Genetic modification of *Herbaspirillum seropedicae* Z67 for the development of mineral phosphate solubilization (MPS) to enhance nitrogen fixation ability.

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BY

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

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

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Date: 12/8/2013

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

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Dedicated to my Beloved my Parents...

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Jitendra wagh

Abbreviations

ATCC	American type culture collection
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D galactopyranoside
IPTG	Isopropyl β-D thio galactopyranoside
LB	Luria broth
SDS	Sodium Dodecyl Sulphate
APS	Ammonium persulfate
Ori	Origin of replication
WT	Wild type
PPC	Phosphoenolpyruvate carboxylase
CS	Citrate synthase
PYC	Pyruvate carboxylase
ICL	Isocitrate lyase
ICDH	Isocitrate dehydrogenase
GDH	Glucose dehydrogenase
G-6-PDH	Glucose-6-phosphate dehydrogenase
GADH	Gluconate dehydrogenase
PEPCk	Phosphoenolpyruvate carboxykinase
PYK	Pyruvate kinase
ME	Malic enzyme
MDH	Malate dehydrogenase
LDH	Lactate dehydrogenase
POX	Pyruvate oxidase
PDH	Pyruvate dehydrogenase
PFK	Phosphofructokinase
PTS	Phosphotransferase system
GFP	Green fluorescent protein
PQQ	Pyrolloquinoline quinone
G-6-P	Glucose-6-phosphate
6-PG	6-phosphogluconate
PEP	Phosphoenolpyruvate
OAA	Oxaloacetate
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
cAMP	Cyclic adenosine monophosphate
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
DTT	1, 4-Dithiothreitol
EMP	Embden–Meyerhof–Parnas pathway

TCA	Tricarboxylic acid
PPP	Pentose phosphate pathway
ETC	Electron transport chain
ED	Entner-Doudoroff
PSM	Phosphate solubilizing microorganisms
P/Pi	Phosphate / inorganic phosphate
HPLC	High performance liquid chromatography
BLAST	Basic local alignment search tool
PCR	Polymerase Chain Reaction
PAGE	Polyacrylamide Gel Electrophoresis
EDTA	Ethylene diamine tetra acetic acid
Tris Tris	(hydroxymethyl) aminomethane buffer
CTAB	Cetyl trimethylammonium bromide
PGPR	Plant Growth Promoting Rhizobacteria
RE	Restriction Endonuclease
nm	Nanometer
ng	Nanogram
µM	Micromolar
kb	kilobase (s)
bp	base pair (s)
kDa	Kilodalton
rpm	Revolutions per minute
OD	Optical Density
DCW	Dry cell weight
mM/M	Millimolar/Molar
µl/ml	Microlitres/millilitres
Tet^r	Tetracycline
Ap	Ampicillin
Km^r	Kanamycin
$Strp^r$	Streptomycin

Note: The full forms of several rarely used abbreviations have been described within the text.

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Chapter 1

Introduction

1.1: Introduction

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

1.2: Global Phosphate Status.

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

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

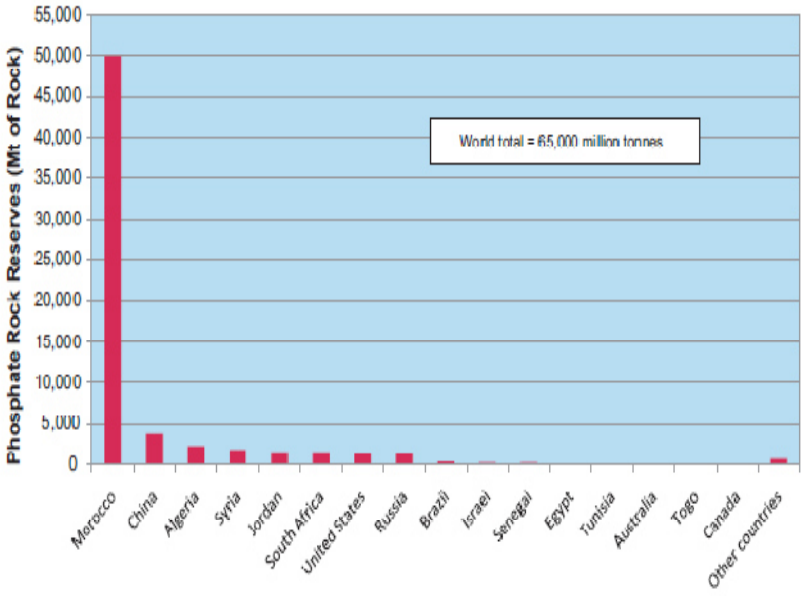


Fig. 1.1: Global distribution of phosphorus reserve

(Cooper et al., 2011).

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

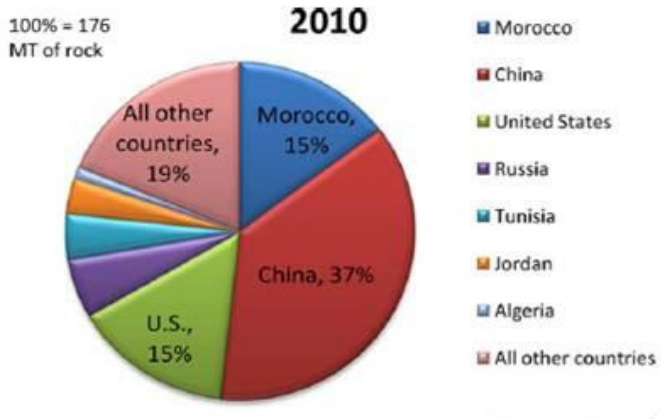


Fig. 1.2: Share of Rock phosphate production in 2010 (Cooper et al., 2011).

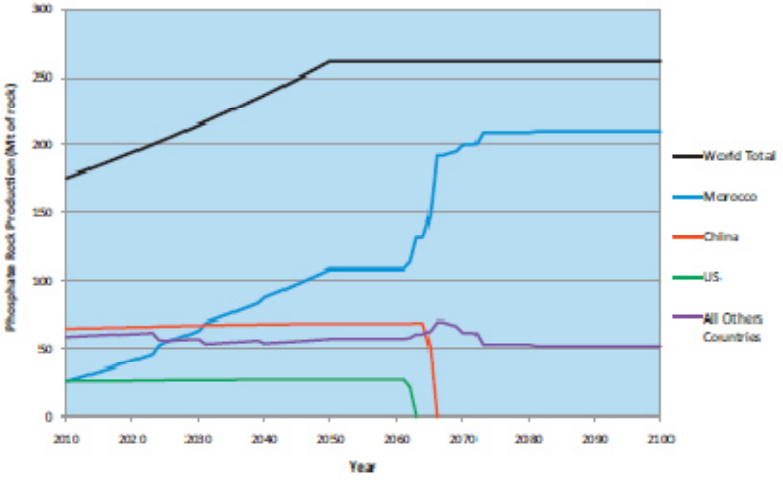


Fig. 1.3: Total global P consumption (Cooper et al., 2011).

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

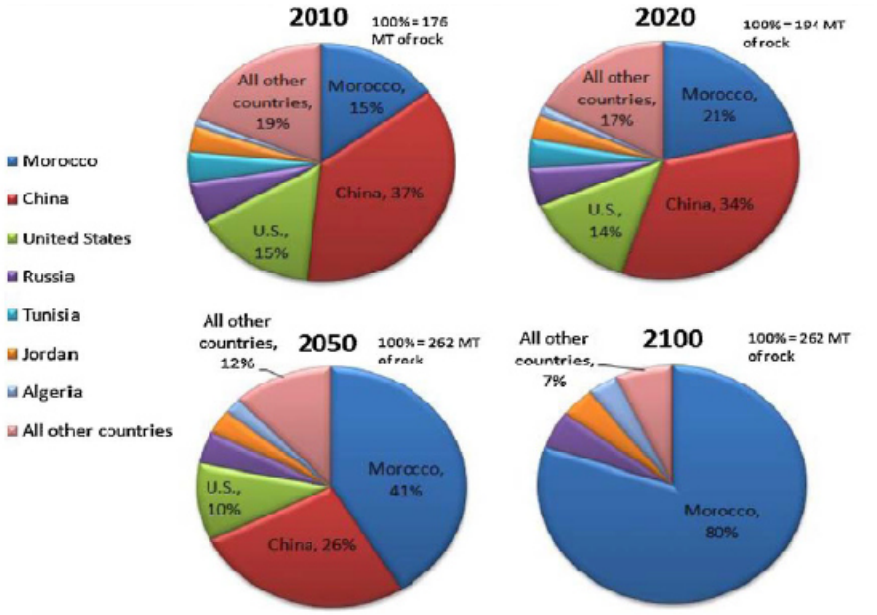


Fig. 1.4: Changing share of rock phosphate production (Cooper et al., 2011).

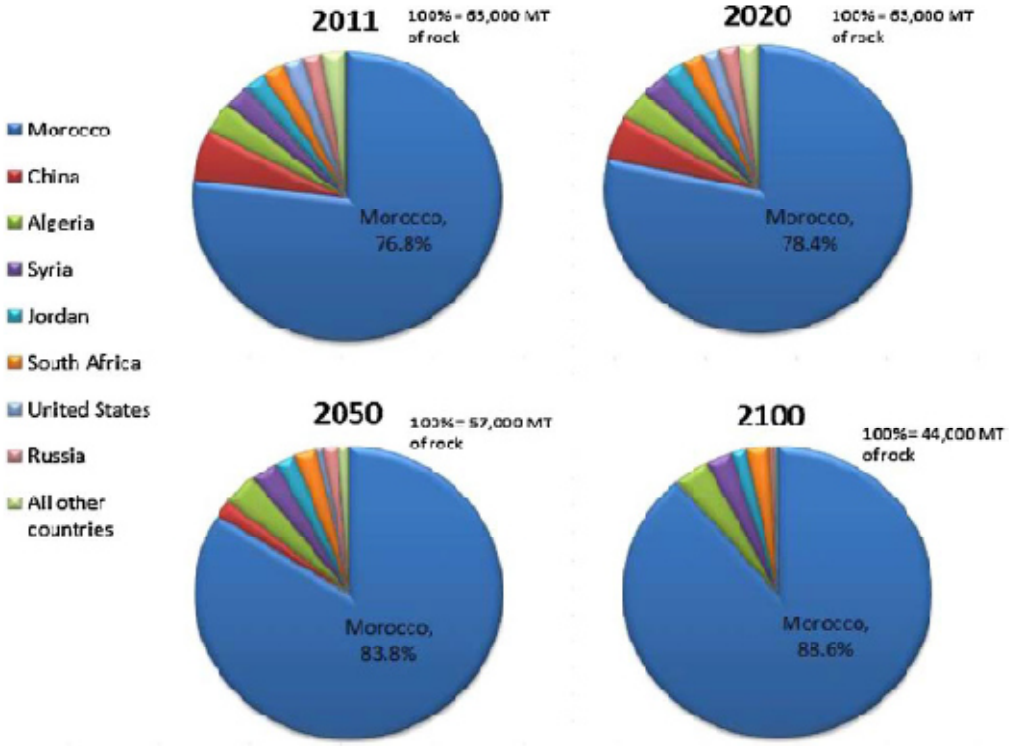


Fig. 1.5: Changing share of rock phosphate reserves (Cooper et al., 2011).

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

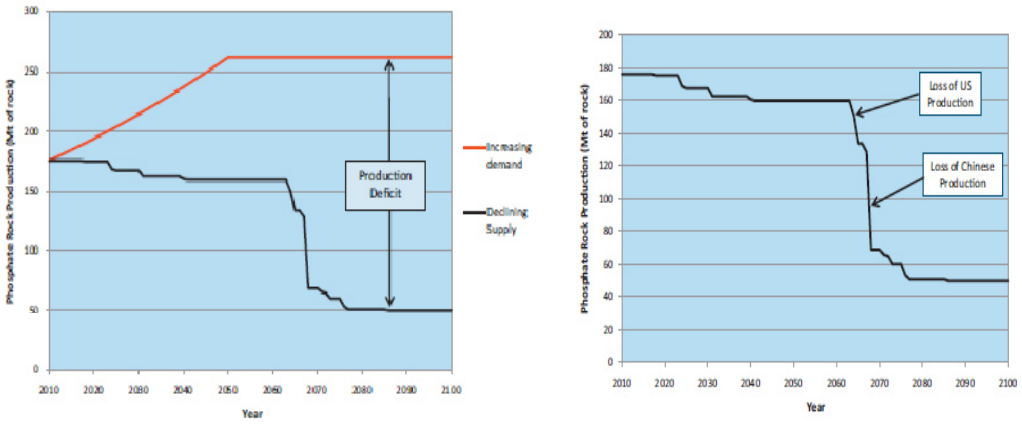


Fig. 1.6: Depletion of resource base of phosphate rock under the Global Orchestration (GO) scenario (Cooper et al., 2011).

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

1.3: Status of phosphorus in soil.

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

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

1.3.1: Alkaline Vertisol.

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

1.3.2: Acidic Alfisols.

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

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

1.4: Phosphorous availability to plants.

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

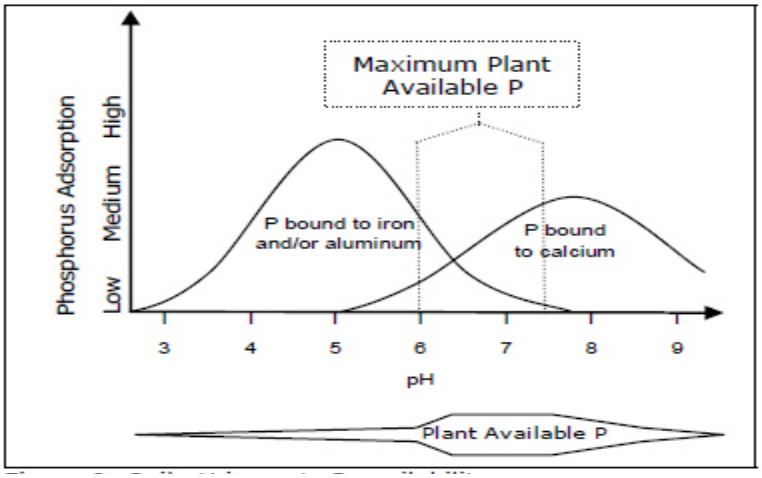


Fig. 1.7: Soil pH impacts P availability (Hyland et al., 2005 Agronomy Fact Sheet Series, Fact Sheet 12).

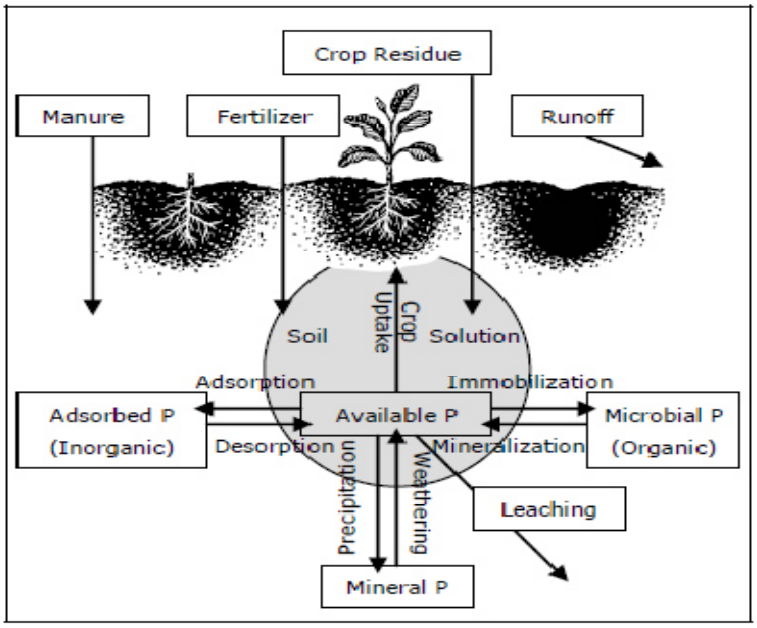


Fig.1.8: Phosphorous cycle

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

1.5: Need for biofertilizers in plant phosphate nutrition

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

1.6: Phosphate Solubilizing Microorganisms.

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

Aereobacter, Flavobacterium, Rahnella, Enterobacter, Azospirillum and *Erwinia.* In general, fungal isolates exhibit greater P-solubilizing ability than bacteria in both liquid and solid media (Kucey, 1983; Gyaneshwar et al., 2002; Khan et al., 2006). Among the fungal genera with this capacity are *Aspergillus, Penicillium,* and *Rhizopus.* The P-solubilizing ability of PSMs also depends on the nature of N source used in the media, with greater solubilization in the presence of ammonium salts than when nitrate is used as N source. This has been attributed to the extrusion of protons to compensate for ammonium uptake, leading to a lowering of extracellular pH (Roos and Luckner, 1984). In some cases, however, ammonium can lead to decrease in P solubilization (Reyes et al., 1999). In addition, other media components were also found to affect the P solubilization ability (Cunningham and Kuiack, 1992). PSMs are integral component of the soil which controls the biofertility of the soil. Numerous microorganisms reported to have MPS ability (Gyaneshwar et al., 1998; Henri et al., 2008; Hameeda et al., 2008; Zaidi et al., 2010). Important genera reported to PSMs include *Bacillus, Enterobactor* and *Pseudomonas* (Illmer and Schinner 1992; Wani et al., 2007a; Archana et al., 2012) while *Penicilium, Aspergillus* and *Rhizopus* are the important fungal genera (Souchie et al., 2006; Pandey et al., 2008). Many other genera also show MPS ability including *Rhodococcus, Arthrobacter, Chryseobacterium, Gordonia, Phyllobacterium, Arthrobacter, Delftia* sp. (Chen et al. 2006), *Azotobacter* (Kumar et al. 2001), *Xanthomonas* (de Freitas et al. 1997), *Pantoea*, and *Klebsiella* (Chung et al. 2005). Some symbiotic nitrogen fixers also showed MPS ability. *Rhizobium leguminosarum* bv. *trifolii* (Abril et al. 2007), *R. leguminosarum* bv. *viciae* (Alikhani et al. 2007) and *Rhizobium* species nodulating *Crotalaria* sp. (Sridevi et al. 2007) showed improvement in plant P content by mobilizing inorganic and organic P from the soil.

1.7: Mechanism of mineral phosphate solubilization.

1.7.1: Acidification.

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

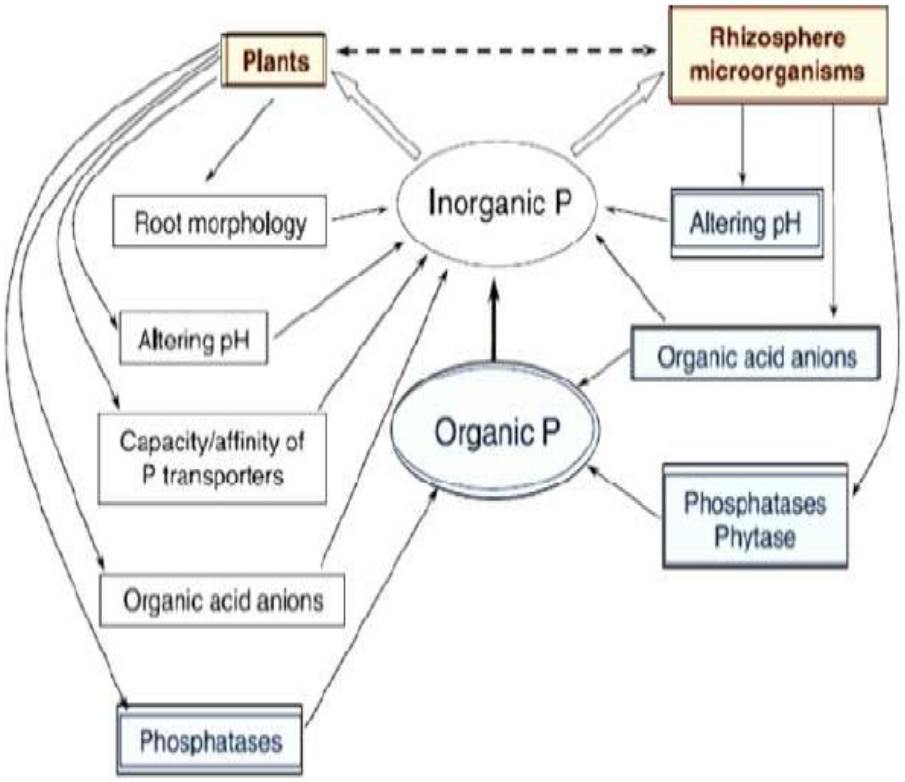


Fig.1.9. Plant- microbe mechanism to increase Phosphorus availability in rhizosphere (Richardson, 1994).

1.7.2: Chelation.

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

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

1.7.3: H^+ excretion.

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

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

A: Nature of organic acids secreted by PS fungi

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

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

Microorganism	Organic acid produced	Reference
Burkholderia cepacia DA23	Gluconic acid	Song et al. (2008)
Pseudomonas corrugata (NRRL B-30409)	Gluconic, 2-ketogluconic acid	Trivedi and Sa (2008)
Citrobacter sp. DHRSS	Acetic and gluconic acid	Patel et al. (2008)
Burkholderia, Serratia, Ralstonia and *Pantoea*	Gluconic acid	Elizabeth et al. (2007)
Bacillus, Rhodococcus, Arthrobacter, Serratia, Delftia,Chryseobacterium, Gordonia, Phyllobacterium, Arthrobacter ureafaciens, Phyllobacterium myrsinacearum,	Citric, gluconic, lactic, succinic and propionic acid	Chen et al. (2006)
Enterobacter intermedium	2-ketogluconic	Hwangbo et al. (2003)
Enterobacter asburiae PSI3	Gluconic, acetic acid	Gyaneshwar et al. (1998)
Bacillus amyloliquefaciens, B. licheniformis, B. atrophaeus, Penibacillus macerans, Vibrio proteolyticus, Xanthobacter agilis, Enterobacter aerogenes, E. taylorae, E. asburiae PSI3*, Kluyvera cryocrescens, Chryseomonas luteola*	Lactic, itaconic, isovaleric, isobutyric, acetic	Vazquez et al. (2000)
Pseudomonas cepacia	Gluconic, 2-ketogluconic	Bar-Yosef et al. (1999)
Bacillus polymyxa, B. licheniformis, Bacillus spp.	Oxalic, citric	Gupta et al. (1994)

1.8: Necessity of genetic modifications for P solubilization.

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

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

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

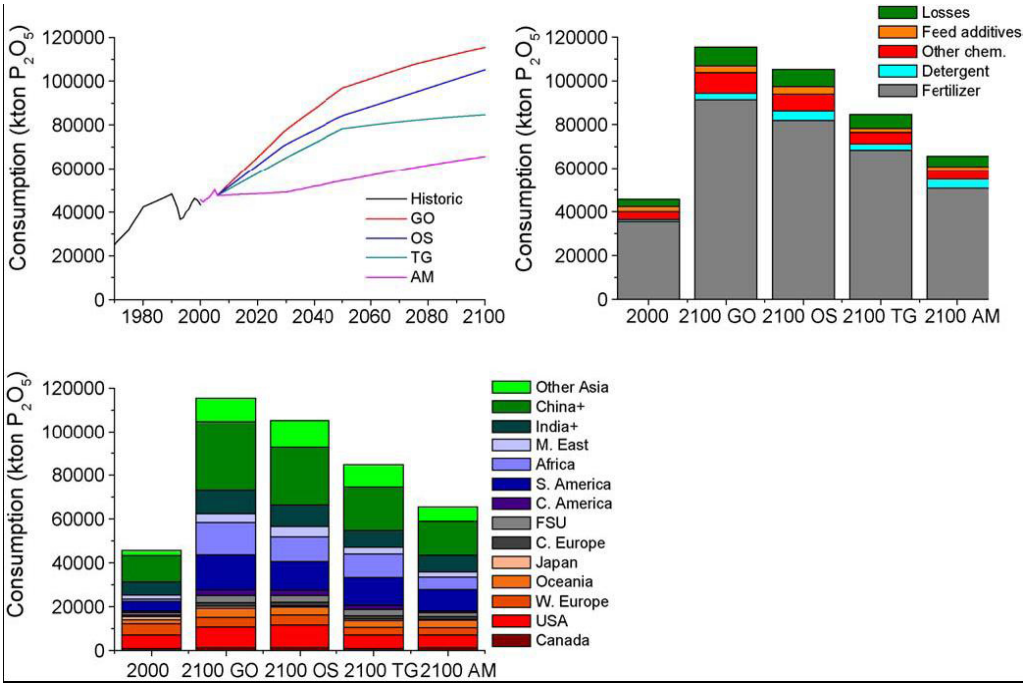


Fig. 1.10. Total global P consumption (a), P use per use category (b), and P use per world region (c), for the four Millennium Ecosystem Assessment (MA) scenarios (Vuuren et al., 2010).

