* mutant exhibits auxotrophy for TCA cycle intermediates like succinate when grown on glucose because glyoxylate bypass that can theoretically substitute the PPC reaction is inactive due to catabolite repression as well the competition of isocitrate dehydogenase (ICDH) and isocitrtae lyase (ICL) for the common substrate isocitrate. The anaplerotic function in *ppc* mutant could be fully restored by overexpressing pyruvate carboxylase (*pyc*) which is otherwise absent in *E. coli* (Gokarn et al., 2000; 2001). Similarly *ppc* gene overexpression in *E. coli* under aerobic conditions reduced acetate formation and a significantly increased biosynthetic efficiency (Farmer and Liao, 1997). Collectively, these results suggest that the anaplerotic reaction in *E. coli* is not optimized for unhampered growth on glucose and that some of the enzymes apart from their classically recognized functions in catabolism, anaplerosis and gluconeogenesis play novel roles in the metabolism of some bacteria.

Although extensive information on the regulatory mechanisms operating at the anaplerotic node is available for *B. subtilis* (Sauer and Eikmanns, 2005), no genetic manipulations are reported at this node. *C. glutamicum* is an aerobe industrially important for production of L-Lysine and L-glutamate (Kiefer et al., 2004). The fact that PEP-Pyruvate-OAA node is crucial for the supply of precursors for amino acid biosynthesis, a lot of focus has been there on the enzymes and their regulations involved at this node. Increase in PYC activity and abolition of PEPCk activity in *C. glutamicum* independently resulted in increased production of TCA cycle-derived amino acids like glutamate and lysine (Sauer and Eikmanns, 2005). The levels of ME activity affected the growth pattern of *C. glutamicum* on lactate but not on glucose or acetate (Gourdon et al., 2000). *C. glutamicum* overexpressing ME accumulated high levels of pyruvate in the medium. *C.*

glutamicum ppc mutant grown under biotin limitation accumulated pyruvate due to which ME functioned in the reverse direction by utilizing pyruvate to replenish the TCA cycle intermediates (Gourdon et al., 2000).

All these bacteria, discussed above, metabolize glucose via the traditional EMP pathway yet exhibit such a diversified anaplerotic node. Pseudomonads, as discussed in earlier sections, are metabolically distinct with respect to non-PTS glucose uptake, two glucose catabolic routes ultimately following the ED pathway and exhibiting strain specific variations in the occurrence of enzymes of central carbon metabolism and hence are likely to display a completely different interplay between the enzymes at the anaplerotic node. On account of the agricultural importance of pseudomonads and metabolic versatility, detailed analysis of prevailing regulations at the PEP-Pyruvate-OAA node by various genetic perturbations would not only add to fundamental knowledge but also might discover novel targets for metabolic engineering.

1.3.3: Metabolic engineering of organic acids for P solubilization.

1.3.3.1: Role of citric acid in P solubilization

The secretion of gluconic acid is the major mechanism of P-solubilization by gram negative bacteria (Goldstein 1995; Kim et al., 1998). The acidification of soil by organic acids depends on both the nature and quantity of the organic acid for e.g. acetic, lactic and succinic at 100 mM bring about a drop in pH of a soil solution from around 9.0 to about 6.0; a similar drop is brought about by only 20 mM of gluconic acid, 10 mM of oxalic acid and even lesser amount of citric and tartaric acids (Gyaneshwar et al. 1998; Srivastava *et al.,* 2006) (**Table 1.8**). Addition of organic acids decreases the pH of the alkaline vertisol soil solution in the order Acetic = Succinic = Lactic < <Gluconic < <Oxalic < Tartaric = Citric and results in P release in a similar order. *Penicillium billai* secretes 10 mM each of citric and oxalic acids (Cunningham and Kuiack, 1992) and has been shown to be effective in releasing P in the field conditions (Asea et al., 1988). On the other hand, *C. koseri* and *B. coagulans* were found to secrete various organic acids in

the range 1-5 mM whereas as the concentration of these acids required to reduce the pH of the soil was 20-50 times more.