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

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

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

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

MPS ability of PSMs depends upon the secretion of organic acids but amount of P release in the medium does not always correlate with the amount of organic acid (Thomas, 1985; Asea et al., 1988). Therefore, nature and strength of organic acid seems to be effective in P solubilization. Oxalic acid is more effective as compared to citric acid in acidic alfisols supplemented with RP but in alkaline vertisol both the acids efficiently release P from RP (Gyaneshwar et al., 1998; Srivastava et al., 2006). Phosphorus biofertilizers in the form of micro-organisms can help in increasing the availability of accumulated phosphates for plant growth (Subba Rao, 1982; Goldstein, 1986; Tandon, 1987; Kucey et al., 1989; Richardson, 1994). In addition, the micro- organisms involved in P solubilization as well as better scavenging of soluble P (P biofertilizers) can enhance plant growth by increasing the efficiency of biological nitrogen fixation, enhancing the availability of other trace elements such as Fe, zinc (Zn), etc. and by production of plant growth promoting substances (Kucey et al., 1989).

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

Table 1.2: Genetic modifications of microorganisms for phosphate solubilization

Gene/Function	**Source**	**Host**	**Mineral P solubilized**	**Organic acid**	**References**
PQQ biosynthesis	*Serratia marcescens*	*E. coli*	TCP	GA	Krishnaraj and Goldstein (2001)
pqqE	*Erwinia herbicola*	*Azospirillum sp.*	TCP	GA ?	Vikram et al. (2007)
pqqED genes	*Rahnella aquatilis*	*E. coli*	HAP	GA	Kim et al. (1998)
Unknown	*E. agglomerans*	*E. coli*		GA ?	Kim et al., 1997
pqqABCDEF genes	*Enterobacter intermedium*	*E. coli* DH5α	HAP	GA	Kim et al., 2003
Ppts-gcd, P gnlA-gcd	*E. coli*	*A. vinelandii*	TCP	GA	Sashidhara and Podille, 2009
gabY Putative PQQ transporter	*Pseudomonas cepacia*	*E. coli* HB101		GA	Babu-Khan et al., 1995
Unknown	*Erwinia herbicola*	*E. coli* HB101	TCP	GA	Goldstein and Liu, 1987
gltA/ citrate synthase	*E. coli* K12	*P. fluorescens* ATCC 13525	DCP	Citric acid	Buch et al., 2009
Unknown	*Synechocystis* PCC 6803	*E. coli* DH5α	RP	Unknown	Gyaneshwar et al., 1998
gad / Gluconate dehydrogenase	*P. putida* KT2440	*E. asburiae* PSI3	RP	GA and 2KG	Kumar et al., 2013

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

1.8.2: Direct oxidative pathway.

1.8.3: Gluconic and 2-ketogluconic acid secretion.

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

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

Pseudomonads are significant due to their plant growth promoting and phosphate solubilizing abilities. Under P-limitation, due to compromised metabolic status, intracellular phosphorylative pathway of glucose oxidation in both the

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

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

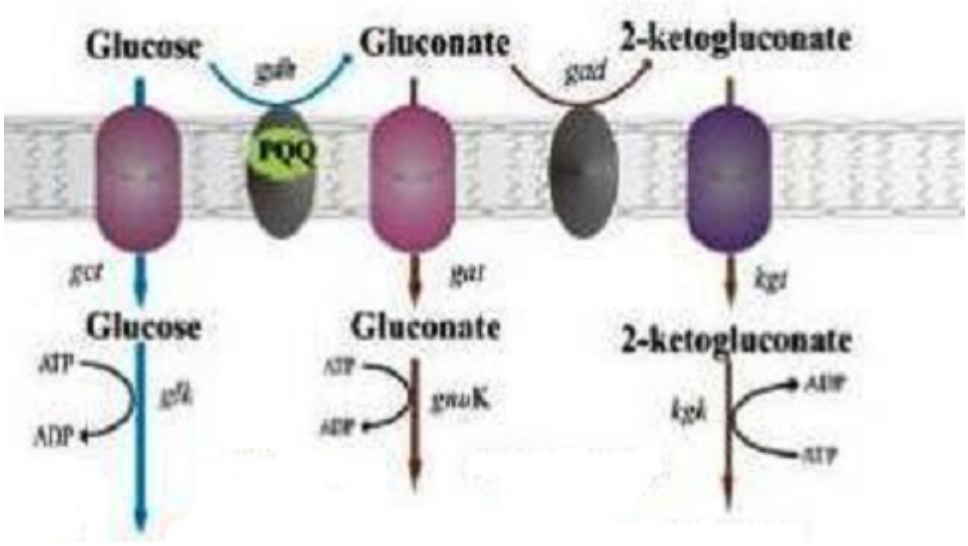


Fig. 1.11: Direct oxidative pathway in pseudomonads

(Archana et al., 2012)

1.8.4: Importance of pyrroloquinoline quinone in abiotic stress.

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

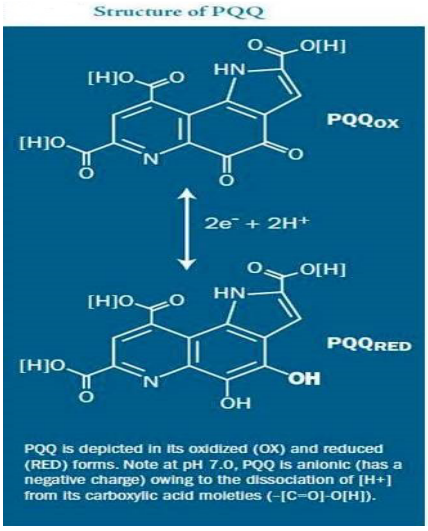


Fig. 1.12: Structure of PQQ (Rucker et al., 2009).

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

Fig. 1.13: PQQ as a redox cycling agent.

PQQ biosynthesis in bacteria involves varying number of genes present in clusters (**Fig.1.10**) (Choi et al., 2008). PQQ biosynthesis is not completely understood but a putative biosynthetic pathway has been proposed on the basis of the functions of conserved genes in bacteria (Puehringer et al., 2008). *Klebsiella pneumonia* possesses *pqqABCDEF* genes which are involved in PQQ biosynthesis (Meulenberg et al., 1992). *pqqA* gene encodes for 23-24 amino acid polypeptide which a substrate for a set of enzymes modifying the glutamate and tyrosine residues leading to PQQ formation (Goosen et al. 1992; Meulenberg et al. 1992; Velterop et al. 1995). PqqB belongs to metallo-β-lactamases and has been suggested to help in the transport of PQQ into periplasm (Velterop et al., 1995). PqqC is a

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

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

Table 1.3: Functions of *pqq* genes

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

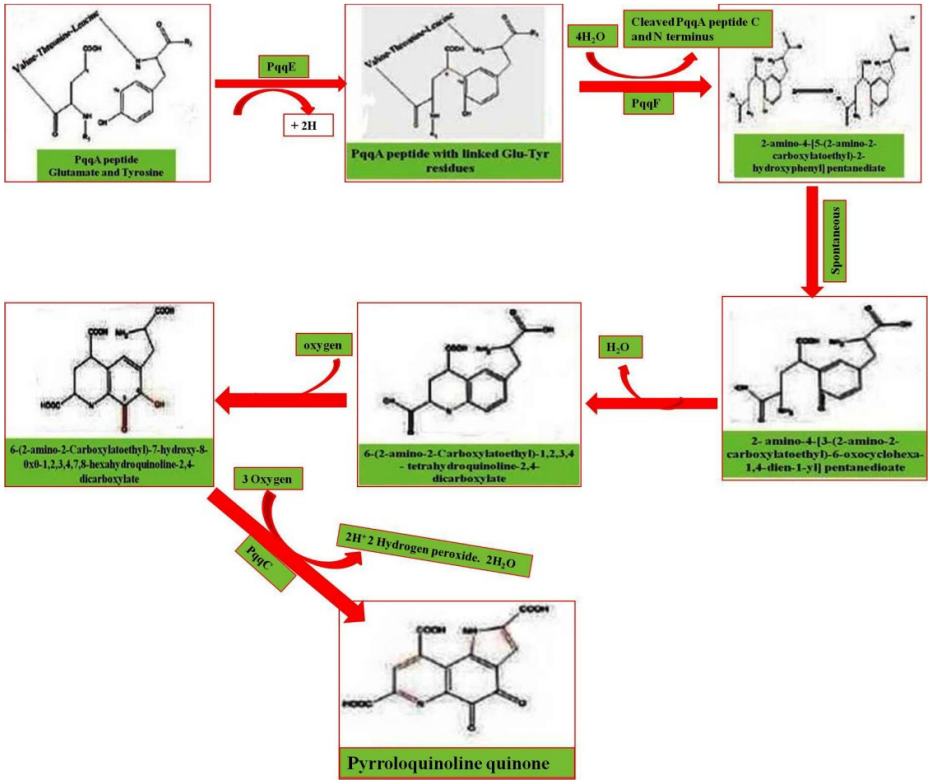


Fig. 1.14: PQQ biosynthesis pathway

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

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

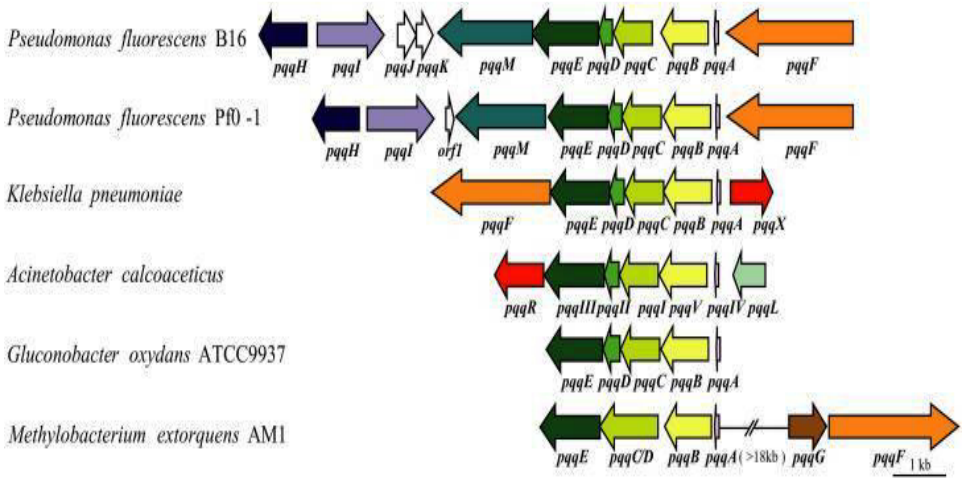


Fig. 1.15: Comparison of the *pqq* gene clusters of *P. fluorescens* B16, P. *fluorescens* Pf0-1, Klebsiella *pneumoniae*, *Acinetobacter calcoaceticus*, *Gluconobacter oxydans* ATCC9937 and *Methylobacterium extorquens* AM1 (Choi et al., 2008).

Species	**Pathogenicity**	***Pqq* genes**
Acinetobacter baumannii ATCC 19606	Opportunistic human pathogen	ABCDE
Acinetobacter hemolyticus ATCC 19194	Rare human pathogen	BCDE

Azoarcus sp. (strain BH72)	Plant symbiont	ABCDEF
Bradyrhizobium japonicum	Plant symbiont	ABCDEF
B*radyrhizobium* sp.(strainBTAi1/ATCC BAA-1182)	Plant symbiont	ABCDEF
Burkholderia cenocepacia (strain HI2424)	Human cystic fibrosis pathogen	ABCDE
Burkholderia cepacia (strain J2315/LMG 16656)	Animal and plant pathogen	ABCDE
Burkholderia glumae (strain BGR1)	Plant pathogen	ABCDE
Burkholderia multivorans (strain ATCC 17616/ 249)	Animal pathogen in mammals	ABCDE
Burkholderia phymatum (strain DSM 17167/ STM815)	Plant symbiont	ABCDE
Colwellia psychrerythraea (strain 34H/ATCC BAA-681)	Plant pathogen	ABCDE
*Cronobacter turicensis (*strain DSM 18703/LMG 23827/z3032)	Neonatal pathogen	ABCDEF
Cupriavidus taiwanensis (strain R1/LMG 19424)	Plant pathogen	ABCDE
Dinoroseobacter shibae (strain DFL 12)	Animal symbiont	ABCDEF
Enterobacter intermedius	Opportunistic pathogen	ABCDEF
Enterobacter sakazakii (strain ATCC BAA-894)	Neonatal pathogen	ABCDEF
Erwinia amylovora (strain ATCC 49946/CCPPB 0273/Ea273/27-3)	Plant pathogen	ABCDEF
Erwinia pyrifoliae	Plant pathogen	ABCDEF
Erwinia tasmaniensis (strain DSM 17950/Et1/99)	Plant commensal	ABCDEF
Gluconacetobacter diazotrophicus (strain ATCC 49037/DSM 5601/PAl5)	Plant symbiont	ABCDE
Granulibacter bethesdensis (strain ATCC BAA-1260/CGDNIH1)	Human pathogen	ABCDE
Grimontia hollisae CIP 101886	Human pathogen	BCDE
K. pneumoniae	Opportunistic pathogen	ABCDEF
Klebsiella sp. 1_1_55	Opportunistic pathogen	BCDEF
Marinobacter algicola DG893	Plant symbiont	ABCDE
Methylobacterium nodulans (strain ORS2060/ LMG 21967)	Plant saprophyte and symbiont	ABCDEF
Methylobacterium populi (strain ATCC BAA-705/ NCIMB 13946/BJ001)	Plant endophyte	ABCDEF
Methylobacterium radiotolerans (strain ATCC	Plant symbiont	ABCDEF

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

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

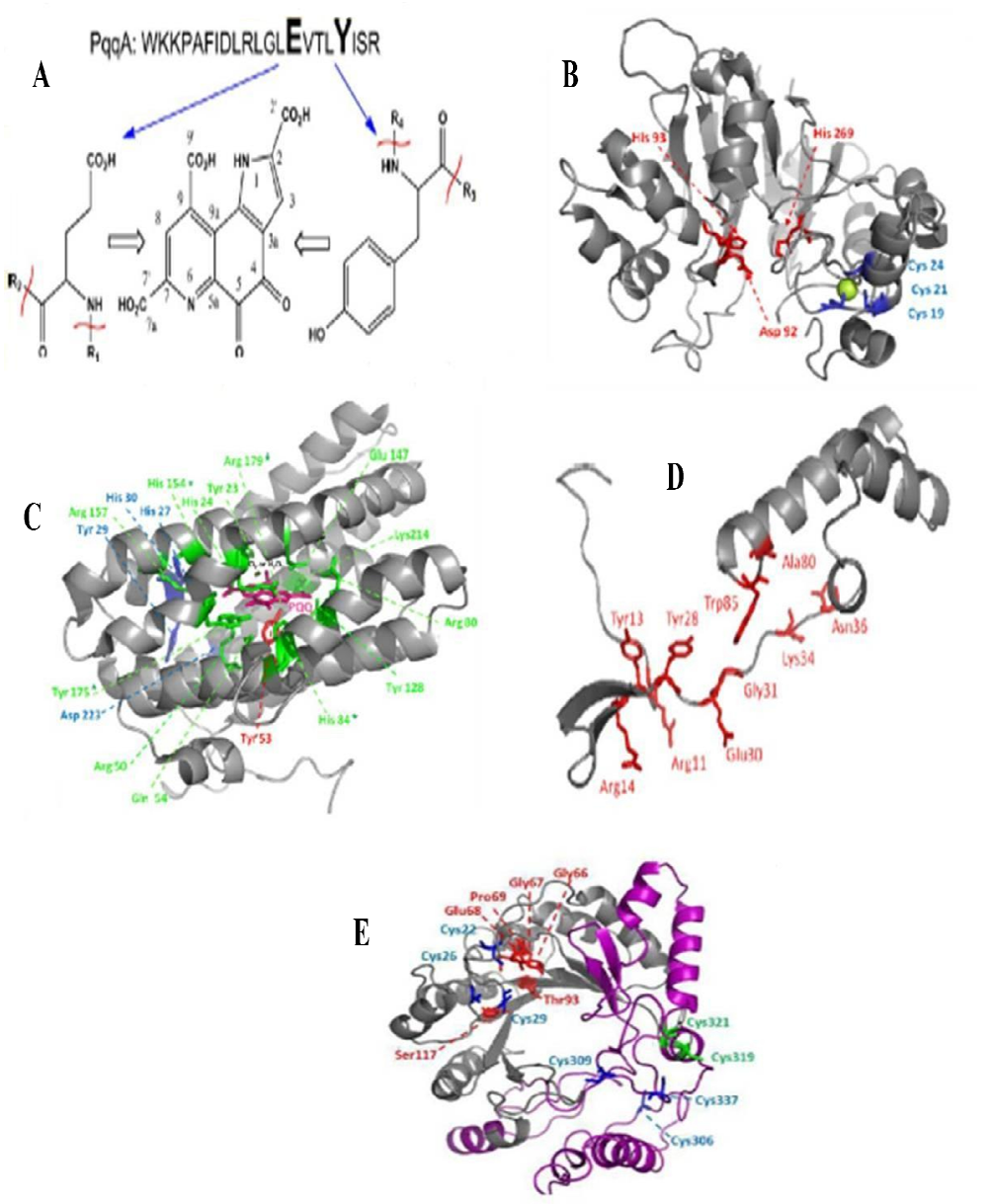


Fig. 1.17: Structures of Pqq genes . A- PqqA, B- PqqB, C- PqqC, D- PqqD and E- PqqE (Shen et al., 2012)

1.9: Citric acid secretion in microorganisms

Citric acid secretion is reported in large number of microorganisms including bacteria, fungus and yeast (Khan et al., 2006). Plants are also known to secrete citrate in root exudates. Bacteria secrete citrate in very low amounts (~ 1 mM) (Gyaneshwar

et al., 1998). *A. niger* is known to produce citrate in molar amounts and thus the organism is used to produce citrate at large scale (Pappagiani, 2007).

1.9.1: Citrate synthase

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

E. coli CS is a mixture of dimers and hexamers of identical subunits, 426 amino acids in length. The dimer–hexamer equilibrium is affected by many factors as pH, ionic strength, and the presence of the allosteric inhibitor, NADH (Tong and Duckworth, 1975; Ayed et al., 1998). Citrate synthase, the ubiquitous enzyme which catalyzes entry of acetyl-coA carbon into the Krebs cycle, is potentially a key control point for energy production. In eukaryotic and Gram-positive bacteria citrate synthase, is dimer of identical subunits. The more complex citrate synthases of Gram-negative bacteria are allosteric hexamers, displaying specific inhibition by NADH and a sigmoid saturation curve for substrate, acetyl-coA (Weitzman and Danson, 1976; Donald et al., 1991).

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

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

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

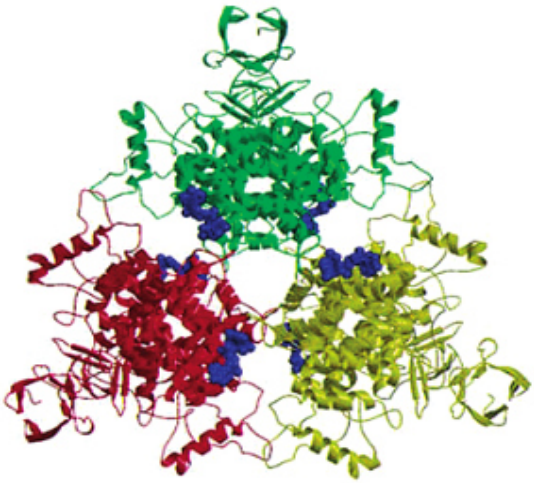


Fig. 1.19: Ribbon structure of NADH bound hexameric *E. coli* F383A type II CS. Green, red and yellow ribbons shows three equivalent dimers of this complex having central 3- fold axis while the location of the six bound NADH molecule (one per subunit) is shown in blue (Maurus et al., 2003).

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

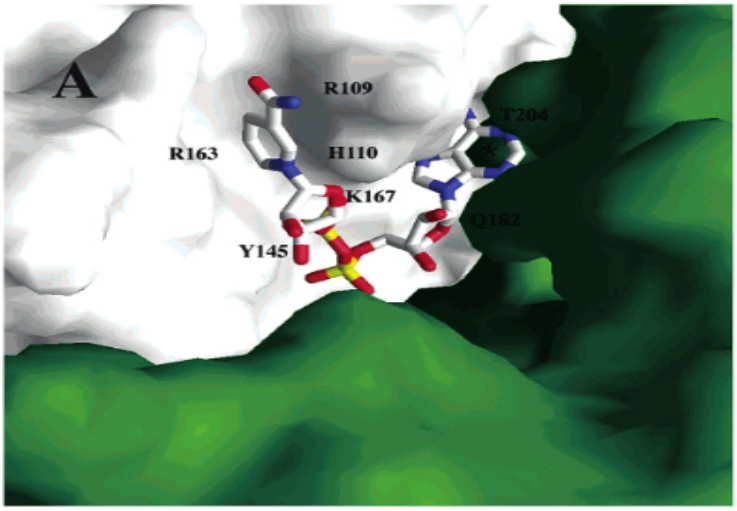


Fig. 1.20: Close-up view of the contour of the enzyme surface in the vicinity of one NADH binding site in hexameric *E. coli* CS (Maurus et al., 2003).

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

R109L variant protein of *E. coli* CS, residue 262-298 refold into a confirmation towards functional acetyl CoA binding site. In wild type and R109L

variant CS structure, residue 316-342 form helix turns helix which acts as helical linker. Hexameric CS shows helical linker region (residue 316-341) structurally links refolded active site polypeptide chain segment region (262-298) to an NADH binding site. However, the NADH binding site involved is not from the same subunit, instead being located across the dimer-dimer interface on an adjacent subunit (**Fig. 1.21**).

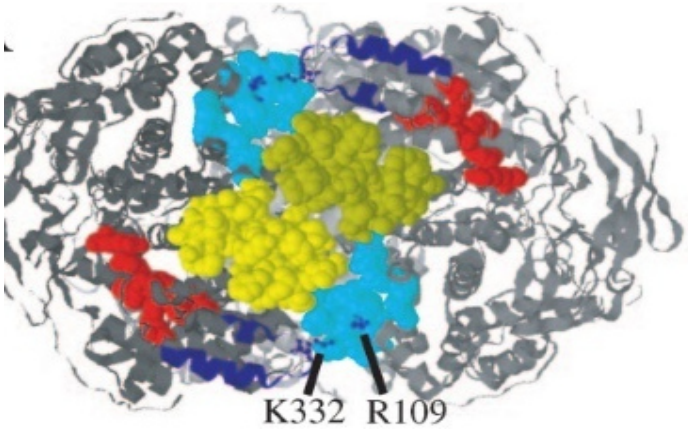


Fig. 1.21: RasMol model of *E. coli* citrate synthase (hexamer). Figure shows perpendicular 3 fold axis that relate dimer subunit and face onto one of the three relatively flat sides. Running through the middle of each side face is a 2-fold axis that relates two NADH binding sites and two active sites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

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

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

active site which in close proximity across the dimer-dimer interface in hexameric form of enzyme.

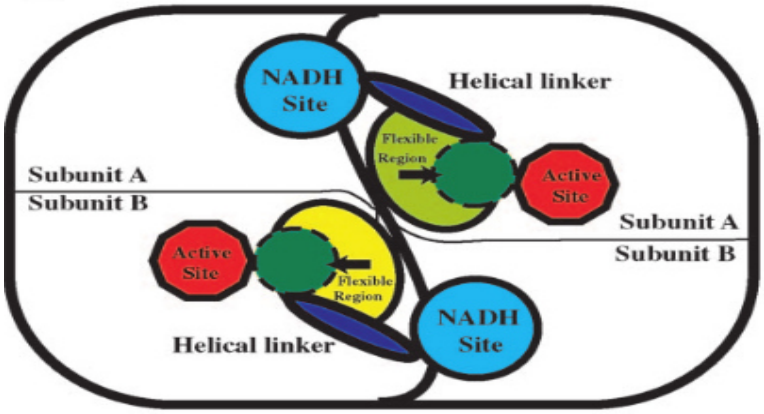


Fig. 1.22: Diagram showing proposed route of communication between NADH binding sites and active sites in the Type II CS of *E. coli* (Stokell et al., 2003).

Replacement of Arg 109 (in the NADH binding site) by Leu is accompanied by substantial changes in position and mobility of two other regions: the "helical linker" (residues 316–342, dark blue) and the "flexible region" (residues 262–298, bright yellow for one dimer, and greenish yellow for the other). A regulatory signal, originating at the NADH binding sites (cyan circles), is transmitted to the 316–342 linker of the other dimer and hence via the flexible 262–298 region to the active site (red decagons; the entry points for the two visible active sites are from the lower left and upper right, respectively). The initial step in this process may be modulated by a charge interaction between Arg and Lys 332. Changes in position and mobility of the 316–342 linkers provide a more suitable surface upon which the flexible 262–298 region can pack. This packing occurs partially in R109L structure leading to refolding a part of the flexible region to form a binding site (green circle) for the substrate, acetyl-CoA. This mechanism suggests how the six NADH binding sites and six active sites in the Type II CS hexamer might communicate so as to induce either the T (inactive) or R (catalytically active) states that are required for the control exerted by NADH on this system.

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

1.9.2: Citrate transporter

Citrate is very abundant in nature and most bacteria have transport proteins in the cytoplasmic membrane for uptake of citrate. The carriers belong to the class of secondary transporters which use the free energy stored in transmembrane electrochemical gradients of ions to drive the uptake of the substrates (Poolman and Konings, 1993). The citrate transporter CitH of *Klebsiella pneumoniae* is driven by the proton motive force and the transporters CitS and CitC of *K. pneumoniae* and *Salmonella* serovars are driven by both the proton motive force and sodium ion

motive force (Lolkema, 2006; 2007). Mechanistically these transporters catalyze coupled translocation of citrate and H^+ and/or Na^+ (symport). However, citrate carriers of lactic acid bacteria take up citrate by an electrogenic uniport mechanism or by exchange with lactate (Lolkema, 2006). These citrate transporters are involved in generation of metabolic energy. A number of structural genes coding for citrate transporters have been cloned, and the primary sequences have been deduced from the base sequences (**Table 1.3**).

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

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

Transporter	Bacteria	Substrates	Transport mode	Reference
CitS	*K. pneumoniae*	citrate	Na^+ symport	Dimorth and Thomer, 1986
CitC	*Salmonella typhimurium*	citrate	Na^+ symport	Ishiguro et al., 1992
CitW	*K. pneumoniae*	citrate, acetate	exchange	Kastner et al., 2002
MleP	*Lactococcus lactis*	malate, lactate	exchange	Molenaar et al., 1993

CitP	*Leuconostoc mesenteroides*	Citrate, lactate	exchange	Marty-Teysset et al., 1995
CimH	*Bacillus subtilis*	Citrate, malate	H^+ symport	Krom et al., 2001
MalP	*Streptococcus bovis*	malate	H^+ symport	Kawai et al., 1997
MaeN	*Bacillus subtilis*	malate	Na^+ symport	Wei et al., 2000

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

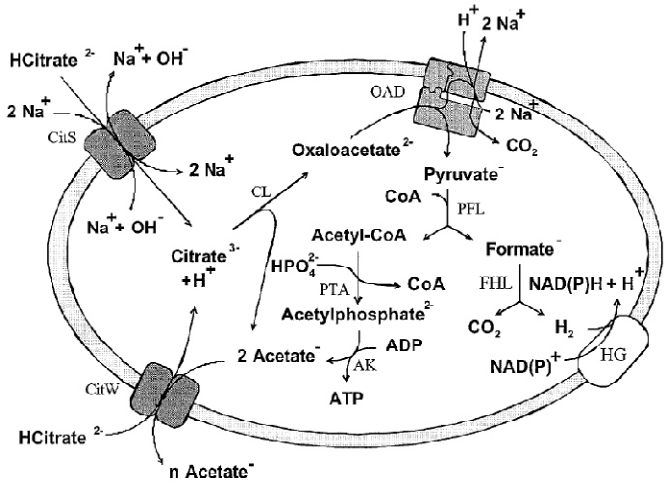


Fig. 1.23: Citrate fermentation pathway in *Klebsiella pneumonia*

(Kustner et al., 2002).

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

1.10: Potassium solubilization.

After phosphate, nitrogen potassium (K) is important macronutrient, deficiency of K in soil limits the plant growth and development as it reduces crop yield (Sparks and Huang, 1985; Richardson et al., 1994). Potassium is tightly bound with metals and the non-exchangeable potassium which is unavailable for plant uptake. Majority of three different forms of potassium are present in soil but they are unavailable for plants (Goldstein, 1994). Feldspar are group of rock forming tectosilicates minerals ($KAlSi_3O_8$ - $NaAlSi_3O_8$ - $CaAl_2Si_2O_8$). These tectosilicates contains three end members. Potassium-Feldspar (K-spar) $KAlSi_3O_8$, Albite $NaAlSi_3O_8$ and Anorthite $CaAl_2Si_2O_8$. However solid three types of feldspar, are characterized on the basis of their forms between solid solutions. Solid solution between K-feldspar and albite are alkali feldspar, solid solution between albite and anorthite are plagioclase, and Barium feldspar. Alkali feldspars- Orthoclase (monoclinic) - $KAlSi_3O_8$, Sanidine (monoclinic) - (K,Na) $AlSi_3O_8$, Microcline (triclinic)- $KAlSi_3O_8$, Anorthoclase (triclinic) -$(Na,K)AlSi_3O_8$.

Plagioclase feldspars- Albite - $NaAlSi_3O_8$, Oligoclase - $(Na,Ca)(Al,Si)AlSi_2O_8$, Andesine - $NaAlSi_3O_8$ -$CaAl_2Si_2O_8$, Labradorite - $(Ca,Na)Al(Al,Si)Si_2O_8$, Bytownite - $(NaSi,CaAl)$ $AlSi_2O_8$, Anorthite - $CaAl_2Si_2O_8$. Barium feldspars are monoclinic ; Celsian $BaAl_2Si_2O_8$, Hyalophane (K,Na,Ba) $(Al,Si)_4O_8$ (Lesure, 1968; Anderson and Burnham 1983; Potter, 1991; Bourne, 1994; Kauffman and Van Dyk, 1994; Potter, 1996; 1997; Drever and Stillings 1997; Walther 1997). The K is in various forms of non-exchangeable and exchangeable K in soil solution (Sparks and Huang, 1985; Mutscher, 1995) (**Fig. 1.24**).

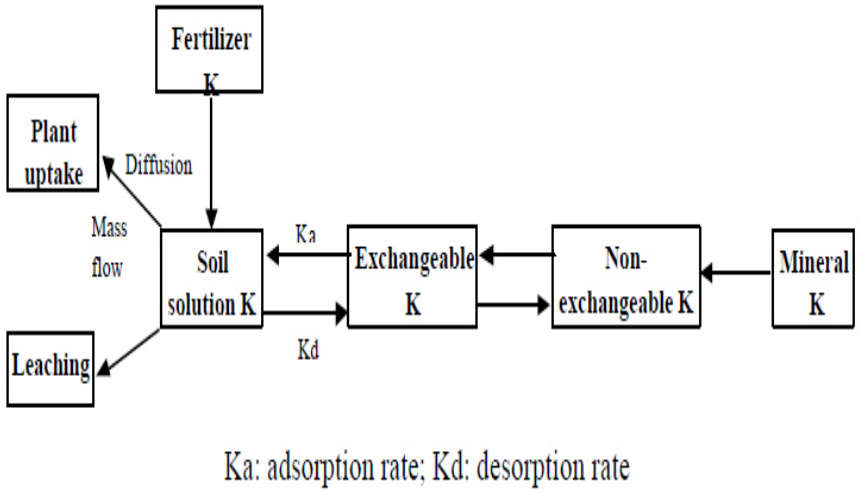


Fig. 1.24: Diagramatic representation of the potassium cycle in soils. Sparks and Huang (1985); Mutscher (1995).

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

1.11: *Herbaspirillum seropedicae* Z67

Herbaspirillum seropedicae is a gram negative belonging to β-group of proteobacteria, vibrioid, has one to three polar flagellae, endophytic nitrogen-fixer. It grows on organic acids under both N-fixing and non-N-fixing conditions. and is associated with many economically important cereals such as rice, sorghum, sugarcane and maize (Baldani et al., 1986; 1996). This endophyte colonizes efficiently the intercellular space and vascular tissues of plants, fixes biological nitrogen and promotes growth (Urquiaga et al., 1992). The endophytic bacteria contributes ~40% of total nitrogen fixation from air (Shrestha and Ladha, 1996; Malarvizhi and Ladha, 1999). The genome sequence of *H. seropedicae* strain SmR1 is available (Pedrosa et al. 2011). This bacterium interacts efficiently with host plants, particularly with members of the family Poaceae, and colonizes in intercellular regions of plants. (**Fig. 1.25**).

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

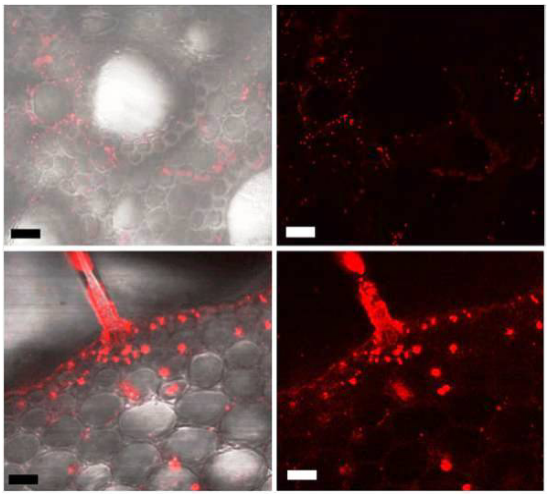


Fig. 1.25: *H. seropedicae* colonization in *Phaseolus vulgaris* (Fabaceae). Transmitted light **(A)** confocal fluorescence microscopy **(B)** cross-section of maize roots
(Monteiro et al., 2012).

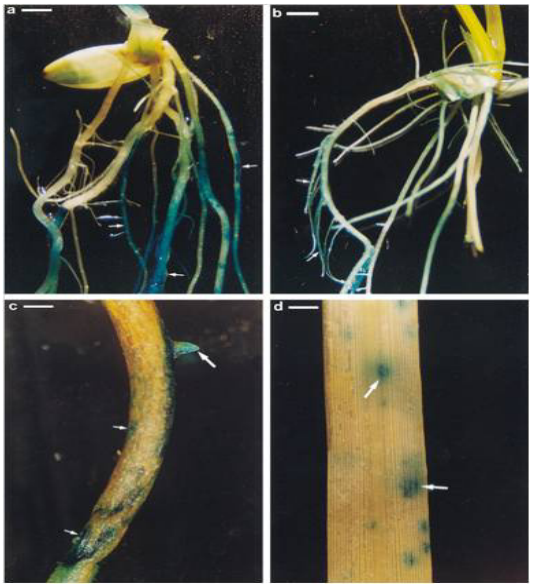


Fig. 1.26: β-Glucuronidase (GUS) staining of rice seedlings with *H. seropedicae* Z67-*gusA* (Gyaneshwar et al., 2002).

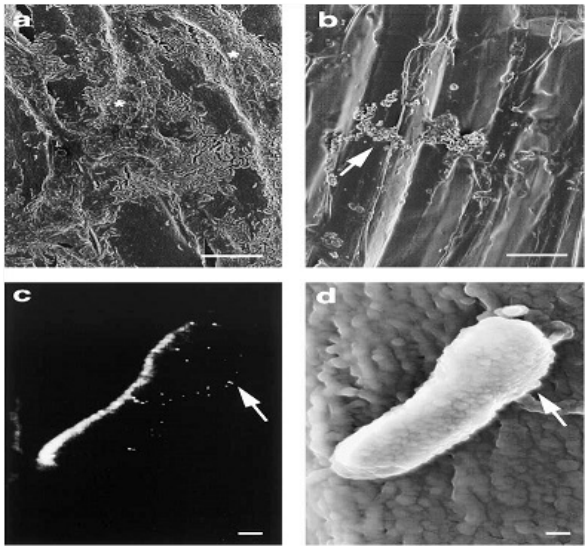


Fig. 1.27: Scanning electron microscope (SEM) images of *H. seropedicae* Z67-*gusA* (Gyaneshwar et al., 2002).

1.11.1: General metabolism

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

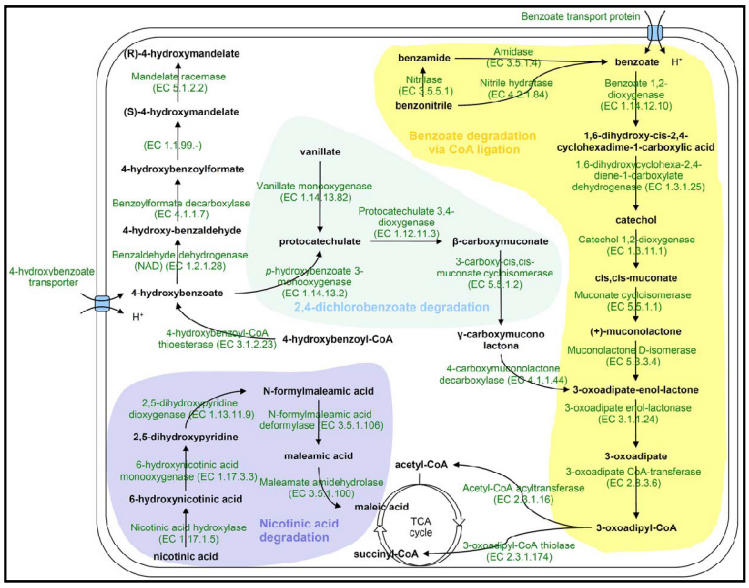


Fig. 1.28: Proposed pathways for aromatic compounds metabolism in *H. seropedicae* SmR1 (Pedrosa et al., 2011).

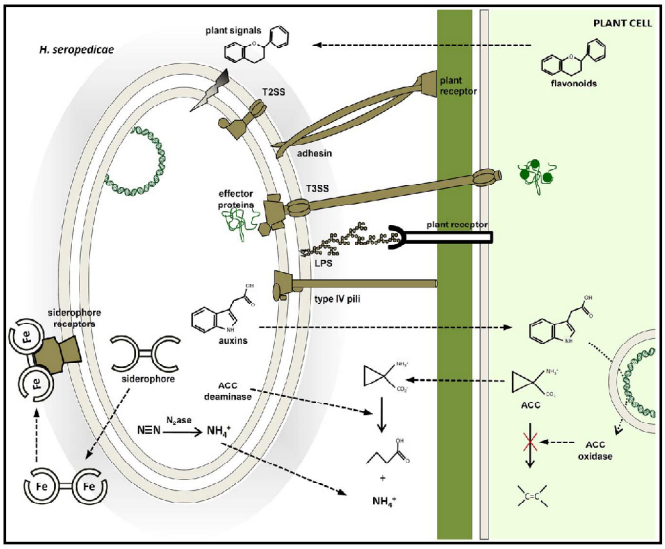


Fig. 1.29: Molecular mechanisms probably involved in plant colonization and plant growth promotion identified in the *H. seropedicae* SmR1 genome (Pedrosa et al., 2011).

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

1.11.3: Nif gene regulation.

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

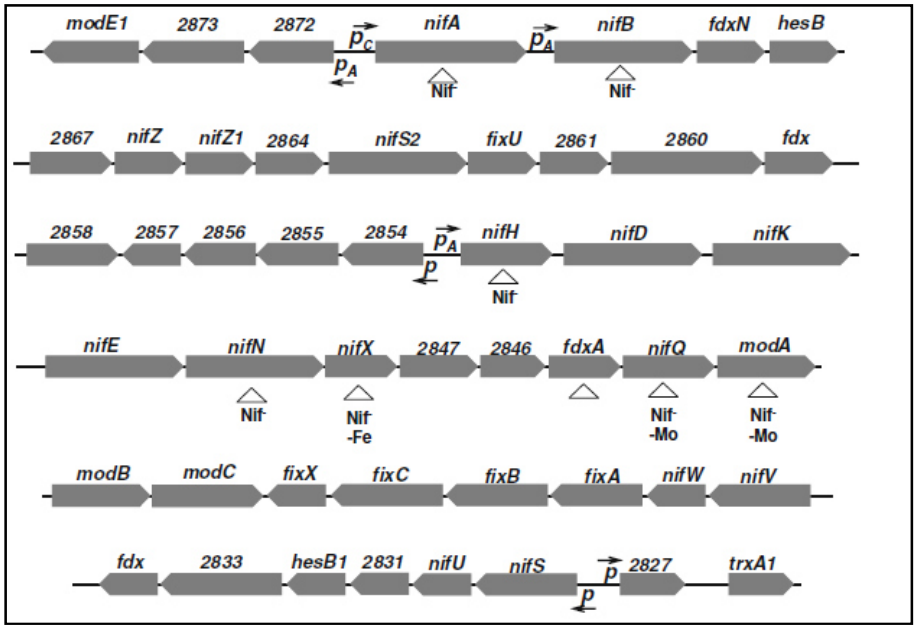


Fig. 1.30: Structural organization of the *nif* and *fix* gene cluster in the *H. seropedicae* genome (Chubatsu et al., 2012).

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

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

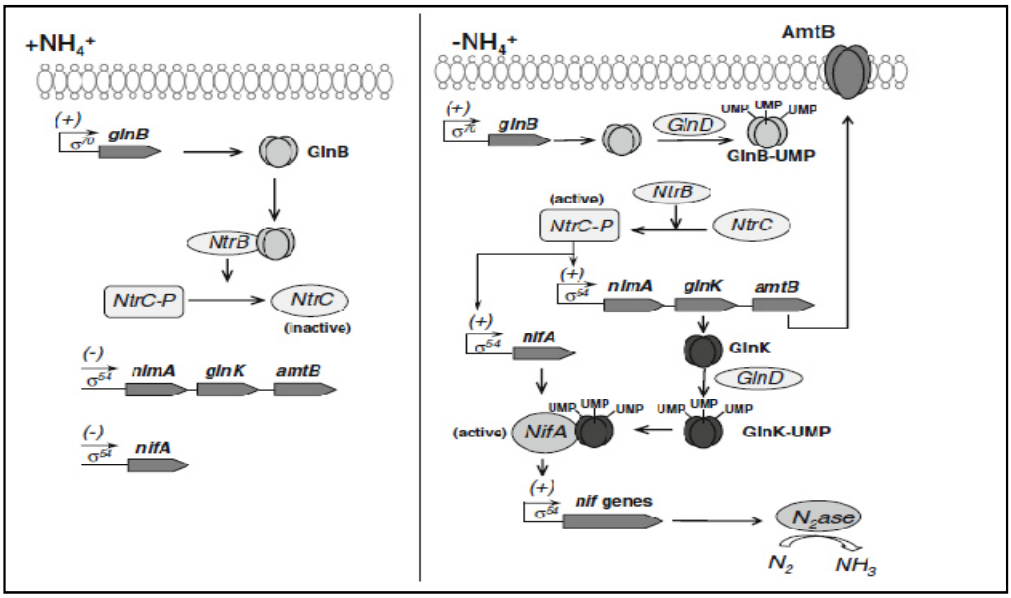


Fig. 1.31: Model of transcriptional regulation of nitrogen fixation in *H. seropedicae* (Chubatsu et al., 2012).

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

Nitrogen fixation is an energy-consuming process; it controls the level of *nif* transcription and by operating the activity of nitrogenase. This post-translational regulation is depending on fixed nitrogen and the utilization of energy which leads to

inactivation of nitrogenase activity. Nitrogenase activity gets inhibited, as the extracellular concentration of ammonium increases. Upon utilization of supplemented ammonium, the activity of nitrogenase is restored. Uridylylated GlnK maintains its cytosolic form under nitrogen-fixing conditions. Under excess ammonium concentration, GlnK gets deuridylylated by GlnD and binds with AmtB in the membrane and nitrogenase is inactivated (Chubatsu et al., 2012) (**Fig. 1.32**).

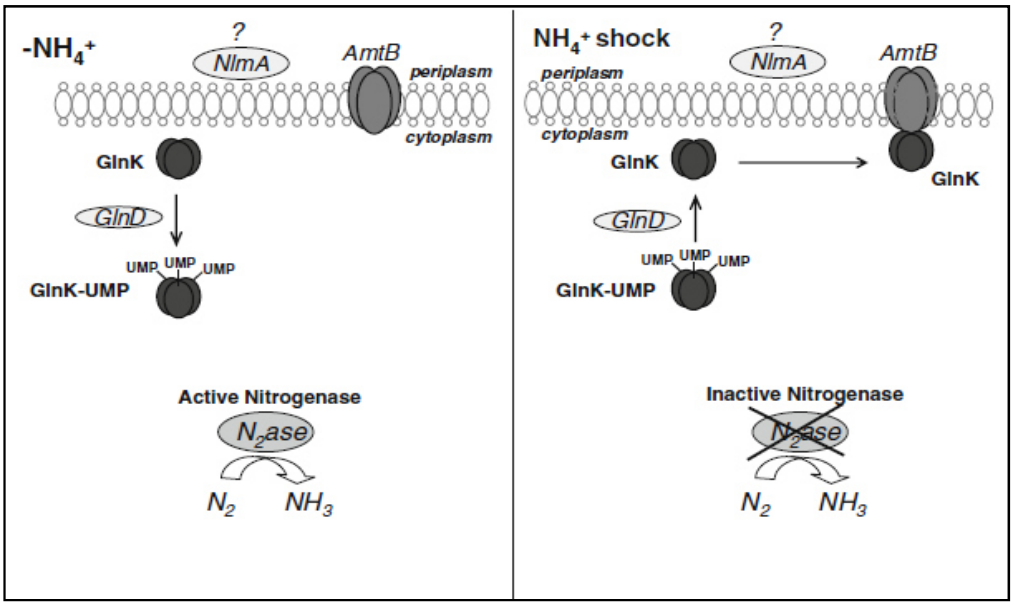


Fig. 1.32: Model of posttranslational control of nitrogen fixation in *H. seropedicae.* (Chubatsu et al., 2012).

1.12: Rational of study.

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

Next to nitrogen, phosphorus (P) is the second major nutrient required for plant development and its availability determines the plant growth and crop yields. On an average, most mineral nutrients in soil solution are present in millimolar amounts; however, P is present only in micromolar quantities. This P is present in the soil as organic and inorganic phosphates. Inorganic P in acidic soils is associated

with Al and Fe compounds whereas Ca phosphates are the predominant form of inorganic phosphates in calcareous soils. Thus, majority of the P is complexed with metal ions rendering it insoluble and thus unavailable for the plant uptake. Moreover, fixation reactions in soil may allow only a small fraction (10–15%) of the P in fertilizers to be taken up by plants in the year of their application. Consequently, farmers are advised to apply several times more phosphorus than the plant needs. Excessive application of P causes environmental and economic problems.