Table 1.8: Organic acids for phosphate solubilisation in different soil types (Srivastava et al., 2006).

Organic acid	Alkaline Vertisol pH >7	Acidic alfisol supplemented With RP pH < 7
Citric acid	10 mM	10-20 mM
Oxalic acid	10 mM	5-10 mM
Gluconic acid	20 mM	50 mM
Tartaric acid	10 mM	20 mM

Citric acid has better chelation properties due to presence of its three –COOH group" s having pKa values of 3.15, 4.77, and 6.40, respectively. Hence, *Rhizobium* strains producing citric acid could be effective as P biofertilizers in alkaline soils

1.3.3.2: Metabolic engineering of rhizobacteria for citric acid secretion.

Rhizobia are well known for nitrogen fixing ability and other PGPR effects. Genome sequence of several bacteria and metabolic data reveals that there are lot of interspecies diversity in terms of occurrence and regulation of enzymes at the central metabolism and PEP-Pyruvate-OAA node. Also *Rhizobium* has shown P solubilization ability. This study is an effort to genetically engineer a stable system for phosphate biofertilizer and to examine its applicability amongst *Rhizobium* spp. *Bradyrhizobium* is very efficient is nitrogen fixation which is more than the plant requirement and releases ammonia which can be used by other crops. Thus could be useful in intercropping systems *B. japonicum USDA110*USDA110 can nodulate several legumes and it has PQQ but not sufficient to function as cofactor for GDH..*M. loti* MAFF303099 nodulates lotus plants and it lacks PQQ. *S. fredii* NGR 234 is a broad host range *Rhizobium* .Thus these three *Rhizobium* strains were selected for the study. Present study describes improvement in citric acid secretion in *B. japonicum USDA110*USDA110, *M. loti* MAFF303099 and to the required amount for P release from soils. Additionally, the genetic manipulations

need be directed to the chromosomal integration as it would lead not only to increased stability but also decrease the metabolic load caused by the presence of the plasmids in the bacterial cell (Buch et al., 2010; Sharma et al., 2011).

P-solubilization was improved by overexpression of PEP carboxylase (*ppc*) and citrate synthase (*cs*) genes in fluorescent pseudomonads. Introduction of genetic perturbations in the glucose metabolic pathway at the anaplerotic node of *Pseudomonas* and at citric acid formation from OAA and acetyl-CoA by the overexpression of *ppc* and *cs* genes, respectively, demonstrated a direct correlation of increased CS activity with citrate accumulation (Buch et al 2010). The resultant ~2-fold increase in CS activity in the *gltA*-overexpressing strain *Pf* (pAB7) enhanced the intracellular and extracellular citric acid yields. Low extracellular citrate levels as compared to the intracellular levels in Pf(pAB7) suggested a probable limitation of efficient citrate transport (Buch et al 2009). Studies done in our laboratory in which overexpression of NADH insensitive *cs* gene in *P. fluorescens Pf* O-1 strains harbouring pY145F led to simultaneous increase in intracellular and extracellular citric acid level. However, the intracellular increase was not proportional to the extracellular increase in citric acid. The reason may be the inefficiency of the native proton dependent citrate transporter.(Adhikary, 2012)

Herbaspirilum seropidecae Z67 (pAB7) and (pJNK3) transformants increased CS activity but citric acid secretion was not significant. *Hs* (pJNK3) secreted 45 mM acetic acid while *Hs* (pJNK4) secreted 2.7mM and 51 mM citric and acetic acids, respectively. *Hs* (pJNK3) and (pJNK4) transformants, released ~80 µM and ~110 µM amount of P, respectively, in buffered medium in both aerobic and microaerobic conditions. These transformants also showed better growth and colonization parameters (Wagh, 2013).