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

Gluconic acid is produced by direct oxidation of glucose *via* membrane bound quinoprotein glucose dehydrogenase (GDH) enzyme. The GDH enzyme is known to exhibit broad substrate specificity in some organisms in that it can also convert other aldose sugars such as xylose, galactose, maltose, etc. apart from glucose to their

corresponding aldonic acids which can also bring about efficient P solubilization. Gluconic acid can further be oxidized to 2-ketogluconic acid by another periplasmic enzyme gluconate dehydrogenase (GAD).

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

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

Solubilization of mineral phosphates by microbes has been well established under laboratory conditions; however, their efficacy in the field studies has been

highly variable. This variability has hampered the large-scale use of PSB in agriculture. Many reasons have been suggested for this variability, but none of them have been extensively investigated. Root exudates are known to serve as a source of reduced carbon compounds which are released in the rhizosphere. Microbes in the rhizosphere utilize root exudates as nutrients and this forms the major basis for rhizosphere colonization

1.13: OBJECTIVES OF THE PRESENT STUDY

The objectives of the present study were:

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

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

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

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

Chapter 2 Materials and Methods

2: Materials and Methods

2.1 Bacterial strains / Plasmids

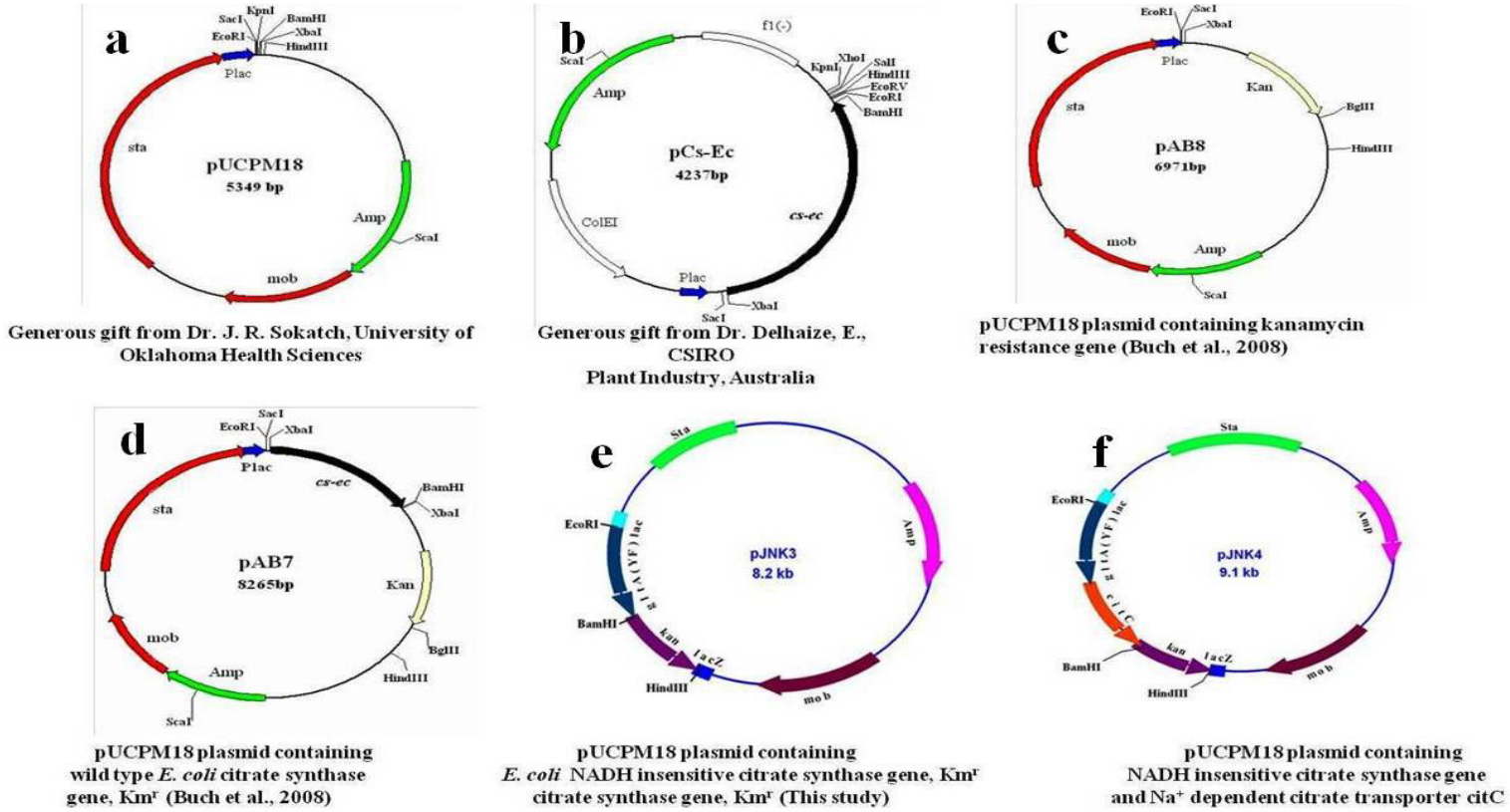
All the plasmids use in the present study and their restriction maps are given in **Table 2.1, Fig. 2.1** and **Fig 2.2.** The wild type and genetically modified *E. coli* and *Herbaspirillum seropediace* Z67 strains are listed in **Table 2.1** *E. coli* DH10B was used for all the standard molecular biology experiments wherever required. *Herbaspirillum seropediace* Z67 strain used in all this study is generous gift from (Dr E.K. James) The *glt*A (citrate synthase gene) mutant of *E. coli* was obtained from *E. coli* Genetic Stock Center (CGSC), Yale University, U.S.A. The NADH insensitive *cs* mutant plasmid was a generous gift from Prof Harry Duckworth, University of Manitoba, Canada. The *pqqE* gene was gifted by Goldstine Department of Biology, California State University at Los Angeles, State University Drive, Los Angeles, California. *pqq* gene cluster of *Acinetobacter calcoaceticus* was gifted by (Dr. Goosen, Molecular Genetcis, University of Leiden, Netherland) and *pqq* gene cluster of *Pseudomonas fluorescens* B16 was generous gift by (Dr. Ingyu Hwanag, Department of Biochemistry, Seoul University, Korea).

Table 2.1: List of plasmids used in the present study.

Plasmid/Strains	Characteristics	Source orReference
E. coli DH10B	Used for maintaining plasmids. *Str^{r}F$^-$ endA1 recA1 galE15 galK16 nupGrpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ$^-$*	(Invitrogen)
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r	Hester et al., 2000
pAB8	pUCPM18 with km^r gene; Ampr, Km^r	Buch et al., 2009
pAB7	*gltA* gene of *E. coli* in pAB8, Apr/Km^r	Buch et al., 2009
pY145F	NADH insensitive *cs** gene	Duckworth, H., 2003
citC	Na$^+$ dependent citrate transporter of *Salmonela typhimorium*	Chapter 3
pJNK3	pUCPM18 with *E. coli* NADH insensitive *cs** gene under *Plac*and km^{r}gene; Ap^r, Km^r	Chapter 3
pJNK4	pUCPM18 with *E. coli* NADH insensitive *cs** gene citrate transporter citC of *Salmonella typhimorium* under *Plac* and km^{r}gene; Ap^r, Km^r	Chapter 3
Hs Z67	Nitrogen fixer, Natural isolate from gramenae plant; Nal^r	Baldani et al., 1986a
Hs (pAB8)	*H. seropedicae* Z67 pUCPM18 with *nptII* gene, Ap^r/Km^r	Chapter 3
Hs (pAB7)	*H. seropedicae* Z67 Nal^{r}with pAB7, Km^r	Chapter 3
Hs (pJNK3)	*H. seropedicae* Z67 Nalr with pJNK3, Km^r	Chapter 3

Hs (pJNK4)	*H. seropedicae* Z67 Nal^r with pJNK4, Km^r	Chapter 3
pBBR1MCS-2	Broad-Host-Range vector; Km^r	Kovach et al., 1995
pMCG898	Containing *pqqE* of *Erwinia herbicola*	Goldstein and Liu; 1987
pJNK1	pBBR1MCS-2 Kmr with 1.8 kb *E. herbicola pqqE* gene	Chapter 4
pSS2	25Kb plasmid contains 5.1-kb of *pqq* gene cluster of *Acinetobacter calcoaceticus*	Goosen et al.,1989
pOK53	35Kb plasmid contains 13.4-kb *pqq* gene cluster of *Pseudomonas fluorescens* B16	Choi et al., 2008
E. coli DH10B	*Str r F- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZ ΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ-*	Invitrogen
Hs Z67	Nitrogen fixing rice endophyte , Nal^r	Baldani et al., 1986a
Hs (pBBR1MCS2)	*H. seropedicae* Z67 with (pBBR1MCS2) Km^r	Chapter 4
Hs (pJNK1)	H. seropedicae Z67 with pJNK1 Km^r	Chapter 4
Hs (pSS2)	*H. seropedicae* Z67 with pSS2 Tet^r	Chapter 4
Hs (pOK53)	*H. seropedicae* Z67 with pOK53Tet^r	Chapter 4
Enterobacter asburiae PSI3	Amp^r secretes gluconic acid	Gyaneshwar et al., 1999
Pseudomonas aeruginosa P4	Secretes gluconic acid	Buch et al., 2008
pSS160	pUC18 plasmid contains 5.1-kb of *pqq* gene cluster of *Acinetobacter calcoaceticus*	Goosen et al.,1989
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r and Gen^r	Hester et al., 2000

pCNK12	pJET2.1 with *gad* operon	Chanchal et al., 2013
pJNK5	*Pqq* gene cluster of *Acinetobacter calcoaceticus* in pUCPM18, Apr and Gen^r	Chapter 5
pJNK6	*gad* operon of *Pseudomonas putida* KT2440 in pJNK5, Apr and Gen^r	Chapter 5
Hs (pUCPM18)	*H. seropedicae* Z67 with pUCPM18 Broad-Host-Range vector; Ap^r and Gen^r	Chapter 5
Hs (pJNK5)	*H. seropedicae* Z67 with pJNK5, Ap^r and Gen^r	Chapter 5
Hs (pJNK6)	*H. seropedicae* Z67 with pJNK6, Ap^r and Gen^r	Chapter 5



Generous gift from Dr. J. R. Sokatch, University of Oklahoma Health Sciences

Generous gift from Dr. Delhaize, E., CSIRO Plant Industry, Australia

pUCPM18 plasmid containing kanamycin resistance gene (Buch et al., 2008)

pUCPM18 plasmid containing wild type *E. coli* citrate synthase gene, Km^r (Buch et al., 2008)

pUCPM18 plasmid containing *E. coli* NADH insensitive citrate synthase gene, Km^r citrate synthase gene, Km^r (This study)

pUCPM18 plasmid containing NADH insensitive citrate synthase gene and Na^+ dependent citrate transporter citC

Fig. 2.1: Restriction maps of the plasmids used in this study. All plasmids are broad host- range vectors used for expression of heterologous overepression of genes under *lac* promoter in *Herbaspirillum seropedicae* Z67

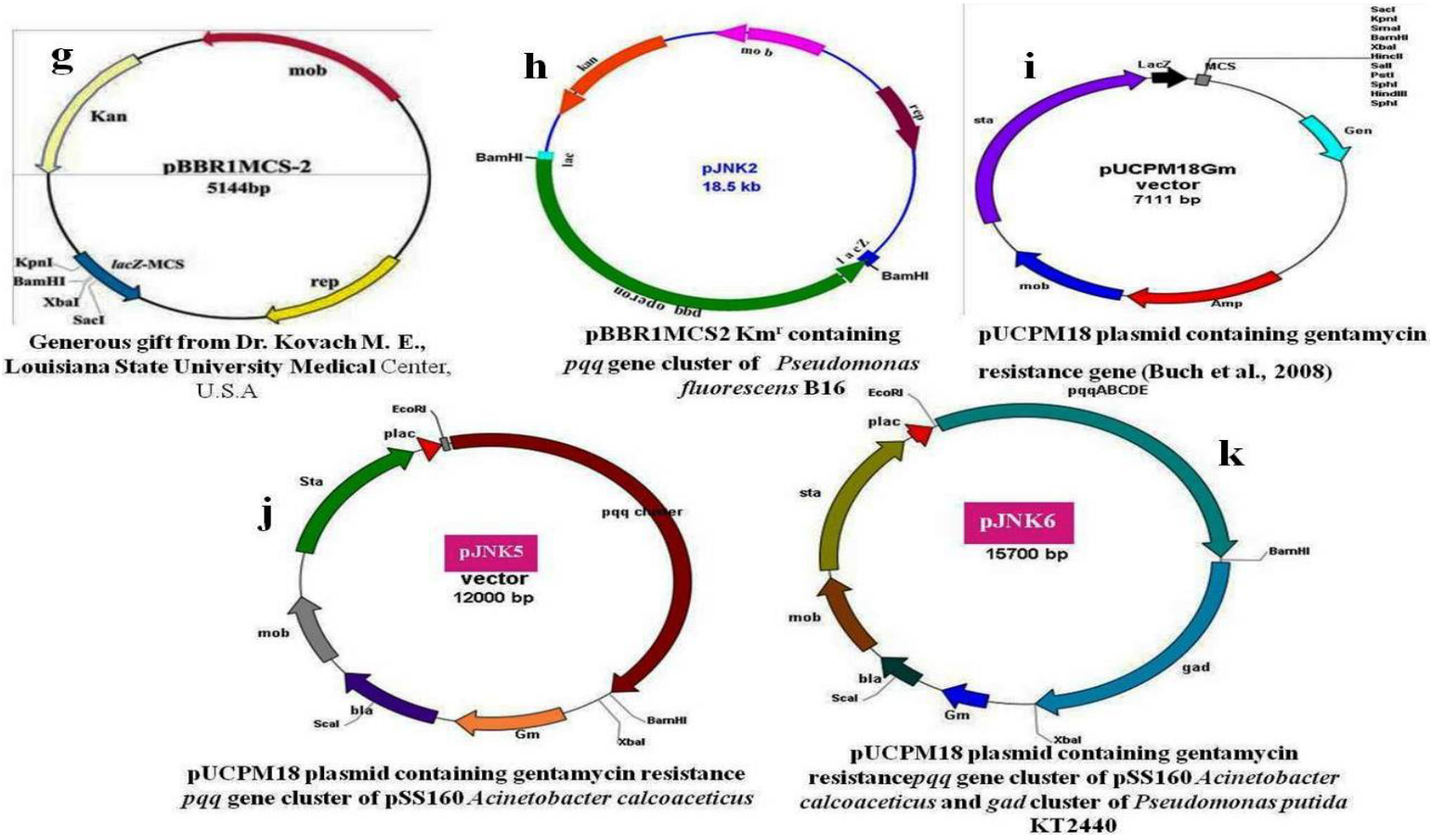


Fig. 2.2: Restriction maps of the plasmids used in this study. All plasmids are broad host- range vectors used for expression of heterologous overepression of genes under *lac* promoter in *Herbaspirillum seropedicae* Z67

2.1.1: pUCPM18 plasmid is derived from a pUC-derivative, pUCP18 plasmid which is stably maintained in both *E. coli* and *Pseudomonas* species (Schweizer, 1991). pUCPM18 was developed from pUCP18 (GenBank accession number: U07164) by incorporating a 750bp mob fragment from pLAFRI (broad host range vector used for genetic analysis of gram-negative bacteria; Vanbleu et al., 2004), in order to enable convenient mobilization within *Pseudomonas* species (Hester et al, 2000). It replicates in *E. coli* using ColE1 origin of replication (*ori*) while in *Pseudomonas* it replicates owing to a pRO1614 derived DNA fragment encoding a putative *ori* and a replication-controlling protein (West et al., 1994).

2.1.2: pBBR1MCS-2 (GenBank accession number: U23751) plasmid conferring kanamycin resistance is originally derived from pBBR1 plasmid of *Bordetella bronchiseptica* (Kovach et al., 1995). This plasmid facilitates cloning due to availability of unique restriction sites and direct selection of recombinant plasmids based on blue white selection strategy. pBBR1MCS based vectors are mobilizable when the RK2 transfer functions are provided in *trans*, are compatible with IncP, IncQ and IncW group plasmids, as well as with ColE1- and P15a-based replicons, can stably replicate in a variety of hosts including *E. coli*, *P. fluorescens*, *P. putida*, *Rhizobium meliloti* and *R.leguminosarum bv. Viciae* and are stably retained *in vivo* for more than 4 weeks in absence of antibiotic selection (Kovach et al., 1995).

2.2: Media and Culture conditions

The *E. coli* strains and pseudomonads were cultured and maintained on Luria Agar (LA) and Pseudomonas Agar respectively (Hi-Media Laboratories, India). *E. coli* cultures were grown at 37ºC while all *H. seropedicae* Z67 and *Pseudomonas* cultures were grown at 30ºC. For growth in liquid medium, shaking was provided at the speed of 200rpm. The plasmid transformants of both *E. coli* and pseudomonads were maintained using respective antibiotics at the final concentrations as mentioned in (**Table 2.2)** as and when applicable. Both *E. coli* and *H. seropedicae* Z67 wild type strains and plasmid transformants grown in 3ml Luria broth (LB) containing appropriate antibiotics were used to prepare glycerol stocks which were stored at -20ºC.

Antibiotic	Rich medium	Minimal medium
Nalidix acid	15 µg/ml	3 µg/ml
Gentamycin*	20µg/ml	5 µg/ml
Tetracycline	30µg/ml	7.5µg/ml
Kanamycin	50µg/ml	12.5µg/ml
Streptomycin	10µg/ml	2.5µg/ml
Trimethoprim	60µg/ml	15µg/ml
Ampicillin*	50µg/ml	12.5µg/ml
Erythromycin	100µg/ml	-
Chloramphenicol*	20µg/ml	-
Spectinomycin	50µg/ml	-

The compositions of different minimal media used in this study are as described below. Antibiotic concentrations in all the following minimal media were reduced to 1/4th of that used in the above mentioned rich media (**Table 2.2**).

Table 2.2: Recommended doses of antibiotics used in this study (Sambrook and Russell, 2001). The antibiotic dozes were maintained same for both *E. coli* and pseudomonads. All the antibiotics were prepared in sterile distilled water or recommended solvent at the stock concentrations of 1000x or 2000X (for antibiotics marked with *) and were used accordingly to have the desired final concentrations.

2.2.1: Bushnell-Haas (BH) medium

The ingredients of BH agar include Ammonium nitrate, 1.0g/L; calcium chloride, 0.02g/L; dipotassium phosphate, 1.0g/L; ferric chloride, 0.05g/L; magnesium sulphate, 0.2g/L; monopotassium phosphate, 1.0g/L and agar, 20.0g/L. The readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions. Sterile sodium benzoate at the final concentration of 1mM was supplemented as the sole carbon source.

2.2.2: Koser's Citrate medium

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

2.2.3: M9 minimal medium

Composition of M9 minimal broth was according to Sambrook and Russell (2001) including Na2HPO4 7H2O, 34g/L; KH2PO4, 15g/L; NH4Cl, 5g/L; NaCl, 2.5g/L; 2mM MgSO4; 0.1mM CaCl2 and micronutrient cocktail. The micronutrient cocktail was constituted of FeSO4.7H2O, 3.5 mg/L; ZnSO4.7H2O, 0.16 mg/L; CuSO4.5H2O, 0.08 mg/L; H3BO3, 0.5 mg/L; CaCl2.2H2O, 0.03 mg/L and MnSO4.4H2O, 0.4 mg/L. Carbon sources used were glucose, xylose, fructose and lactose as and when required. For solid media, 15g/L agar was added in addition to above constituents. 5X M9 salts, micronutrients (prepared at 1000X stock concentration) and carbon source (2M stock) were autoclaved separately. Fixed volumes of these were added aseptically into pre-autoclaved flasks containing distilled water to constitute the complete media with the desired final concentrations. Volume of water to be autoclaved per flask was calculated by subtracting the required volumes of each ingredient from the total volume of the media to be used.

2.2.4: HEPES buffered medium

The media composition included HEPES (pH=8.0), 100mM; NH4Cl, 10mM; KCl,10mM; MgSO4, 2mM; CaCl2, 0.1mM; micronutrient cocktail; Glucose, 100mM and phosphate (P) sources (Sharma et al., 2005). 1mg/ml Senegal Rock phosphate (RP) or KH2PO4 were used as insoluble and soluble P sources respectively. Each ingredient was separately autoclaved at a particular stock concentration and a fixed volume of each was added to pre-autoclaved flasks containing sterile distilled water (prepared as in Section 2.2.3) to constitute complete media.

2.2.5: Pikovskaya's (PVK) Agar

The media composition included Ammonium sulphate, 0.5g/L; Calcium phosphate, 5.0g/L; Dextrose, 10.0g/L; Ferrrous sulphate, 0.0001g/L; Magnesium Engineering the glucose metabolism of *Pseudomonas* spp. by heterologous expression of phosphoenol pyruvate carboxylase (PPC) and citrate synthase (CS) genes sulphate, 0.1g/L; Manganese sulphate, 0.0001g/L; Potassium chloride, 0.2g/L; Yeast extract, 0.5g/L and agar, 15.0g/L. Dextrose was substituted by same amount of xylose and fructose as and when mentioned. The readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions.

2.2.6: Murashige-Skoog's Medium

Murashige-Skoog's (MS) medium was composed of macro elements and micro elements. The macro elements included CaCl2.2H20, 0.440g/L; KH2PO4, 0.17g/L; KNO3, 1.9g/L; MgSO4.7H2O, 0.37g/L; NH4NO3, 1.65g/L and 10g/L agar. The micro elements included the essential trace elements. Micronutrients and desired carbon source (autoclaved separately), were added aseptically to reconstitute the complete media. The readymade micronutrient was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions while the macronutrients were reconstituted as and when required.

2.2.7: JNFb medium for *Herbaspirillum seropedicae* Z67

JNFb medium consists of l-malic acid, 5.0; K_2HPO_4, 0.6; KH_2PO_4, 1.8; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.02; trace element solution ($Na_2MoO_4 \cdot 2H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.235 g; H_3BO_3, 0.28 g; $CuSO_4 \cdot 5H_2O$, 0.008 g; $ZnSO_4 \cdot 7H_2O$, 0.024 g; distilled water, 1000 ml), 2.0 ml; bromthymol blue (0.5% aqueous solution (dissolve in 0.2N KOH)), 2.0 ml; FeEDTA (1.64% solution), 4.0 ml; vitamin solution (biotin, 0.01 g; pyridoxine, 0.02 g; distilled water, 1000 ml), 1.0 ml; KOH, 4.5; pH adjusted to 5.8 with KOH. Bring the final volume to 1000 ml with distilled water. For a semisolid medium, add 1.75 to 1.9 g agar/l (agar should be dissolved before distribution into vials); for a solid medium, add 15.0 g agar/l. (Baldani et al., 1996)

2.3: Molecular biology tools and techniques

2.3.1: Isolation of plasmid and genomic DNA

2.3.1.1: Plasmid DNA isolation from *E. coli* and *Herbaspirillum seropedicae* Z67.

The plasmid DNA from *E. coli* and *H. seropedicae* Z67 was isolated by the boiling alkali lysis method, (Sambrook and Russell, 2001). Engineering the glucose metabolism of *H. seropedicae* Z67. by heterologous expression of and citrate synthase (CS) genes , PQQ gene clusters and GAD operon.