The 2-hydroxycarboxylate transporter (2HCT) family of secondary transporters are transporters for citrate, malate and lactate. Na^+ coupled citrate transporters like CitS of *Klebsiella pneumoniae* and CitC of *Salmonella enterica* are highly specific for citrate (Lolkema, 2006).Earlier studies in the lab have shown that, among them, *Salmonella typhimurium* Na^+ dependant citrate transporter is a better citrate transporter (Adhikary, 2012).

Currently there is more interest in modifying the chromosome itself than in plasmid-based gene manipulation, which necessitates selection pressures such as antibiotic supplementation and is not feasible in agriculture practices. Plasmids can be lost from the host cell more easily than genes found on the chromosome by a variety of mechanisms.

1.4: RATIONALE

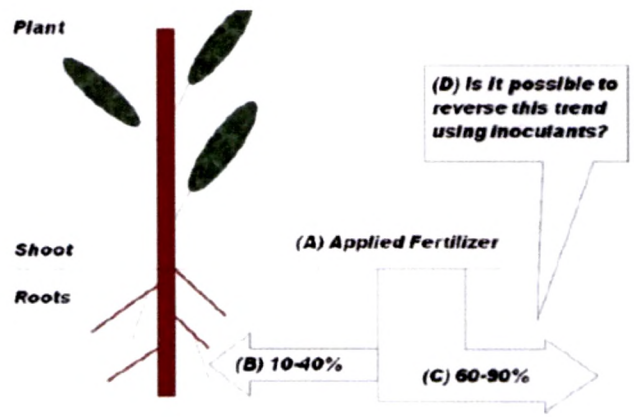


Fig. 1.30: Model for improved plant nutrient use efficiency with inoculants (Adesemoye et al., 2009).

The part of the applied fertilizer that is lost could be in the range of 60% to 90% of the original amount of fertilizer or manure applied (Hardy and Eaglesham 1995; Rowarth 1997; Hood et al. 1999; Gyneshwar et al. 2002; Barlog and Grzebisz 2004; Kleinman et al. 2005). Examples of the route of nutrient loss include N leaching, P fixation, and nutrient run-off among others. Then, the question being asked is whether it is possible to reverse the trend of (i) loosing high percentage of applied fertilizer and (ii) applying large amounts of fertilizers by supplementing reduced fertilizer with inoculants while maintaining plant growth and high yield comparative to the use of full recommended fertilizer rates?

Under any given condition, the metabolic network and its regulatory circuits synchronize in order to balance the catabolic and anabolic reactions to meet the requirements of energy and biomass. For smooth functioning of the metabolism, there are

several check-points existing in the system at the junction of any two metabolic pathways. These branch points are sufficiently flexible so as to maintain a tight control over the carbon flux through a particular path under a particular condition .CS had been assumed to be an important control point for determining the metabolic rate of the cell and the carbon flux at the anaplerotic node (PEP levels) which could directly determine the flux through TCA cycle (Peng et al., 2004).

Under diverse soil and agro-climatic conditions, the organisms with phosphate solubilizing abilities have proved to be an economically sound alternative to the more expensive superphosphates and possesses a greater agronomic utility (Khan et al., 2007; Xiao et al., 2009). Emphasis is therefore, being placed onto the possibility of greater utilization of unavailable phosphorus forms wherein the phosphate solubilizing microbes could play a pivotal role in making soluble phosphorus available to plants (Khan et al., 2010).

Key questions –

1. What would be the effect of incorporation of *ppc*, *cs* and *citrate transporter* genes on the biosynthesis and secretion of citrate in Rhizobia?
2. If citric acid is secreted, whether the amount is sufficient for solubilization of rock phosphate in media conditions mimicking alkaline vertisols?
3. Can citric acid secretion promote the plant growth promotion and nodulation efficiencies of Rhizobia?
4. Can metabolic engineering strategy for citric acid be applied to different Rhizobia?