2.3.1.2: Isolation of large size plasmid DNA from *Herbaspirillum seropedicae* Z67

The large size of ~117kb TOL plasmids of *H. seropedicae* Z67 was isolated, by the protocol described for the isolation of plasmid (Ramos-Gonzalez et al., 1991). Single colonies of the *Herbaspirillum* were inoculated in 3ml of LB and were allowed to grow at 30ºC under shaking conditions (200rpm). 0.5ml of freshly grown cultures was centrifuged at 9, 200x *g* for 2 minutes and the pellet was re-suspended homogenously in 200µl of sterile STE [20% sucrose in 25mM Tris-Cl containing 25mM EDTA (pH=8.0) and 1mg/ml lysozyme]. Complete lysis of the cells was achieved by adding 100µl of alkaline SDS [0.3N NaOH containing 2% SDS]. After gentle mixing, the resulting viscous solution was incubated at 55ºC for 5 minutes to minimize the chromosomal DNA contamination. Following this the solution was treated with equal volume of phenol-chloroform and after gentle mixing was subjected to centrifugation at 9, 200x *g* for 10 minutes. The aqueous layer recovered was subjected to chloroform extraction, centrifugation at 9, 200x *g* for 10 minutes, recovery of the aqueous phase and finally DNA precipitation using 0.6 volumes of isopropanol. The DNA was allowed to precipitate at room temperature for ~30-40 minutes, washed with 70% ethanol and finally dissolved in 30µl Tris-EDTA (TE) buffer (Sambrook and Russel, 2001). Of this, 15µl DNA solution was subjected to agarose gel electrophoresis using 1% agarose (Section 2.4.4).

2.3.2: Transformation of plasmid DNA

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

The transformation of plasmids in *E. coli* using MgCl2-CaCl2 method and blue white selection of the transformants using IPTG and X-Gal (as and when applicable) was carried out according to Sambrook and Russell (2001).

2.3.2.2: Electroporation of plasmid DNA in *Herbaspirillum seropedicae* Z67

Plasmid transformation in *Herbaspirillum seropedicae* Z67 was done by electroporation as described by (Unge et al., 1998) *H. seropedicae* Z67 cells grown till early stationary phase, cells were harvested (at 4^{o}C) in sorvall washed 4 times with sterile distilled water and resuspended at 10^{10} cells ml^{-1}. Plasmid DNA of 0.5 µg $µl^{-1}$ was added in competent cells in electroporation cuvette. After 10 min of incubation on ice, the DNA was electroporated into cells using Eppendorf Electroprater system. System was adjusted to 2.5 kV ans 186 Ω. The cells were immediately transformed to 1 ml LB and incubated for 45 min at 30^{o}C, and then cells were plated on respective antibiotic for the selection of plasmid.

2.3.3: Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% agarose (containing 1µg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer at 5v/cm for 0.5-2h. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator.

2.3.4: Restriction enzyme digestion analysis

0.5-1.0µg DNA sample was used for each restriction enzyme digestion. 1-3U of the restriction endonuclease (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10µl. The reaction mixture was incubated overnight at 37°C. The DNA fragments were visualized by ethidium

bromide staining after electrophoresis on 0.8% agarose gels and were subsequently photographed. In case of double digestion, a compatible buffer for the two REs was essentially checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers.

2.3.5: Gel elution and purification

The DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band. The agarose piece was weighed in a sterile microcentrifuge tube and was solubilized in 2.5 volumes of 6M sodium iodide (NaI, freshly made) [e.g. for 200mg of agarose piece, 500µl of NaI was added]. Once completely dissolved, 15µl of silicon dioxide suspension (50% w/v, stored at 4°C) was added and was incubated at room temperature for 15-20 minutes. The DNA bound to silica was recovered by centrifuging at 9, 200x *g* for 2 minutes; the pellet was washed twice with 70% ethanol, dried and finally re-suspended in 20-30µl sterile double distilled water. The microcentrifuge tube was incubated at 55°C for 10 minutes to allow complete dissociation of DNA from silica beads into the solution and then was subjected to centrifugation at 9,200x *g* for 2 minutes. The resultant supernatant was gently recovered using sterile micropipette tip and was transferred to fresh sterile tube. The purification efficiency was checked by subjecting 2µl DNA solution to gel electrophoresis and visualizing the sharp DNA band of desired size. The purified DNA was used for ligation experiments only if >50ng/µl DNA was recovered after purification.

2.3.6: Ligation

The ligation reaction was usually done in 10µl volume containing the following constituents: Purified vector and insert DNA (volume varied depending on the respective concentrations); 10X T4 DNA Ligase buffer, 1µl; T4 DNA ligase (MBI Fermentas), 0.5- 1.0U and sterile double distilled water to make up the volume. The cohesive end ligation reaction was carried out at 16°C for 12-16h. The vector to insert

molar ratio (molar concentrations calculated by the under mentioned formula) of 1:4 was maintained, with a total of 50-100ng of DNA in each ligation system.

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

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2.3.7: Polymerase Chain Reaction (PCR)

The PCR reaction set up was based on the guidelines given in Roche Laboratory Manual. The assay system and the temperature profile used are described in **Table 2.6.**

Assay system used		**Temperature Profile**	
Sterile DDW	38 µl	Initial denaturation	94°C- 5 min
dNTP(10mM with 2.5mM each)	3 µl	Denaturation	94°C- 30 sec
Reverse Primer 20pmoles	1 µl	Annealing	Varies from 55-62°C for 30sec.*
Forward Primer 20pmoles	1 µl	Elongation	72°C for 45sec-2.5min*
Template DNA (100ng/µl)	1 µl		
Taq/20A PCR buffer (10X)	5 µl	Final Elongation	72°C- 10 min
Taq DNA/XT20 Polymerase (1 unit/µl)#	1.0 µl	For plasmid	(30 cycles)
Total System	**50 µl**	For genomic DNA	(40 cycles)

Table 2.3: PCR conditions used in the present study.

PCR amplifications were performed in Techne TC-312 thermal cycler. *Exact primer annealing temperature and primer extension time varied with primers (designed with respect to different templates) and has been specified in the text as and when applicable. Processivity of Taq polymerase is ~1000 bases per min. Taq DNA polymerase and its buffer, dNTPs and primers were obtained respectively from Bangalore Genei Pvt. Ltd., India, Sigma Chemicals Pvt. Ltd. and MWG Biotech. Pvt.

Ltd, India, respectively and were used according to manufacturer's instructions. The theoretical validation of the primers with respect to absence of intermolecular and intramolecular complementarities to avoid primer-primer annealing and hairpin structures and the appropriate %G-C was carried out with the help of online primer designing software Primer 3. The sequence, length, and %G-C content of primers are subject to variation depending on the purpose of PCR and will be given as and when applicable in the following chapters. The PCR products were analyzed on 1.0% agarose gel along with appropriate molecular weight markers (Section 2.4.4)

2.4: Aromatic hydrocarbon utilization

Aromatic hydrocarbon utilizing ability of *Herbaspirillum seropedicae* Z67 strain was checked by monitoring the growth at 30°C on BH Agar (Section 2.2.1) containing 1mM of Na-benzoate as sole carbon source. Kanamycin was used at the final concentration of 7.5µg/ml. Accompanying phenotype of green fluorescence (attributed to expression of GFP) was monitored under UV light.

2.5: Lactose and Citrate utilization

Ability of the *Herbaspirillum seropedicae* Z67 to utilize citrate as carbon source and inability to assimilate lactose (Lessie and Phibbs, 1984) was exploited as a selection tool for pseudomonads in several experiments. Growth at 30°C on Koser's Citrate medium (Kiyohara et al., 1982) and M9 minimal medium containing 100mM lactose agar plates was monitored to indicate citrate and lactose utilizing abilities.

2.6: P-solubilization phenotype

Fresh culture of *H. seropedicae* Z67 transformants obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30ºC was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed thrice with sterile normal saline and used to characterize P-solubilizing ability of the native as well as the *H. seropedicae* transformantswere monitored for (i) di-calcium phosphate (DCP) solubilziation on Pikovaskya's (PVK) agar (Pikovskaya, 1948),(ii) HEPES buffered

RP-Methyl red as pH indicator dye and 1.5% agar (HRP) agar plates. HRP medium is similar to TRP medium with 50 mM HEPES pH 8.0 was used instead of 100 mMTris pH 8.0 (Gyaneshwar et al., 1998). Culture was aseptically spotted on the HRP agar plates and was allowed to dry completely followed by incubation at 30°C for 2-4 days. P solubilization was determined by monitoring the zone of clearance on PVK agar and red zone on the HRP Methyl red agar plates.

2.7: Potassium soluiblizing assay.

Fresh culture of *Hseropedicae* Z67 transformants obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30ºC was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed thrice with sterile normal saline and used to characterize K-solubilizing ability of the native as well as the *H. seropedicae* transformants were monitored for (i) Feldspar (Aleksandrov agar plate) (Hu *et al.*, 2006), (ii) HEPES buffered Rock Phosphate and Feldspar - Methyl red as pH indicator dye and 1.5% agar (HRPF) agar plates. HRPF medium is similar to TRP medium with 50 mM HEPES pH 8.0 was used instead of 100 mM Tris pH 8.0 and Feldspar was used with Rock phospahte (Gyaneshwar et al., 1998). Culture was aseptically spotted on the HRPF agar plates and was allowed to dry completely followed by incubation at 30°C for 2-4 days. K solubilization was determined by monitoring the zone of clearance on Aleksandrov agar plate and red zone on the HRPF Methyl red agar plates.

(Aleksandrov agar medium constituted 1% glucose, 0.5% Yeast extract, 0.05% $MgSo_4.7H_2O$, 0.0005% $FeCl_3$, 0.01% $CaCO_3$, 0.2% $CaPo_4$ and 0.5% Feldspar, 3 % agar) .

2.8: Mutant Complementation Phenotype.

E. coli cs (W620) mutants were used to confirm the functionality of the cloned *cs* genes, respectively. The recombinant plasmids containing *cs* genes independently under *lac* promoter were transformed into respective mutants. The *cs* transformants were selected on streptomycin and kanamycin. These transformants were grown on M9 minimal medium with 0.2% (11.11mM) glucose as carbon source in presence and absence of 340µg/ml sodium glutamate, under shaking conditions at 30°C for *E. coli*

W620 at 37°C for. Kanamycin, and streptomycin were used at the final concentrations of 12.5µg/ml, 7.5µg/ml and 2.5µg/ml (1/4th of the concentration used for growth in LB broth). Both *ppc* and *cs* mutants exhibited glutamate auxotrophy (Izui et al., 1986; Underwood et al., 2002) as they could not grow on glucose as the sole carbon source in the absence of glutamate supplementation.

2.9: Physiological experiments

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

2.9.1: Inoculum preparation

The inoculum for M9 and HEPES minimal media containing rock phosphate were prepared by growing the *H. seropedicae* Z6 overnight at 30°C in 3ml LB broth. Inoculum for the buffered RP broth (HRP) was prepared by growing the *H. seropedicae* Z6 overnight at 30°C in 10ml of M9 minimal medium. Cells were harvested aseptically, washed twice by normal saline, and finally re-suspended in 1ml normal saline under sterile conditions. Freshly prepared inoculum was used for all the experiments.

2.9.2: Growth characteristics and pH profile

Growth parameters and pH profile of the native as well as *H. seropedica*e transformants were determined using HRP medium with RP as P source (Gyaneshwar et al., 1998). Growth curve and pH profile was carried in 150ml conical flasks containing 30ml of relevant media. The initial O.D.600nm was about 0.09-0.15. The batch culture studies were performed under aerobic conditions in Orbitek rotary shaker maintained at 30°C with agitation speed kept constant at 200 rpm. 1ml samples were aseptically harvested at regular intervals and were subjected to analytical techniques. Microaerobic condition was maintained in flask by addition of ¾ volume of medium in 150 ml flask as per the method of Boesten and Priefer (2004). Flasks were sealed with rubber septum and incubated at 30^{o}C by shaking at 200 r.p.m. and the syringe was used to take out samples.

2.10: Estimation of Plant growth promoting factors

2.10.1: Culture conditions for EPS production and quantification

Fresh overnight grown culture of *Hs* Z67 transformants as well as native obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30ºC was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed thrice with sterile normal saline and used to for inoculation in 150ml flask containing 30ml M9 minimal medium supplemented with 50mM glucose as sole carbon source, cultures were grown at 30ºC for 24h used to examine the EPS production. EPS was extracted using (EDTA) method described by (Tapia et al., 2009). Bacterial cells were centrifuged at 14,000 rpm for 20min supernatant was used for EPS extraction. EDTA (2% v/v) was added to supernatant for precipitation and kept at 4^0C for 2h, EPS obtained in each extraction were separated by centrifugation from solution and under 4^0C. EPS containing free cells solution was obtained by filtrated using nitrocellulose of 0.22 mm of pore size (Millipore). Filtered solution was used for EPS estimation. The phenol-sulphuric method was followed for total EPS estimation EPS (expressed as µg/ml) (Dubois et al., 1956) reaction mixture contained 1ml of EPS solution,1ml of aqueous phenol and 5ml of concentrated H_2SO_4 was mixed properly and kept for 15-20minutes, absorbance was measured at 490nm, the amount of EPS was determine against glucose as standard.

2.10.2: Bioflim assay

Samples were inoculated in test tube containing 3ml M9 minimal medium containing 50mM glucose concentration, Bacterial cultures were incubated under aerobic conditions in Orbitek rotary shaker maintained at 30°C for 24h with agitation speed kept constant at 200rpm. Growth medium was discarded after 24h from each test tube; to remove the unattached bacteria with test tubes were washed with Phosphate Buffer Saline (PBS). Biofilm were fixed with 2 ml of 99% methanol, tubes were left empty to dry. Crystal violet 2 ml of 1% was used for staining of adhered cells with 5 minutes incubation. Surplus stain was rinsed by distilled water for 5 times. Tubes were air dried and the dye attached to cells was dissolved in 1.5 ml of 33% glacial acetic acid. Biofilm formation was detected by simple tube method and estimated by measuring optical density (OD) at 570nm spectrophotometer assay described by Mathur et al., (2004).

2.10.3: Indole acetic acid (IAA) production and estimation

Overnight grown all cultures were pulled in sterile 1.5ml eppendorf in laminar, aspetic conditions were maintained , samples were washed 3 times by normal saline, these samples were used for inoculation of IAA production, cultures were inoculated in 150ml conical flask containing 30ml M9 minimal medium with L-tryptophan (0.1%) and one set without L-tryptophan with 50mM glucose as carbon source , flask were incubated at 30^0C orbital shaker 200rpm shaking for 24h, further samples were screened for IAA production, experiment was carried out in triplicates. Cell-free supernatant was obtained by centrifugation at 9000 × g for 20 minutes; supernatant was used for IAA extraction method described by (Sinha and Basu, 1981). To the 10 ml of supernatant, 2 ml of salkowski's reagent (1 ml of 0.5 M $FeCl_3$ + 50 ml of 35% perchloric acid) was added and incubated for 30 mintues under darkmess. Pink colour formed was determined colorimetrically at 540 nm as IAA production.

2.11: PQQ determination

PQQ production was estimated using the method of Rajpurohit et al., (2008). *Hs* Z67 transformants and other bacteria were grown for 36 h on M9 minimal medium containing 50 mM glucose. Cell supernatant was digested with 50% acetonitrile at 65°C for 2 h. The mixture was centrifuged at 15,000 *g* for 10 min; the clear supernatant was collected and dried with a concentrator under a vacuum. The residue was dissolved in 50% *n*-butanol at 1 mg/ml, and PQQ was extracted at 50°C overnight. The clear supernatant was dried under a vacuum and dissolved in 100% methanol The identity of the PQQ was ascertained by comparing the with standard PQQ on spectrofluorometer Fluorescence was monitored at *ex* 360 and *em* 480 nm.

2.12: Analytical techniques

Modified Lowry's method (Peterson 1979) was used for total protein estimation. *H. seropedicae* Z67 native as well as transformants increased in cell growth density was determined at OD600 (spectrophotometer). Increase in culture growth, pH drop indicates organic acid secretion and phosphate solubilisation from rock phosphate in medium, End point culture supernatants samples were filtered through 0.2 μm nylon membranes further they were used for Phosphate estimation by the ascorbate method (Ames 1964) same samples were used for HPLC analysis to

detect organic acid levels, retention time of acids were determined under above mentioned conditions. Standards organic acids were used for quantification (equipment from Shimadzu , India) HPLC was performed using a C18 column operated at room temperature with 0.01 M H_2SO_4 as mobile phase at a flow rate of 0.7 ml min^{-1}, while for citric acid estimation the same column was operated at room temperature using mobile phase of 20mM Na_2HPO_4 with 2.5% acetonitrile at a flow rate of 1.0 ml min^{-1} and the column effluents were monitored using a UV detector at 210 nm (Buch et al., 2009, 2008) and measuring left over glucose using the GOD-POD kit (Enzopak, Reckon Diagnostics Pvt. Ltd., India). Physiological parameters were calculated like growth rate, biomass yield on glucose and specific glucose reduction rate as described by (Buch et al., 2009, 2008). Graph Pad Prism (version 3.0) and Microsoft Excel were used for statistical analysis of the parameters. Each parameter has been represented as mean ± SD or mean ± SEM as specified in the figure legends.

(i) Specific growth rate (h-1):

$$k = \frac{(\text{Log10Nt1-Log10Nt2}) \times 3.3}{(t1-t2)\ (h)}$$ where,

N1 and N2 are the number of cells at time t1 and t2 respectively and (t1-t2) is the corresponding time interval in hours. 3.3 is the factor derived from the formula-number of generations (n) = (Log10N-Log10N0)/Log102. The number of cells was calculated from O.D.600nm using the correlation 1 O.D.600nm = 1.5x109 cell/ml (Koch et al., 2001)

(ii) Specific total glucose utilization rate (QGlc):

$$QGlc = \frac{\Delta\text{Glucose (t1-t2) (g/L)}}{\Delta\text{dcw (t1-t2) (g/L) x Time interval (t1-t2) (h)}}$$ where,

ΔGlucose (t1-t2) is the amount of glucose consumed over the time interval t1-t2; **Δ**dcw (t1-t2) is the difference in the dry cell weight (dcw) of the cells over the time interval t1-t2. QGlc is expressed as g glucose utilized/g dcw/h). Dry cell weight was calculated using the correlation 1 O.D.600nm = 0.382mg/ml (Bugg et al., 2000).

(iii) Biomass yield

$$Ydcw/Glc = \frac{\Delta dcw\ (t1\text{-}t2)\ (g/L)}{\Delta Glucose\ (t1\text{-}t2)\ (g/L) \times Time\ interval\ (t1\text{-}t2)\ (h)}$$ where,

All the parameters were as described for Specific glucose utilization rate. Ydcw/Glc is expressed as g of dry cell weight produced/ g glucose utilized/h.

(iv) Organic acid yield

$$Organic\ acid\ yield = \frac{Amount\ of\ organic\ acid\ produced\ (g/L)}{Total\ glucose\ utilized\ (g/L) \times dcw\ (g/L)}$$ where,

The amount of total glucose utilized was obtained by deducting the value of residual glucose concentration from the initial glucose concentration supplied in the medium. The difference between the total glucose utilized and gluconic acid produced was considered as glucose consumed. Hence, the total glucose utilized and not glucose consumed was taken into account for calculating specific glucose utilization rate. The statistical analysis of all the parameters was done using Graph Pad Prism (version 3.0) software and microsoft Excel.

Dry cell mass (dcw) = O.D600nm x 0.382, where 0.382 is the factor correlating O.D600 was with dry cell weight (Bugg et al., 2000)

2.13: Enzyme assays

2.13.1: Preparation of cells and cell free extracts

Glucose grown cells under above mentioned minimal media conditions were harvested in appropriate growth phase from 30ml of cell culture by centrifugation 9,200x *g* for 2 minutes at 4°C, citrate synthase (CS), the preparation of cell free extracts for, CS, assays was carried out according to Kodaki et al (1985) The cell pellet was washed once with 80 mM phosphate buffer (pH=7.5) followed by resuspension in same buffer containing 20% glycerol and 1mM DTT. The cells were then subjected to lysis by sonicating for maximum 1-1.5 minute in an ice bath, followed by centrifugation at 9,200x *g* at 4°C for 30 minutes to remove the cell debris. The supernatant was then used as cell-free extract for the enzyme assays. The whole cell preparation for GDH assay was done by washing the harvested cells (mid-late log phase cultures) thrice with normal saline to remove the residual glucose of the medium and resuspending in 0.01M phosphate buffer (pH 6.0) with 5mM MgCl2.

2.13.2: Enzyme Assay Protocols

2.13.2.1: GDH assay

GDH (D-glucose phenazine methosulphate oxidoreductase, (EC 1.1.5.2) was determined spectrophotometrically by following the coupled reduction of 2,6-dichlorophenolindophenol (DCIP) at 6_{00} nm (Quay et al., 1972). Molar absorbance of DCIP was taken as 15.1 mM^{-1} cm^{-1} at pH 8.75. The reaction mixture included: Tris-Cl buffer (pH 8.75), 16.66 mM; D-glucose, 66 mM; DCIP, sodium salt, 0.05 mM; phenazine methosulfate, 0.66 mM; sodium azide, 4 mM; whole cells, and distilled water to 3.0 ml.

2.13.2.2: Gluconate dehydrogenase assay (GADH)

Hs natives as well as transformants were grown on M9 and RP-containing minimal medium with 50 mM D-gluconate as the C source, Additionally RP-containing minimal medium contains 50mM HEPES buffer, pH 8.0, Cells were harvested (5000g for 10 min) after the pH dropped below 5.5, washed with sterile saline, and resuspended in 50 mM Tris-HCl, pH 8.75, and the whole-cell suspension was used as the source of enzyme in GADH assays. Spectrophotometrically method was followed for estimation

of GADH activity according to Matsushita et al., (1982). Substrate of the GADH enzyme was done with D-gluconate, in the assay mixture. GADH activity was defined as nanomoles of 2, 6-dichlorophenolindophenol (DCIP) reduced per minute using D-gluconate, as substrates, respectively. Enzyme activity is defined as one unit of specific enzyme activity was defined as the amount of protein required to convert one nanomole of substrate per minute per milligram of total protein.

2.13.2.3: CS assay.

CS (4.1.3.7) activity was estimated by following the absorbance of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) at 412nm which would change due to its reaction with sulfhydryl group of CoA (Serre, 1969). The assay mixture per cuvette contained following ingredients in 1.0 ml: Tris-HCl (pH=8.0), 93 mM; acetyl CoA, 0.16 mM; OAA, 0.2 mM; DTNB, 0.1 mM and cell lysate. The reaction was started by addition of OAA. Molar absorbance coefficient was taken as 13.6 $mM^{-1}cm^{-1}$ at 412 nm. The rate of increase in absorbance was used to calculate CS activity. All the enzyme activities were determined at 30°C and were expressed per mg total protein. Total protein concentration of the crude extract as well as whole cell suspensions was measured by modified Lowry's method (Peterson, 1979) using bovine serum albumin as standard. Corrections were made for Tris buffer. Enzyme activities were calculated using following formula

$$\text{Specific enzyme activity (U)} = \frac{\Delta A_y \text{ nm/min}}{€ \times \text{enzyme (sample) aliquot (ml)} \times \text{Total protein (mg/ml)}}$$

where,

ΔA_y nm is the difference in the absorbance at any given wavelengths (y nm) and € is the millimolar extinction coefficient at *y* nm.

One unit of enzyme activity was defined as the amount of protein required to convert 1 nmole of substrate per minute unless stated in the figure legend.

2.14: Hydrophonic plant study with rice. (*Oryza sativa*).

The hydroponic experiment was designed in three replications according (Crestani et al., 2009). Rice (*Oryza sativa*, Gujarat-17) seeds were surface sterilized in 70% ethanol for 1 min and in 1% sodium hypochlorite (NaClO) for 20 min, and then rinsed 5- 6 times with sterile distilled water and then allowed to germinate for 72 h on moist filter paper in petriplates kept in the dark at 30^{o}C placed Uniform grown seedlings with 5 mm radicles were transferred to nylon nets placed on top of beakers containing 500 ml hydroponics nutrient solution for rice suggested by (Miyamoto et al., 2001), Stress of cadmium ($CdCl_2$) in µM and sodium chloride (NaCl) in mM was added independently in hydroponic containing nutrient medium. Exogenously PQQ of 100 nM was also added independently to set of stress given plants (Choi et al., 2008). After 10 day of treatment plants were removed and rinsed with tap water and used for catalase assay, superoxide dismutase assay, root length and shoot length was measured to know the effect of stress on rice plants.

2.15: Pot experiments - Interaction with (*Oryza sativa* Gujarat-17).

2.15.1: Plant Inoculation Experiments:

Pure bacterial cultures were grown in nutrient broth at 30^{o}C, centrifuged, and diluted to a final concentration of 10^8 CFU/ml in sterile distilled water. Rice (*Oryza sativa*, Gujarat-17) seeds were surface sterilized in 70% ethanol for 1 min and in 1% sodium hypochlorite (NaClO) for 20 min, and then rinsed 5- 6 times with sterile distilled water and then allowed to germinate for 24 h on moist filter paper in petriplates kept in the dark at 30^{o}C.and then immersed in a bacterial suspension for 5 h. Germinated seeds were sown in plastic bags filled with 1Kg of autoclaved, locally collected soil. Before sowing seeds in bags initial physical and chemical properties in experimental of soil is presented in (**Table.4).**The plants were grown at 30^{o}C under natural day light and were irrigated with 50 mL of sterile distilled water every day. The experiment was conducted on six replicates (three bags per replicate, two plants per bag) for each treatment and was completely randomized. The plants were harvested after 30 days and growth parameters (plant height and plant biomass) were recorded. Total N, was measured by micro Kjeldahl's method, byVepodest-

Automatic Digestion, Distillation and titration system. Total P content was measured by Vanadomolybdo phosphoric acid yellow color method, and Total K content was measured by Flame photometer.

Table 2.4: Physical and chemical properties of experimental soil used for *H. seropedicae* Z67 transformants

E.C	pH	Total phosphorus (%)	Total nitrogen (%)	Total potassium (%)
0.63	7.86	19	16	65

2.15.1.1: Antioxidant Enzymes / ROS scavenging enzyme activity:

(1) Superoxide Dismutase (SOD)

Two hundred mg of leaves were homogenized in a pre-chilled mortar and pestle under ice cold condition using 3.0 ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.4), 1 mM EDTA and 1% (W/V) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay (Costa et al.2002).

Total SOD (EC 1.15.1.1) activity was measured spectrophotometrically based on inhibition in the photochemical reduction of nitroblue tetrazoilum (NBT). The 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8),13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 0.1 ml enzyme extract, riboflavin was added last (Van Rossun et al., 1997).After addition of all these components and mixing, test tubes were placed on stand 30 cm below a light source consisting of four 15-w fluorescent lamps. The photochemical inhibition was allowed to happen for 10 minutes and stopped by switching off the light source. The photoreduction in NBT was measured as increase in absorbance at 560 nm. Blanks and controls were run the same way but without illumination and enzyme, respectively. One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% in a reaction mixture. Enzyme unit of SOD was calculated according to formula given by Constantine and Stanley (1977).

$$\text{SOD unit} = \frac{\text{Control O.D. (without enzyme)}}{\text{Sample O.D.}} - 1 \times \frac{1}{\text{(g) conc. Enzyme}}$$

$$\text{SOD U/g protein} = \frac{\text{SOD unit}}{\text{protein mg/g}}$$

(2) Catalase

Two hundred mg of acetone powder homogenized with a pre-chilled mortar and pestle under ice cold condition in 2.0 ml of extraction buffer, containing 0.1 M sodium phosphate buffer (pH 7.2) with the addition of 1 mM EDTA and 1% (w/v) polyvinylpyrolidone (PVP) and a pinch of activated charcoal. The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants were used for the assay (Mahatma et al., 2011). Total catalase (EC 1.11.1.6) activity was determined in the supernatants by measuring the decrease in absorption at 240 nm as H2O2 (= 39.4 mM-1 cm-1) got consumed according to the method of Aebi (1984) and enzyme activity expressed as mmol H_2O_2 oxidized min^{-1} g^{-1}protein. The 3 ml assay mixture contained 50 mM sodium phosphate buffer (pH 7.0), 30mM H_2O_2 and 50 µl enzyme extract. Enzyme unit of CAT was defined as:

$$\text{Mmol/min/g protein} = \frac{\text{O.D. } \Delta}{\varepsilon \times \text{protein mg/g} \times \text{Enzyme conc. (g)}}$$

Where, ε = Extinction coefficient, Enzyme conc. (g) = Amount of enzyme in 3 ml reaction mixture

2.15.1.2: Estimation of Water Soluble Protein Content

Protein concentration of each enzyme extract was estimated by method of Lowry et al., (1951).

(a) Reagents for Lowery's Method

(i) Solution A: 2% Na2CO3 in 0.1 N NaOH

(ii) Solution B: (a) 1% CuSO4.5H2O solution

(b) 2% sodium potassium tartarate solution

Working solution of

B : Prepared fresh before use by mixing equal volume of solution B (a) and B (b).

(iii) Solution C: Prepared fresh before use by mixing 50 ml of solution A and 1 ml of working solution of B.

(iv) Solution D: Folin & Ciocalteu reagent (1N).

(b) Procedure (Folin Lowery's Method)

Enzyme extracts (25 µl) were taken in test tube and volume was made up to 1 ml with millipore water. A tube with 1 ml of water served as blank. Five ml of solution C was mixed by vortexing and kept for 10 min. Then 0.5 ml of solution D (Folin & Ciocalteu reagent) was added and vortexed. The tubes were allowed to stand at room temperature for 30 min. Absorbance was read at 660 nm. A standard curve was prepared using bovine serum albumin (BSA) in the concentration range of 10-80 µg.

Chapter 3

3.1: Introduction

Citrate synthase (CS) is a ubiquitous enzyme that catalyzes the first committed step of tricarboxylic acid (TCA) cycle involving condensation of oxaloacetate (OAA) and acetyl-CoA to form citrate. Hence it is the key enzyme governing the carbon flux into the TCA cycle which plays a dual function in the production of cellular energy and biosynthetic precursors under aerobic conditions and only latter under anaerobic conditions, respectively (Park et al., 1994).

Rhizobacteria mainly secret organic acids such as citric, oxalic, gluconic, 2-ketogluconic, lactic, malic, succinic, tartaric and acetic for mineral phosphate solubilizing (MPS) ability (Archana et al., 2012). Solubilization of mineral P from soils is depends on the nature and amount of the acid which varies from 10 to 100 mM from strong to weak organic acid (Gyaneshwar et al., 1998). *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium, Rahnella, Enterobacter, Citrobacter, Azospirillum* and *Erwinia* are known to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate (TCP), dicalcium phosphate (DCP), hydroxyapatite (HAP) and rock phosphate (RP) (Khan et al*.,* 2006). Among all organic acids, citric acid is one of the most efficient organic acids in mineral P solubilization due to its good chelating and strong acid properties (Kpomblekou et al., 1994; Liu et al., 2012).

Rice (*Oryza sativa*) is the staple food for one third of world population. Nitrogen and Phosphate are key nutrients which limit agricultural production and supplementation of P and N chemical fertilizers significantly enhanced plant growth and development (Gyaneshwar et al., 2002; Tilman et al. 2002; Ladha et al. 2003). Alternative to application of chemical fertilizers is the use of nitrogen fixing and plant growth promoting rhizobacteria (PGPR). Inoculation of microbial consortium of P solublizing *Bacillus megaterium* and N fixing free bacteria like *Azotobacter* have been found to be more effective than individual members (Aditya et al., 2009).

Herbaspirillum seropedicae is a diazotrophic endophyte which colonises the external and internal tissues of economically important plants gramineous (*Poaceous*) plants, such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*) rice, as well as some non-*Poaceaous* plants (Baldani et al. 1986; Olivares et al. 1996; Weber et al. 1999). Under field conditions, *H seropedicae*

inoculation stimulates root and shoot growth and rice seed germination (Pereira et al. 1988). However, these effects are highly variable which depend on the rice variety and environmental conditions (Malarvizhi and Ladha1999; Gyaneshwar et al. 2002; James et al. 2002).

Citric acid production in *A. niger* was increased by 45% through increasing the influx of glucose (Guebel and Torres, 2001). Overexpression of phospho fructokinase (*pfk*), pyruvate kinase (*pk*) and citrate synthase (*cs*) genes in *A. niger* improved citrate production (Ruijter et al. 1997; 2000). Isocitrate lyase (*icl*) gene over expression in *Yarrowia lipolytica* altered citrate/isocitrate ratio towards citric acid (Forster et al., 2007a). Additionally, invertase gene expression enabled *Y. Lipolytica* to secrete high levels of citric acid in presence of sucrose. Similarly, *B. subtilis icd* mutant in early stationary phase accumulated ~15 fold higher intracellular citrate levels as compared to the wild type (Matsuno et al., 1999). Metabolic studies on citric acid producing fungi, yeasts, and *E. coli* demonstrated that high citric acid levels could be attained on glucose and depending on the host metabolism.

Disruption of *icd* gene of *E. coli* has been reported to secrete citric acid levels up to 3.4 mM (Aoshima et al., 2003; Kabir and Shimizu, 2004). Although citric acid was increased, the mutant showed glutamate auxotrophy. However, citric acid secretion is difficult to explain as *E. coli* K strain does not possess citrate transporter (Hall, 1982). Citric acid secretion was also found in *Bacillus subtilis icd* and *Streptomyces coelicolor* aconitase mutants (Matsuno et al., 1999; Viollier et al., 2001). Since these mutants show glutamate auxotrophy, they are not suitable for field conditions.

Increase in CS activity was postulated to be a better strategy for citric acid production in *E. coli* rather than isocitrate dehydrogenase (*icd*) mutation which reduces biomass and growth (Aoshima et al., 2003). Overexpression of *E. coli cs* gene in *Pseudomonas fluorescens* ATCC 13525 yielded millimolar levels of intracellular and extracellular citric acid (Buch et al., 2009). The amount of citric acid produced by *P. fluorescens* over expressing *E. coli cs* gene was similar to that secreted by the phosphate solubilizing *Bacillus coagulans* and *Citrobacter koseri* on glucose (Gyaneshwar et al., 1998). However, the levels were insufficient for releasing P from soils (Gyaneshwar et al., 1998; Srivastava et al., 2006). With a view to increase the flux through the *anaplerotic node* for increasing oxaloacetate levels, phosphoenol

pyruvate carboxylase (*ppc*) gene of *Synechococcus elongatus* was over-expressed in fluorescent pseudomonads. Overexpression of *ppc* enhanced cellular biomass, glucose catabolism through intracellular phosphorylative pathway and resulted in increased gluconic, pyruvic and acetic acids but citric acid was not detected (Buch et al., 2010). When *ppc* and *gltA* (gene for citrate synthase) were over expressed, the biomass yield was increased but citric acid secretion was not observed (Buch et al., 2009).

E. coli has Type II CS which is a hexamer of identical subunits, is strongly and specifically inhibited by NADH (Weitzman, 1996). This allosteric regulation of CS may result in tight homeostasis of citrate levels *in vivo* and this might explain why *cs* overexpression did not result in high accumulation of citrate (Buch et al., 2009). Gram positive bacteria, on the other hand, possess Type I CS which is insensitive to NADH inhibition, but it is inhibited by ATP (Stokell et al., 2003). Several specific point mutations in the NADH binding pocket of *E. coli* CS are reported extremely weak inhibition by NADH (Stokell et al., 2003). Citrate transport in *Enterobacteriaceae* family is mainly mediated by cation dependent or ATP dependent transporters (Lolkema, 2006). The *Klebsiella pneumonia* Na^+-dependent citrate carriers CitS and *Salmonella serovars* CitC belong to 2-hydroxycarboxylate family and are driven by both the proton motive force and sodium ion motive force (Ishiguro et al., 1992; Lolkema, 2006). The *K. pneumonia* citrate transporter CitH is driven by the proton motive force (Lolkema, 2006). The objective of present study was to investigate the effect of over expressing *E. coli cs** and *S. typhimurium citC* genes in *H. seropedicae* Z67 on citric acid secretion, MPS ability and growth promotion of rice plants.

3.1.1: Rational of the present study

Objective of the present study was to determine the MPS ability of *H. seropedicae* Z67 in field conditions, which is slowed down by low accessibility of carbon sources, high buffering capacity of soils. The improvement in the MPS ability was investigated by targeted genetic manipulation of the central carbon metabolic pathway towards citrate secretion by over expression of NADH insensitive citrate synthase (*cs**) of *E. coli* and *S. typhimurium* Na^+ dependent citrate transporter (*citC*) gene.

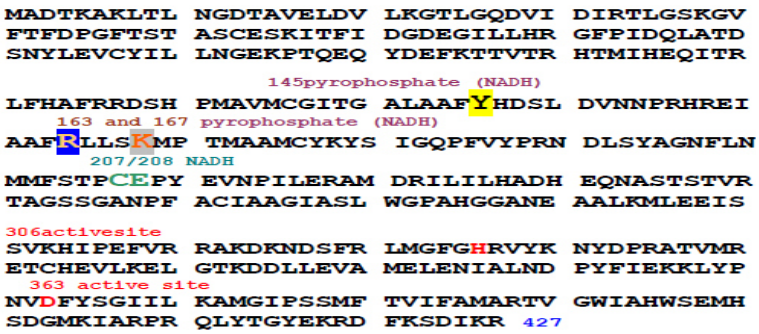


Fig. 3.1: *E. coli cs* protein sequence showing the regulatory variants

Table 3.1: NADH binding and inhibition by variant citrate synthases (Stokell et al., 2003)

Variant	Kd (µM)	Ki (µM)	Maximum inhibition (%)
Wild type	1.6±0.1	2.8±0.4	100±10
R109L	1.16±.04	1.7±0.2	96±6
H110A	5.2±0.2	121±11	97±3
T111A	6.6±0.2	80±15	87±6
Y145F	**>100**	**790±210**	**100±3**
R163L	**5.81±.04**	**400±80**	**77±8**
K167A	**4.1±0.2**	**630±130**	**100±10**
Q128A	6.1±0.5	18±3	100±7
N189A	6.9±0.8	242±26	96±5
T204A	10.2±0.4	165±36	100±7

The present study demonstrates the effect of overexpression of NADH insensitive *cs* Y145F on citric acid accumulation and secretion by *H. seropedicae* Z67.

3.2: The experimental plan of work includes the following-

3.2.1: Bacterial strains used in this study

Plasmid/Strains	Characteristics	Source or Reference
E. coli DH10B	Used for maintaining plasmids.	(Invitrogen)
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r	Hester et al., 2000
pAB8	pUCPM18 with Km^rgene; Ampr, Km^r	Buch et al., 2009
pAB7	*gltA* gene of *E. coli* in pAB8, Apr/Km^r	Buch et al., 2009
pY145F	NADH insensitive *cs** gene	Duckworth, H., 2003
citC	Na^+ dependent citrate transporter of *Salmonelatyphimorium*	This study
pJNK3	pUCPM18 with *E. coli* NADH insensitive *cs** gene under *Plac* and km^r gene; Ap^r, Km^r	This study
pJNK4	pUCPM18 with *E. coli* NADH insensitive *cs** gene citrate transporter citC of *Salmonella typhimurium* under *Plac* and km^r gene; Ap^r, Km^r	This study
P. putida BBC443 pTOL	TOL*gfp*mut3b plasmid; Km^r	Christensen et al.,1998
Hs Z67	*H. seropedicae* Z 67 with TOL*gfp*mut3b plasmid; Km^r	
Hs Z67	Nitrogen fixer, Natural isolate from gramenae plant; Nal^r	Baldani et al., 1986a
Hs (pAB8)	*H. seropedicae* Z67 pUCPM18 with *nptII* gene, Ap^r/Km^r	This study
Hs Z67 (pAB7)	*H. seropedicae* Z67 Nal^r with pAB7, Km^r	This study
Hs Z67 (pJNK3)	*H. seropedicae* Z67 Nal^r with pJNK3, Km^r	This study

Hs Z67 (pJNK4)	*H. seropedicae* Z67 Nal^r with pJNK4, Km^r	This study

Table 3.2: List of bacterial strains used. Detailed characteristics of these strains are given in **Table 2.1; 2.2.** Parent strains and the transformants of *E. coli* and *H. seropedicae* Z67 were respectively grown at 37ºC and 30ºC with variations in antibiotic concentrations for rich and minimal media as described in (Section **2.2).**

3.2.2: Expression of *lac* promoter in *H. seropedicae* Z67

P. putida BBC443 contained a modified pTOL plasmid (~117Kb) conferring several properties to the host, like toluene degradation, kanamycin resistance, and green fluorescence due to green fluorescence protein (GFP) cloned under *lac* promoter (**Table 3.2**; Christensen et al., 1998). Green fluorescence due to GFP expression was monitored in *H. seropedicae* Z67 on Luria Agar as well as BH Agar containing Na-benzoate as sole carbon source **(Section 2.4).** *H. seropedicae* Z67 transformants with pTOL plasmid were selected on kanamycin and the positive *P. putida* KT2440 -TOL plasmid transformants were checked for kanamycin resistant growth and presence GFP mediated fluorescence on BH agar containing Na-benzoate. IPTG was not used to induce *lac* promoter in any of the above experiments.

3.2.3: Construction of *H. seropedicae* Z67 stable plasmid containing *E. coli* NADH insensitive *cs* gene under *lac* promoter

Plasmid pBR322 containing NADH insensitive *E. coli* cs (*gltA*) gene was obtained by restriction digestion of 1281 bp of Y145F.

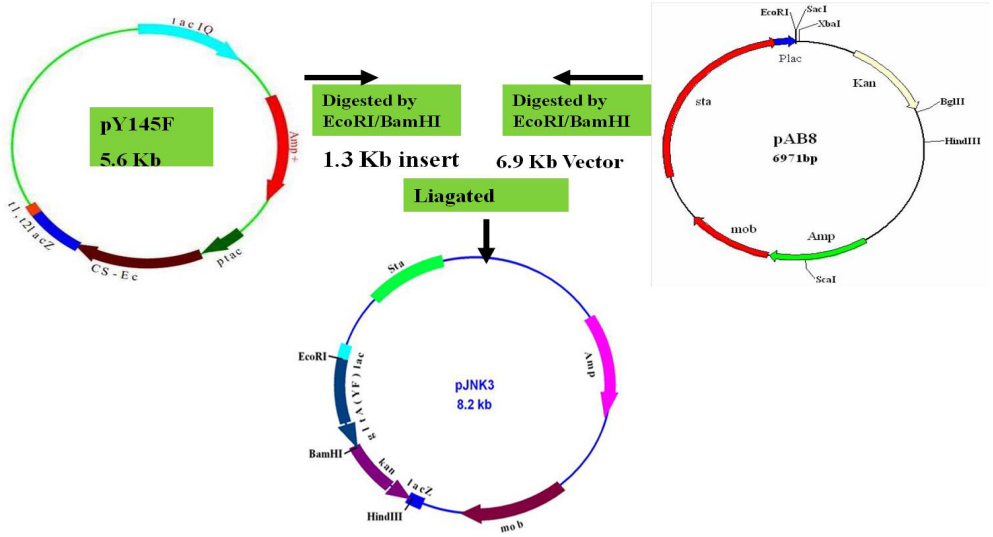


Fig. 3.2: Schematic representation of construction of *H. seropedicae* Z67 stable vectors containing NADH insensitive *E. coli cs* gene under *lac* promoter.

3.2.4: Incorporation of NADH insensitive *E. coli cs* and Na^+ dependent citrate transporter (*citC*) gene of *Salmonella typhimurium* under *lac* promoter in pUCPM18.

PCR amplification of pBR322 plasmid containing NADH insensitive *E. coli cs* (*gltA*) gene are carried out using gene specific primer (**Table 3.5**) to get an amplification of 1281 bp of Y145F

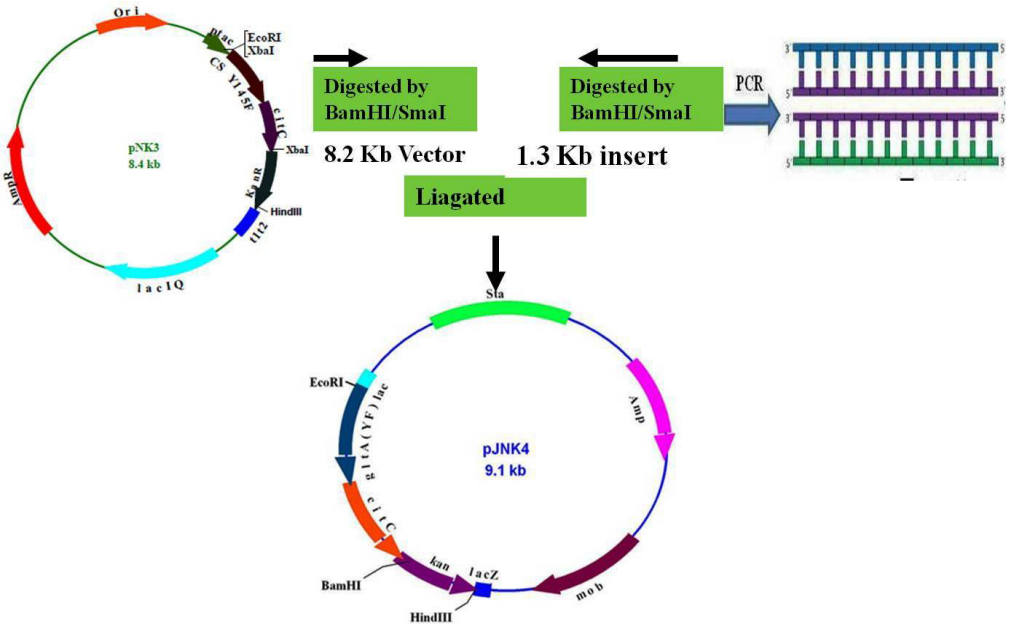


Fig. 3.3: Schematic representation of construction of plasmid containing NADH insensitive *E. coli cs* gene and citrate transporter (*citC*) gene under *lac* promoter.

E. coli NADH insensitive *cs* (pY145F) gene cloned in pBR322 was a generous gift from Dr. H. W. Duckworth, University of Manitoba, Canada. The *E. coli* NADH insensitive *cs* gene was ligated in pUCPM18 Km^r was selected using Kanamycin and further designated as pJNK3. *S. typhimurium citC* gene was obtained by polymerase chain reaction amplification using gene specific primer pair. Underline sequence shows BamHI/ *Sma*I restriction enzyme site used for cloning. PCR amplification was done using *Pfu* polymerase (Thermo scientific, USA) from plasmid pBKS-citC. Amplicon of 1.3 kb size, containing *citC* gene, was digested with *Bam*HI, gel eluted, purified, ligated in *BamHI*/*Sma*I digested and gel purified plasmid pJNK3. The resultant construct containing citrate operon was designated as pJNK4. The construct was confirmed by restriction digestion pattern. Further plasmids pAB8 as vector control, pAB7, pJNK3, pJNK4 were transformed by electroporation in *H. seropedicae* Z67 as described by Unge et al. (1998).

Table 3.3: Specific primer pair for Na^+ dependent citrate transporter (*citC*) gene.

Primer		Tm	GC%
FP-	5'GGATCCCGCACGGAGGAATCAACTTATGAC CAACATGACCCAGGCTTC3'	**65**	**54.2**
RP-	5'CCCGGGTTACACCATCATGCTGAACACGAT GC3'	**66.5**	**56.3**

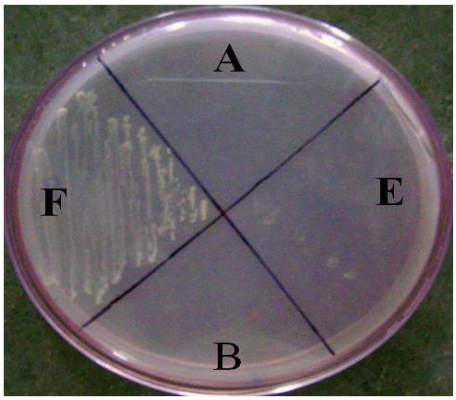
GGATCCCG- BamHI **CCCGGG -SmaI**

3.3: RESULTS.

3.3.1: Use of *lac* promoter for constitutive expression of desired genes in *H. seropedicae* Z67.