Thus the present study dealt with monitoring the effect of overexpressing *ppc* gene of *Synechococcus elongatus* PCC 6301, *E. coli cs*, NADH insensitive Y145F *cs* gene and NADH insensitive Y145F *cs* along with Na^+ dependant citrate transporter genes on production of organic acid and mineral phosphate solubilizing ability in two different *Rhizobium* strains: *Bradyrhizobium japonicum* USDA 110 and *Mesorhizobium loti* MAFF303099. This transgenic *Rhizobium* having P solubilizing activity could be

effective as N and P biofertilizers. Part of the study also dealt with monitoring the effect of genomic integration of *yc* operon containing *E. coli* NADH insensitive *cs* along with *S. typhimurium citC, vgb* and *egfp* gene on production of organic acid and mineral phosphate solubilizing ability in *Bradyrhizobium japonicum* USDA 110, *Mesorhizobium loti* MAFF303099 and *Sinorhizobium fredii* (NGR 234). Preliminary plant experiment was done to see the effect of *S. fredii* NGR 234 genomic integrant on growth promotion of Mung bean plants. The overall strategy of the present study is depicted in **Fig. 1.31**.

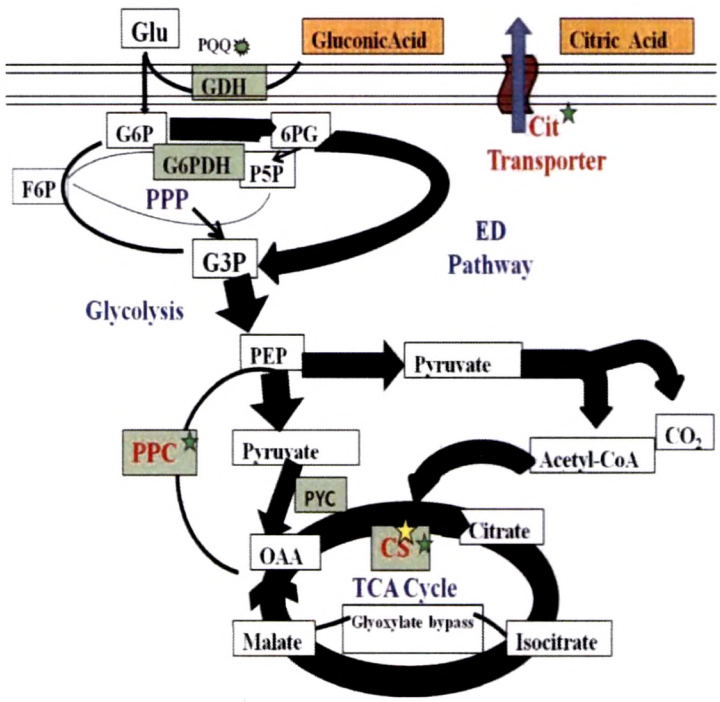


Fig. 1.31: Metabolic basis for designing the genetic modifications in *Rhizobium* strains. - Presence of PQQ is species dependent; .

Shows overexpression of *ppc,cs* and *citC* genes

Shows overexpression of NADH insensitive *cs* gene

1.5: Objectives of the present study

The objectives of the present study were defined as follows-

1. Effect of constitutive overexpression of *ppc* gene of *Synechococcus elongatus* PCC 6301 on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF030669

2. Effect of constitutive overexpression of *E. coli cs* gene on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF030669

3. Effect of overexpression of *E. coli* NADH insensitive Y145F *cs* gene on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF030669

4. Effect of overexpression of *E. coli* NADH insensitive Y145F *cs* and *Salmonella typhimurium* Na^+ dependant citrate transporter on production of organic acid in *B. japonicum* USDA110 and *M. loti* MAFF030669

5. Effect of genomic integration of *E. coli* NADH insensitive *cs* along with *Salmonella typhimurium* Na^+ dependent citrate transporter with *vgb, egfp* on production of organic acid in *B. japonicum* USDA110, *M. loti* MAFF030669 and *S.fredii* NGR 234.

6. Effect of *Sinorhizobium fredii* NGR 234 genomic integrant containing *E. coli* NADH insensitive *cs* along with *S. typhimurium citC, vgb* and *egfp* gene cluster on growth promotion of Mung bean plants.