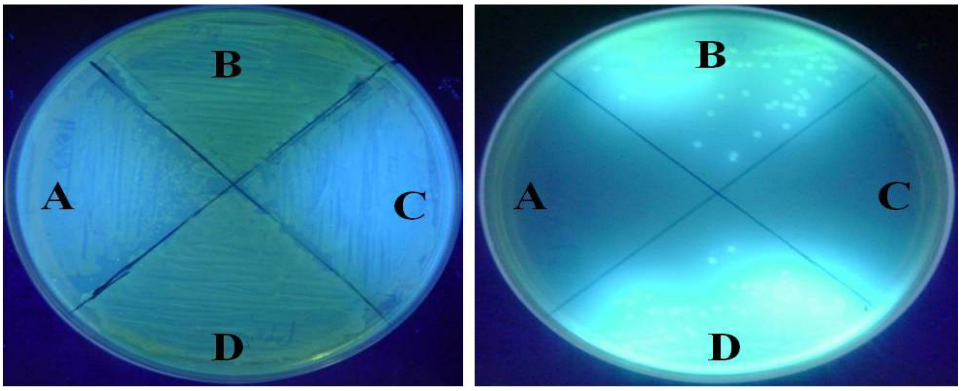
Lactose utilization of the *H. seropedicae* Z67 strain was checked on M9 minimal medium supplemented with lactose as sole carbon source. *H. seropedicae* Z67 and *E. coli* JM101 (used as negative control) could not grow on lactose whereas *Enterobacter asburiae* PSI3 showed good growth (utilizes lactose; used as a positive control) as shown in (**Fig 3.4**). *P. putida* KT2440 containing TOL*gfp*mut3b plasmid could grow and fluoresce due to GFP expression from *lac* promoter on rich media like Luria Agar as well as BH agar with Na-benzoate as sole carbon source in the presence of kanamycin. On the contrary *H. seropedicae* Z67 lacking pTOL plasmid grew on Luria Agar but could not fluoresce while failed to utilize Na-benzoate (**Fig. 3.5**). On the other hand, conjugal transfer of pTOL from *P. putida* BBC443 to *H. seropedicae* Z67 not only imparted the ability to utilize Na-benzoate as the sole carbon source in presence of kanamycin and but also conferred GFP expression as evident from green fluorescence (**Fig. 3.5**). These results confirmed that *lac* promoter was constitutively functional and thus could be used to express foreign genes in *H. seropedicae* Z67

Fig. 3.4: Lactose utilization of *H. seropedicae* Z67 strain on M9 minimal medium.



A - *H. seropedicae* Z67 W.T., **B** - *H. seropedicae* pTOL, **E** – *E. coli* JM1O1 negative control, **F** – *Enterobacter asburiae*

Fig. 3.5: pTOL mediated Na-benzoate utilization and GFP expression in *H. seropedicae* Z67.



Growth and GFP mediated fluorescence of **(a) A** - *H. seropedicae* Z67 W.T on Luria Agar, **(b) B** - *H. seropedicae* (+TOL*gfp*mut3b plasmid) on Luria agar, **C**- *P.putida* KT2440 and **D** - *P. putida* KT2440 (+TOL*gfp*mut3b plasmid), on BH Agar containing Na-benzoate as sole carbon source and 7.5µg/ml kanamycin.

3.3.2: Construction and functionality of pJNK3 and pJNK4 plasmids containing P_{lac}*cs** gene and P_{lac}*cs*citC* operon.

*E. coli cs**gene was obtained as 1.3Kb DNA fragment by digesting pY145F plasmid with EcoRI and BamHI enzymes. The *cs**fragment was gel eluted, purified and ligated to purified EcoRI-BamHI digested pUCPM18 (Km^r) plasmid (6.9Kb) resulted in pJNK3 plasmid (8.2Kb) containing *cs** gene under *lac* promoter. Plasmid pJNK3 was digested with *BamHI*/*Sma*I and ligated to the PCR product of *citC* to form pJNK4 (9.2Kb). All plasmids were confirmed by restriction digestion analysis. Functionality of *cs** gene in these plasmids was determined by complementation of *E. coli* W620 glutamate auxotrophy while CitC function was monitored by the growth on citrate as sole carbon source.

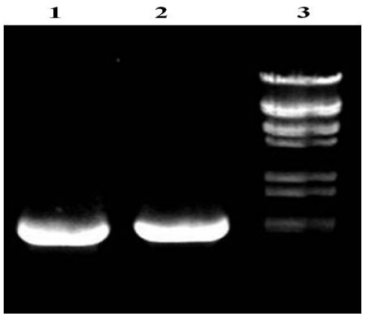


Fig. 3.6: PCR amplification of *S. typhimurium* Na^+ dependent citrate transporter (*citC*) gene.

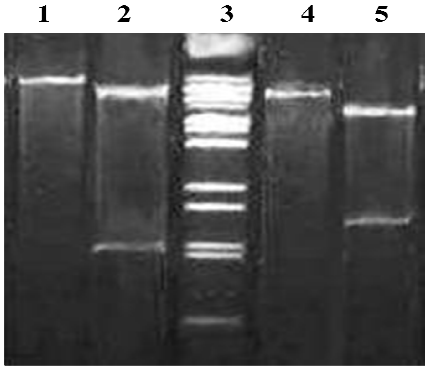


Fig. 3.7: Restriction digestion pattern for pAB8 and pAB7. Lane 1: pAB7 linearized with BamHI (8,265bp); Lane 2: pAB7 digested with XbaI (1,294bp, 6,971bp); Lane 3: Molecular Weight Marker (MWM)-Lambda DNA cut with BstEII; Lane 4: pAB8 linearised with XbaI (6,971bp); Lane 5: pAB8 digested with EcoRI-HindIII (5,298bp, 1,673bp)

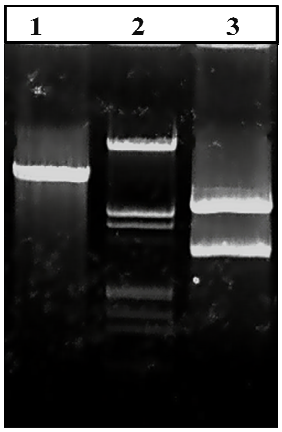


Fig. 3.8: Restriction digestion pattern for pUCPM18 Km^r containing NADH insensitive *cs* gene Lane 1: pJNK3 linearized with BamHI 8,265bp); Lane 2: Molecular Weight Marker (MWM)-Lambda DNA cut withEcoRI/HindIII Lane 3: pJNK3 digested with EcoRI-HindIII (5.310 bp, 2.900 bp)

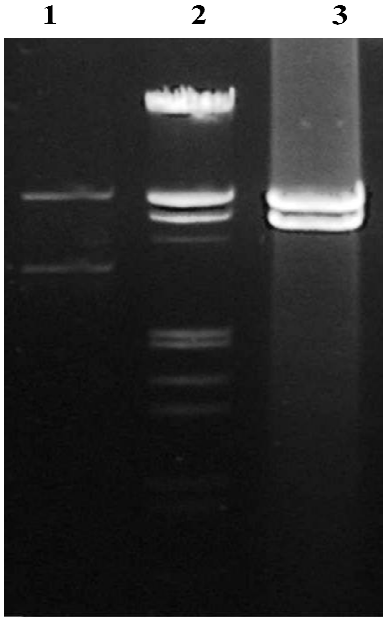


Fig. 3.9: Restriction digestion pattern for pUCPM18 Km*r* containing NADH insensitive *cs* gene and of Na^+ dependent citrate transporter (citC) of *Salmonella typhimurium* Lane 1: pJNK4 digested by XbaI (6.5 Kb, 2.6 Kb); Lane 2: Molecular Weight Marker (MWM)-Lambda DNA cut with EcoRI/HindIII ; Lane 3: pJNK4 digested with EcoRI-HindIII(5.210 bp, 3.900 bp)

3.3.3: *E. coli cs* mutant complementation

E. coli cs mutant strain harboring the pAB7, pAB8, pJNK3 plasmids were subjected to growth on M9 minimal medium containing glucose as carbon source in presence and absence of glutamate. *E. coli cs* mutant strain harboring pAB7 and pJNK3 could grow on glucose when induced with 0.1mM IPTG without glutamate supplementation unlike the controls (pAB8) (**Fig. 3.10**).

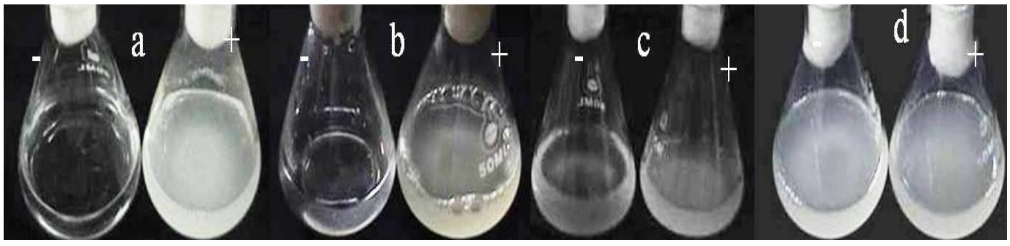


Figure 3.10: Complementation of *E. coli* W620 mutant phenotype by wild type and NADH insensitive *cs* plasmids. **a**: *E. coli* W620-deletion mutant of *cs* gene **b**: E. coli W620 with pAB8 plasmid **c**: *E. coli* W620 with pAB7 plasmid **d**: *E. coli* W620 with pJNK3 plasmid. All plasmid bearing strains are induced with 0.1mM IPTG. Growth is monitored on M9 medium with 0.2% glucose (Section) +/– at the corners of each image indicates absence and presence of 340µg/ml glutamate in the medium, respectively.

3.3.4 Growth and MPS ability of *H. seropedicae*Z67 transformants of pJNK3 and pJNK4 plasmids.

Hs (pAB8), *Hs* (pAB7), *Hs* (pJNK3), and *Hs* (pJNK4) transformants were obtained by electroporation (Eppendorf electroporator). *Hs* (pJNK3) and *Hs* (pJNK4) showed good P solubilization on Pikovaskya's agar and on 50 mM HEPES buffer methyl red plates with very good acidification on 50 mM glucose (**Fig. 3.11**).

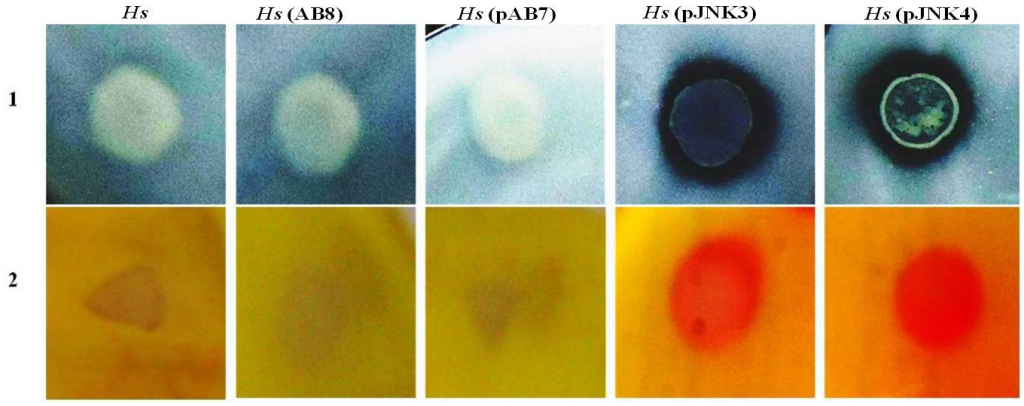


Fig. 3.11: MPS phenotype. 1-Pikovskaya's medium 2 - HRP medium containing 50 mM glucose and 50 mM HEPES rock phosphate (1 mg/ml)

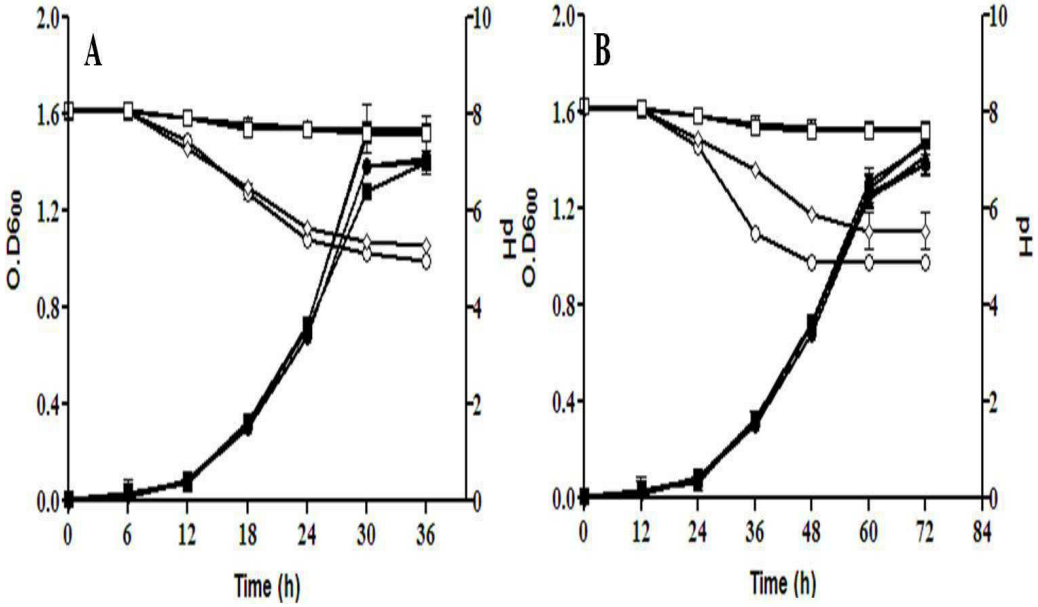


Fig. 3.12: Growth and pH profile. Extracellular pH (□, Δ, ▽, ◊, ○) and growth profile (■,▲,▼,◆,●) of *H. seropedicae* Z67 transformants on glucose 50 mM and HEPES 50mM rock phosphate(1 mg/ml) in medium. □, ■, *Hs* (wild type); Δ, ▲, *Hs* (pAB8); ▽,▼, *Hs* (pAB7) ; ◊, ◆, *Hs* (pJNK3), & ● ,○, *Hs* (pJNK4). A –Aerobic condition and B - Nitrogen free medium OD_{600} and pH values at each time point are represented as the mean ± SD of 3 independent observations

3.3.5 Effect of NADH insensitive (*cs**) and Na^+ dependent citrate transporter on growth, biomass and glucose utilization of *H. seropedicae* Z67 transformants grown on HRP minimal medium with 50mM glucose as C source.

Growth rates of *H. seropedicae* Z6 and the transformants *Hs* (pAB7), *Hs* (pJNK3) and *Hs* (pJNK4) were similar on HRP medium (**Table 3.4**). Total glucose depletion of *Hs* (pJNK3) and *Hs* (pJNK4) was ~3.38 and ~ 3.4 folds higher as against native strain and *Hs* (pAB7), respectively, on HRP medium. On the other hand, the specific glucose utilization rate of *Hs* (pJNK3) and *Hs* (pJNK4) was ~2.35 and ~ 2.68 folds higher than wild type and *Hs* (pAB7), respectively, on HRP medium**.** However, glucose consumption was almost similar in all *H. seropedicae* Z67 transformants when

compared with wild type. In contrast, biomass yield of *Hs* (pJNK3) and *Hs* (pJNK4) was decreased by ~ 2.3 and ~ 2.6 folds on HRP medium when compared with wild type. *Hs* (pAB7) showed ~1.5 fold increase in biomass yield as compared with native strain**.** When inoculated in HRP minimal medium, pH change was not found in *Hs* (pAB7) but *Hs* (pJNK3) and *Hs* (pJNK4) showed good growth and a drop in pH up to 5.0 within 36 h in the presence of glucose (Fig. 3.12). *Hs* (pJNK3) and *Hs* (pJNK4) released P up to ~80.16 µM, ~110.24 µM, on 50 mM glucose (**Table 3.4**).

Table 3.4: Physiological attributes and metabolic record of *H. seropedicae* Z 67 transformants grown on HRP minimal medium with 50mM glucose as C source under aerobic condition.

Bacterial Strain	Growth rate μ (h^{-1})[a]	Total glucose depleted (mM)[b]	Glucose consumed (mM)[b]	Biomass yield $Y_{dcw/Glc}$[a] (g /g)	Sp. glucose utilization rate Q_{Glc}[a] ($g.g\ dcw^{-1}.h^{-1}$)	CS activity (U)		Phosphate released. P (μM)[b]
						M9 minimal medium	HRP medium	
Hs	0.25 ±0.00	13.60 ± 0.55	13.60 ± 0.55	0.051 ± 0.007	0.54 ± 0.07	12.90± 1.51	17.43± 0.96	UD
Hs (pAB8)	0.34 ±0.01	12.93 ± 1.68	12.93 ± 1.68	0.066 ± 0.016	0.43 ± 0.08	12.10 ± 1.31	17.13± 2.05	UD
Hs (pAB7)	0.30 ± 0.05	12.33±1.22^{ns}	12.33 ± 1.22^{ns}	0.075 ± 0.020	0.38 ± 0.10^{ns}	18.50 ± 0.60	26.16 ± 1.20	UD
Hs (pJNK3)	0.32 ± 0.01	46.66±0.66***	46.66 ±0.66***	0.022 ± 0.002**	1.27± 0.13***	34.50 ± 3.95***	51.70 ± 3.17***	80.16 ± 3.58
Hs (pJNK4)	0.32 ± 0.00	47.00±0.95***	47.00 ± 0.95***	0.019 ±0.0005**	1.45 ± 0.042***	36.03 ± 3.20***	53.89 ± 2.86***	110.24± 2.76

The results are expressed as Mean ± SEM of readings from 3 independent observations. *** P<0.001, ns=non-significant (as compared to wild type culture controls) ; [a]Determined from mid log phase of each experiment. [b]*D*etermined at the time of pH drop (36 h).

Table 3.5: Physiological attributes and metabolic record of *H. seropedicae* Z67 transformants grown under micoaerobic condition on HRP minimal medium with 50mM glucose as C source.

Bacterial Strain	Growth rate μ (h^{-1})[a]	Biomass yield $Y_{dcw/Glc}$[a] (g /g)	Sp. glucose utilization rate Q_{Glc}[a] (g.g $dcw^{-1}.h^{-1}$)	Phosphate released P (μM)[b]
Hs	0.11 ± 0.01	0.025 ± 0.00	0.27 ± 0.03	
Hs (pAB8)	0.11 ± 0.00	0.021 ±0.00	0.33 ± 0.07	
Hs (pAB7)	0.12 ± 0.00	0.027 ±0.00	0.27 ± 0.09	
Hs (pJNK3)	0.12 ± 0.00	0.0088 ±0.00***	0.78 ± 0.03***	77.60 ± 1.37
Hs (pJNK4)	0.13 ± 0.00	0.0078 ±0.00***	0.88 ± 0.04***	116 ± 3.46

The results are expressed as Mean ± SEM of readings from 3 independent observations. *** P<0.001, ns=non-significant (as compared to wild type culture controls) ; [a]Determined from mid log phase of each experiment. [b]*D*etermined at the time of pH drop (72 h).

Under N free HRP medium, growth rates of *Hs* (pAB7), *Hs* (pJNK3) and *Hs* (pJNK4) were similar as compared with wild type culture (**Table 3.5**). On the other hand, the specific glucose utilization rate of *Hs* (pJNK3) and *Hs* (pJNK4) was ~2.5 and ~ 3 folds higher than wild type and *Hs* (pAB7), respectively, on N free HRP medium**.** In contrast, biomass yield of *Hs* (pJNK3) and *Hs* (pJNK4) was decreased by ~ 2 folds on N free HRP medium when compared with wild type. *Hs* (pAB7) showed similar pattern of biomass yield as compared with native strain**.** Additionally, no change in pH was found in *Hs* (pAB7) but *Hs* (pJNK3) and *Hs* (pJNK4) showed good growth and decreased the pH up to 5.0 within 72 h in the presence of glucose and solubilized RP (**Fig. 3.12**). Native culture and *Hs* (pAB7) neither showed acidification nor solubilized RP whereas *Hs* (pJNK3) and *Hs* (pJNK4) released P up to ~77.60 μM and ~116 μM, respectively, on 50 mM glucose (**Table 3.5**). Extent of P release in microaerobic condition is similar to that of aerobic conditions.

3.3.6 CS activity and organic acid secretion in *H. seropedicae* Z67 transformants.

H. seropedicae Z67 and *Hs* (pAB7) had12.9 U and 14.98 U of CS activity, respectively, in M9 medium while *Hs* (pJNK3) and *Hs* (pJNK4)showed ~1.8 and ~1.9 folds increase in CS activity, respectively (**Table 3.4**). Similar extent of increase in CS activity was found in HRP medium.

Citric acid secretion was not found in native culture and *Hs* (pAB7) while 0.39 mM and 2.79 mMcitric acid secretion was observed with *Hs* (pJNK3) and *Hs* (pJNK4) (**Fig. 3.13**). Acetic acid was also secreted by all *Hs* transformants. Acetic acid level in the native strain was (mM), while *Hs* (pAB7) were more and highest levels (~ up to 50 mM) in *Hs* (pJNK3) and *Hs* (pJNK4). Similar pattern of yields of citric acid and acetic acids were found in *H. seropedicae* Z67 transformants.

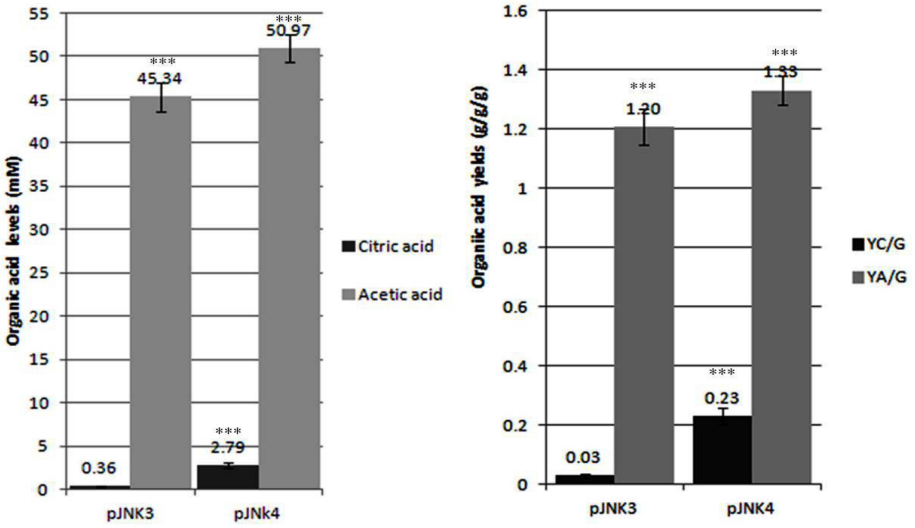


Fig. 3.13: Organic acid production from *H. seropedicae*Z67 transformants.

3.3.7 Influence of phosphorous levels on IAA, EPS and biofilm production.

Hs native culture and *Hs* transformants did not show significant change in EPS production under aerobic and under microaerobic condition (**Fig. 3.14 A, B**). However, biofilm formation was increased by ~4.5 fold in *Hs* (pJNK3) *Hs* (pJNK4) under both N-sufficient aerobic and N-free microaerobic conditions when compared with wild type culture (**Fig. 3.14 C & D**). IAA production was also enhanced in *Hs* (pJNK3) and *Hs* (pJNK4) to ~1.4 and ~1.7 fold, respectively, under aerobic conditions than that of native culture, *Hs* (pAB8) and *Hs* (pAB7) (**Fig. 3.14 E**). All *Hs* transformants showed almost 40% decrease in IAA under N free microearobic conditions as compared to aerobic conditions (**Fig. 3.14 F**).

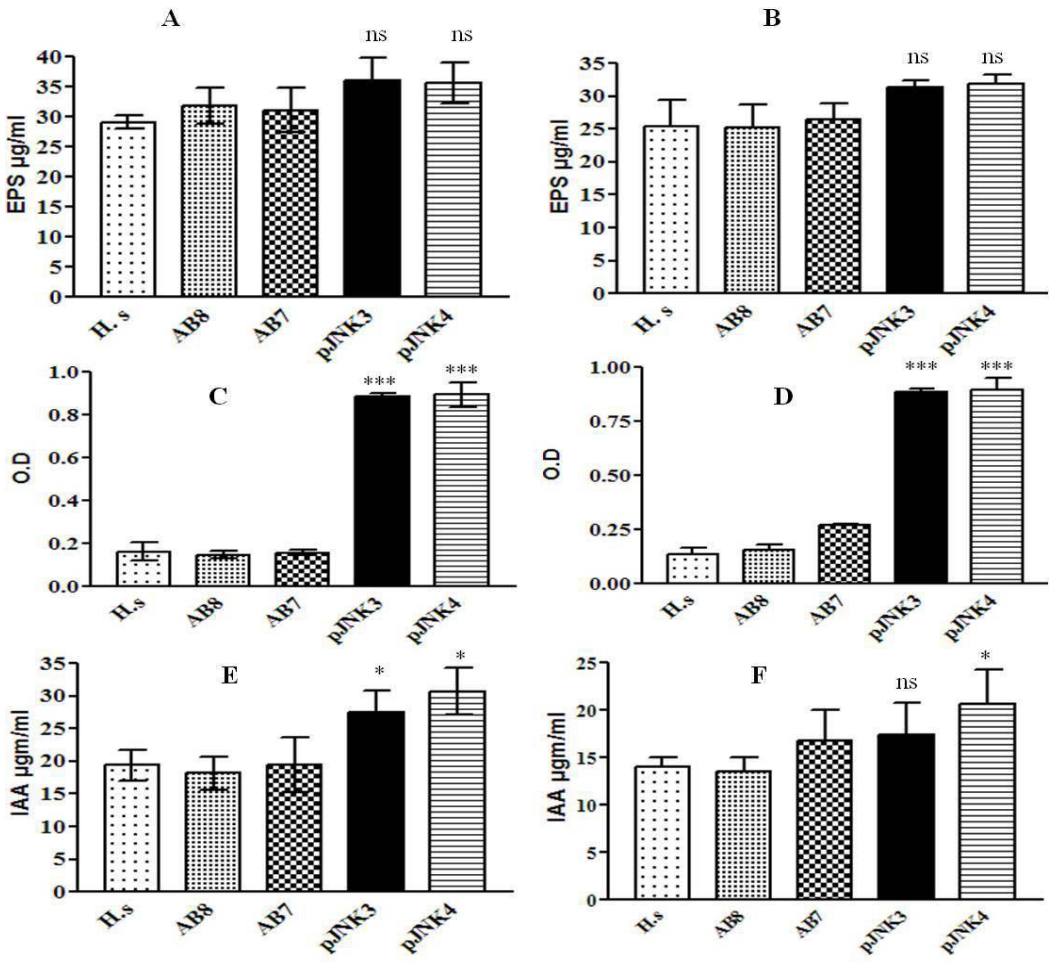


Fig. 3.14: Plant growth promoting factors. The results are expressed as Mean ± SEM of readings from 3 independent observations, ***P < 0.001, ** P < 0.01, * P < 0.05 and ns=non-significant (as compared with wild type culture controls) ; **A, C** and **E** under aerobic **, B, D** and **F** under microaerobic condition

3.3.8 Effect of *Hs* (pJNK3) and *Hs* (pJNK4) on the nutrient status of Rice plants

Rice plant shoot length, root length, fresh weight, dry weight, N, P and K content, in rice plants were monitored after 30 days after inoculation (**Table 3.7 and Table 3.8**). The shoot length of *Hs* native strain and *Hs* (AB7) inoculated rice plants showed ~1.3 fold increase when compared with control plants without any culture, while *Hs* (pJNK3) and *Hs* (pJNK4) showed ~1.2 and ~1.5 folds increase when compared with native strain. Root length was increased in both *Hs* and *Hs* (AB7) by

~1.8 folds when compared with control plants, while *Hs* (pJNK3) and *Hs* (pJNK4) showed ~1.6 fold increase in root length as compared with *Hs* plants. Fresh weight of plants inoculated with *Hs* and *Hs* (AB7) increased by ~1.9 folds when compared with control set, while *Hs* (pJNK3) and *Hs* (pJNK4) showed significant increase in fresh weight by ~1.3 and ~1.4 folds when compared with *Hs* treated plants. Total chlorophyll content was found similar in control, *Hs* and *Hs* (AB7), while both *Hs* (pJNK3) and *Hs* (pJNK4) increased by ~1.5 folds when compared with control and native strain. Nitrogen content in plants leaves was significantly increased with *Hs* and *Hs* (pAB7) by ~1.6 folds as compared with control plants while N levels in *Hs* (pJNK3) and *Hs* (pJNK4) showed ~1.4 and ~1.7 folds increase, respectively, when compared *Hs* plants. N levels in roots in *Hs* and *Hs* (pAB7) showed ~1.4 folds increase against control, while *Hs* (pJNK3) and *Hs* (pJNK4) showed ~1.4 folds and ~1.7 folds increase when compared with *Hs*. Phosphorus content in leaves and roots in both *Hs* (pJNK3) and *Hs* (pJNK4) by ~3.87 folds and ~ 4.35 folds, respectively, when compared against *Hs* plants. K content of leaves and roots was increased in *Hs* (pJNK3) and *Hs* (pJNK4) by almost ~2 folds when compared against *Hs*.

Table 3.6: Physical and chemical properties of experimental soil used for *H. seropedicae* Z67 transformants

E.C	pH	Total phosphorus (%)	Total nitrogen (%)	Total potassium (%)
0.63	7.86	19	16	65

Table 3.7: Effects of *H. seropedicae* transformants on plant fresh weight, dry weight, plant height leaf chlorophyll content at 30 DAI.

H. seropedicae **transformants**	**Shoot length (cm)**	**Shoot/Root ratio**	**Root length/Plant (cm)**	**Root/Shoot ratio**	**Total plant fresh weight /Plant (mg)**	**Total plant Dry weight /Plant (mg)**	**Total Leaf chlorophyll, Content (mg/g fr wt.)**
Control	21.66 ± 2.51	1.91 ± 0.48	11.66 ± 2.08	0.54 ± 0.16	105.33 ± 5.18	10.03 ± 0.96	2.86 ± 0.17
Hs	29.66 ± 1.52	1.38 ± 0.39	22.33 ± 4.72	0.75 ±0.19	201.11 ±18.90	16.11 ± 0.48	3.310 ± 0.27
Hs (pAB8)	27.33 ± 4.16	1.36 ± 0.08	20.00 ± 2.00	0.73 ±0.04	191.22 ±16.98	14.44 ± 1.16	3.050 ± 0.05
Hs (pAB7)	28.66±1.52	1.59 ± 0.14	18.00 ± 1.00	0.62 ±0.05	212.22± 10.88	19.88 ± 0.45	3.07 ± 0.31
Hs (pJNK3)	35.33 ± 3.05***	0.98 ± 0.14	36.33 ± 3.78***	1.03 ±0.14	265.88 ± 3.25***	31.55 ± 1.49***	4.51 ± 1.01***
Hs (pJNK4)	42.33 ± 2.51***	1.13± 0.09	37.33 ± 3.05***	0.88 ±0.07	294.88 ± 0.28***	37.22 ± 2.27***	4.72 ± 0.86***

The results are expressed as Mea n± SEM of readings from 6 plants of treatments. *** P<0.001, ns=non-significant (as compared with wild type culture inoculant and controls plants).

Table 3.8: N, P, K levels in rice plants after 30 DAI with *H. seropedicae*Z67 transformants.

Hs transformants	N content in leaves (%)	N content in roots (%)	P content in leaves (%)	P content in roots (%)	K content in leaves (%)	K content in roots (%)
Control	0.95±0.02	0.44±0.03	0.035±0.002	0.023±0.002	0.60±0.05	0.37±0.01
Hs	1.34±0.02 ***	0.92±0.03 ***	0.062±0.003 ***	0.045±0.003 ***	1.09±0.09 ***	0.85±0.08 ***
Hs (pAB8)	1.36±0.04	0.83±0.03	0.047±0.004	0.035±0.003	1.14±0.05	0.81±0.07
Hs (pAB7)	1.62±0.03	0.91±0.02	0.074±0.003	0.054±0.003	1.17±0.16	0.84±0.09
Hs (pJNK3)	1.91±0.6 ***	1.47±0.04 ***	0.24±0.004 ***	0.094±0.003 ***	2.03±0.01 ***	1.21±0.07 ***
Hs (pJNK4)	2.32±0.11 ***	1.55±0.04 ***	0.27±0.008 ***	0.097±0.002 ***	2.06±0.06 ***	1.35±0.05 ***

The results are expressed as Mea n± SEM of readings from 6 plants of treatments. *** P<0.001, ns=non-significant (as compared with wild type culture inoculant and controls plants).

Fig. 3.15: Effect of Hs transformants in rice plants. 1- Control, 2- *Hs* native, 3-*Hs* (pAB8), 4-*Hs* (pAB7), 5-*Hs* (pJNK3), 6-Hs (pJNK4)

3.4 Discussion

Organic acids such as acetate, citrate, oxalate, gluconate and 2 ketogluconate are responsible for mineral phosphate solubilisation (Hazen et al., 1991; Srivastava et al., 2006; Archana et al, 2012). Secretion of gluconic (GA) and 2-ketogluconic acids (2KG) is known to be mediated by direct oxidative pathway in the periplasm and governed by nutritional and environmental factors. Strategy of increasing the activities of GDH and GAD enzymes has been successful in enhancing the secretion of these acids (Zaidi et al., 2009; Kumar et al., 2013). On the other hand, citric acid secretion was detected bacteria with decrease in the enzymes of TCA cycle pathway. Manipulation of TCA cycle for citric acid is complex in nature due to the central role in catabolic and anabolic pathway. Oxaloacetate, which is a substrate for CS enzyme, along with pyruvate and phosphoenolpyruvate forms the central components of the metabolic network known as *anaplerotic node* (Sauer and Eikmanns, 2005). Previously, genetic modifications related to the *anaplerotic node* was carried out in

fluorescent pseudomonads resulting in secretion of citric acid up to 1.2 mM (Buch, 2008; 2009). These modifications have also diverted the glucose catabolism from phosphorylative pathway towards direct oxidative pathway leading to gluconic acid secretion.

H. seropedicae Z67 primary metabolism is similar to that of fluorescent pseudomonads but differs by the absence of PQQ biosynthesis genes (Pedrosa et al., 2011). Thus, glucose catabolism is only occurs by phosphorylative pathway. Overexpression of *cs* in *H. seropedicae* Z67 has increased CS activity by ~ 1.9 which is similar to that found in *P. fluorescens* 13525 but citric acid secretion was not detected while 1.2 mM level was secreted in *P. fluorescens* 13525 (Buch et al., 2009). This could be attributed to the lesser CS activity in *H. seropedicae* Z67 (pAB7) (26 U) as against 110 U in *P. fluorescens* 13525 transformant. Acetic acid was found in both the transformants with *P. fluorescens* 13525 was slightly higher which is in agreement with efficient metabolism of fluorescent pseudomonads (Rojo, 2010).

Over-expression of NADH insensitive (*cs**) in *Hs* (pJNK3) resulted in enhanced CS activity ~51 U, while citric acid secretion was too less 0.36 mM. CS activity of *Hs* (pJNK3) was less than ~2 folds to that of *Pf* (pAB7) (Buch et al., 2009). Both these bacteria had similar effects as amount of citric acid secreted ids directly related to citric acid secretion while *Pf* (pAB7) also secreted high amount of gluconic acid. On the other hand, *Hs* (pAB7) secreted high acetic acid (~45 mM) while *Pf* (pAB7) had secreted only 15 mM. Thus, in *Hs* (pAB7) increase in CS activity diverted pyruvate towards acetic acid secretion. High citric acid levels inside *P. fluorescens* 13525 and *H. seropedicae* Z67 appear to have similar consequences on the metabolism. Catabolism of citric acid appears to be limited either by isocitrate dehydrogenase or by 2-ketoglutarate dehydrogenase enzymes. Diversion of glucose in *P. fluorescens* 13525 towards direct oxidative pathway and acetic acid in *H. seropedicae* Z67 could help in generation of ATP. In *P. fluorescens* 13525, during the conversion of glucose to gluconic acid pyrroloquinoline quinone (PQQ) gets reduced in the periplasm and is oxidized by supplying electrons to cytochrome C resulting in 1 ATP per molecule (Dijkstra et al., 1989). Since *Hs* does not possess PQQ as cofactor for GDH, acetic acid formation can also lead to ATP generation

during the conversion of acetly CoA to acetate *via* acetylphosphate, involving acetate kinase which catalyzes the second reaction. Additionally, increase in acetic acid secretion by *Hs* (pJNK3) could result in increased carbon dioxide levels which in turn form oxaloacetate by pyruvate carboxylase (Papagianni, 2007).

CS activity of *Hs* (pJNK4) is similar to *Hs* (pJNK3) but secreted ~2.79 mM citric acid which is ~2.3 fold more than *Pf* (pAB7). Thus, enhanced citric acid secretion could be attributed the CitC transporter but not to the overexpression of *cs** gene. Both *P. fluorescens* 13525 and *H. seropedicae* Z67 contain H^+-citrate transporter but it is not efficient in efflux of citrate. Although *H. seropedicae* Z67 contains Na^+ dependent citrate transporter, overexpression of *citC* gene has improved citric acid secretion. The active transport system for citrate secretion appears to be the main rate-determining factor in citrate overproduction by yeasts (Anastassiadis and Rehm, 2005). Thus, both *P. fluorescens* 13525 and *H. seropedicae* Z67 appears to require high CS activity coupled with an efficient efflux system for citric acid secretion.

Hs (pAB7), *Hs* (pJNK3) and *Hs* (pJNK4) had no significant change in growth and glucose depletion rates which were similar to that of pseudomonas and *E. coli* (Walsh and Koshland, 1985; Buch et al., 2009). In *Hs* (pAB7), whose biomass yield was unaltered while *gltA* overexpression in *E. coli* increased the maximum cell dry weight by 23% (De Maeseneire et al., 2006; Buch et al., 2009). In contrast, the biomass was decreased in *Hs* (pJNK3) and *Hs* (pJNK4) which correlated with high acetic acid secretion. In *E. coli,* high acetic acid secretion has detrimental effect on growth (Wolfe, 2005). *Hs* transformants also showed similar extent of MPS ability under both aerobic and microaerobic nitrogen fixing conditions suggesting that levels of secreted citric acid was not influenced by the oxygen status. Similar results were observed in *A. brasielense* by overexpression of *pqqE* for gluconic acid secretion which solubilized tricalcium phosphate on N free medium (Vikram et al., 2007).

Significant contribution in plant growth promotion is shown by plant growth-promoting bacteria by producing factors (EPS, Biofilm and IAA) responsible for plant growth promotion in adverse conditions (Vicente et al., 2012).

Herbaspirillum is known to produce IAA (Radwan et al., 2002). Improved IAA levels, EPS production and biofilm in HRP medium could help in better colonization of *H. seropedicae* Z67 transformants as the bacteria known to form mucilaginous and a fibrillar material in maize and sorghum root surfaces (Gyaneshwar et al. 2002; Roncato-Maccari et al. 2003b). EPS plays an important role during the colonization of the protoxylem vessels of sorghum and sugarcane by *H. seropedicae* and *H. rubrisubalbicans* (James et al. 1997; Olivares et al. 1997; James and Olivares 1998). Biofilm formation is required for the attachment in plant tissues (Baldotto et al., 2011: Santacecilia et al., 2012). Microbial growth and biofilms production is known to be increased by phosphorous (Lehtola et al. 2002; Chu et al. 2005; Fang et al., 2009). The production of EPS is directly correlated with biofilm formation as EPS can protect the cells External addition of phosphorous in water also enhanced biofilm formation (Mohamed et al., 1998). EPS productivity was increased in *Bacillus cereus* GU812900 by increasing nitrogen and phosphate (Bragadeeswaran et al., 2011).

Plant inoculation studies demonstrated the positive effect of *Hs* transformants on growth of rice plants which is cumulative effect of improved N and P status combined with increased IAA and EPS levels. PSB are known to enhanced yield in rice and maize (Tiwari et al*.,* 1989; Pal, 1999). Similar effects are found with wheat plant increased in yield and P uptake when inoculated with *Rhizobium* with phosphate solubilizing bacteria. (Afzal and Bano, 2008). Co-inoculation of N_2 fixing and phosphate solubilizing bacteria showed similar growth improvement in sorghum and wheat (Algawadi and Gaur, 1992; Belimov et al., 1995; Galal, 2003).

Conclusion

This study revealed that overexpression of NADH insensitive citrate synthase (*cs**) and combination of NADH insensitive citrate synthase (*cs**) with Na^+ dependent citrate transporter (*citC*) in *H. seropedicae* Z67 leads to citric acid secretion, enhanced mineral phosphate solubilization and improved growth of rice plant (*Oryza sativa* Gujarat - 17). Thus, genetic manipulations could be useful in the development of better plant growth promoting rhizobacteria.

Chapter 4

4.1: Introduction

H. seropedicae is an endophytic nitrogen-fixing bacterium which colonizes roots of economically important crops such as rice, sugarcane, and wheat (Pedrosa et al., 2001; da Silva et al., 2003; Monteiro et al., 2003). *Herbaspirillum* bacteria have been isolated from the interior of roots, stem, and leaves of many Graminaceous plants without any harmful effect on plants (Baldani et al., 1992; DSbereiner *et al.,* 1994; Olivares *et al.,* 1996). *Herbaspirillum* spp. has the pectinolytic and cellulolytic enzymes that are essential for the cell wall degradation of plant during infection (Elbeltagy et al., 2001; Gyaneshwar et al., 2002). *H. seropedicae* increased biomass and N content of rice plants equivalent to the treatment with 40 kg of N/ha (Pereira and Baldani, 1995).

Nitrogen fixation in field conditions is limited by the availability of P and co-inoculation of nitrogen fixing bacteria with phosphate solubilizing microorganisms (PSMs) have increased growth and N status of plants (Gull et al., 2004; Elkoca et al., 2007; Valverde et al., 2007; Kumar and Chandra, 2008). *Rhizobium* species with high ability to nodulate and fix nitrogen was isolated from common bean *Phaseolus vulgaris* was able to solubilize phosphate (Abril et al. 2003). *Rhizobium leguminosarum* showing P-solubilizing ability has shown to increase the growth of maize and lettuce (Chabot et al., 1996). Phosphate solubilizing *Mesorhizobium meditteraneum* increased growth, N, P, Ca, Mg, and K in chick pea and barley (Peix et al., 2001). Some rhizospheric organisms have the ability to solubilize insoluble phosphate through organic acid production, such as citric and gluconic (Khan et al., 2011; Archana et al., 2012). In Gram negative bacteria, gluconic acid is generated by the direct oxidation of glucose that is mediated by a membrane bound glucose dehydrogenase (GDH) which possesses pyrroloquinoline quinone (PQQ) as cofactor (Goldstein, 1995).

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

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

E. coli, Azospirillum, Rhizobium and *Herbaspirillum* do not encode PQQ biosynthesis genes in their genome but possess glucose dehydrogenase (GDH) apoprotein. Incorporation of *pqq* gene clusters in *E. coli* resulted in PQQ biosynthesis and active GDH enzyme (Goosen et al., 1989; Meulenberg et al., 1990; Khairnar et al., 2003; Yang et al., 2010). Interestingly, **e**xpression of *Erwinia herbicola pqqE* gene alone in *E. coli* HB101 and *Azospirillum* resulted in active GDH enzyme and secreted gluconic acid (Liu et al., 1992; Vikram et al, 2007). Similarly, expression of *Rahnella aquatilis pqqED* genes also resulted in gluconic acid secretion in *E. coli* (Kim et al., 1998). The mechanism of PQQ biosynthesis either in *pqqE* or *pqqED* gene transformants of *E. coli* is not clear as *pqqABCDE* genes are necessary for PQQ biosynthesis (Choi et al., 2008; Shen et al., 2012). Thus, the present study investigates the effect of incorporation of *E. herbicola pqqE* and *pqq* gene clusters from *Acinetobacter calcoaceticus* and *Pseudomonas fluorescens* in gluconic acid secretion in *H. serpedicae* Z67 and monitors its effect on mineral phosphate solubilization (MPS) ability.

4.1.1: Rational of study

H. seropedicae Z67 contain apo GDH enzyme but does not possess *pqq* genes for which is cofactor of GDH for gluconic acid production, through direct oxidative pathway. *H. seropedicae* Z67 does not secrete organic acid and thus does not show MPS ability. In present study, we demonstrated the effect of over expression of three different *pqq* genes (*pqqE* of *E. herbicola*, *pqq* gene cluster of *A. calcoaceticus* and *pqq* gene cluster of *pqq* gene cluster of *Pseudomonas fluorescens* B16). Over expression and their effect on MPS ability and PQQ secretion which is known as plant growth promoter.

4.2: EXPERIMENTAL DESIGN

4.2.1: Bacterial strains used in this study

Plasmid/Strains	Characteristics	Source or Reference
pBBR1MCS-2	Broad-Host-Range vector; Km^r	Kovach et al., 1995
pMCG898	Containing *pqqE* of *Erwinia herbicola*	Goldstein and Liu; 1987
pJNK1	pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	This study
pSS2	25Kb plasmid contains 5.1-kb of *pqq* gene cluster of *Acinetobacter calcoaceticus*	Goosen et al., 1989
pOK53	35Kb plasmid contains 13.4-kb *pqq* gene cluster of *Pseudomonas fluorescens* B16	Choi et al., 2008
E. coli DH10B	Used to maintain plasmids for routine use	Invitrogen, USA
Hs	Nitrogen fixing rice endophyte , Nal^r	Baldani et al., 1986a
Hs (pBBR1MCS2 Km^r)	*H. seropedicae* Z 67 with (pBBR1MCS2) Km^r	This study
Hs (pJNK1)	*H. seropedicae* Z 67 with pJNK1 Km^r	This study
Hs (pSS2)	*H. seropedicae* Z 67 with pSS2 Tet^r	This study
Hs (pOK53)	*H. seropedicae* Z 67 with pOK53Tet^r	This study
Enterobacter asburiae PSI3	Amp^r secretes gluconic acid	Gyaneshwar et al., 1999
Pseudomonas aeruginosa P4	Secretes gluconic acid	Buch et al., 2008

Table. 4.1: Bacterial strains used in this study

4.2.2: Bacterial strains, plasmids, media and culture conditions.

E. coli and *H. seropedicae* Z67 strains and plasmids used for this work have been listed in (**Table 1**). DNA manipulation and molecular biology experiments were performed using *E. coli* DH10B as the host strain using standard protocols (Sambrook and Russell, 2001). Plasmid bearing derivatives of *E. coli* DH10B was routinely grown at 37^0C on Luria Bertani (L.B) and maintain on Luria Agar (L.A) plate with 40 μg ml^{-1} streptomycin and *Hs* was grown on JNFb plate at 30 °C with 10 μg ml^{-1} Nalidixic acid and Gentamycin 20 μg ml^{-1}, respectively, *H. seropedicae* Z67 was routinely grown at 30^0C on Luria Bertani (L.B) and maintained on semisolid JNFb medium (Baldani et al., 1992) was used at pH 5.8 at 30°C. *H. seropedicae* Z67 form small moist colonies with blue centers.

4.2.3: DNA manipulation

Plasmids pMCG898 containing *pqqE* was gifted by (Dr. A. H. Goldstein). Appropriate method was used for subcloning of *pqqE* in broad host range vector pBBR1MCS2 Km^r under *lac* promoter in EcoRI/BamHI site, as described by Sambrook et al. (1989), which resulted in pJNK1 Km^r and transformed into *E. coli* DH10B, followed by selection on LB agar medium containing kanamycin, further plasmids pBBR1MCS2 Km^r as vector control, pJNK1 Km^r, pSS2Tet^r and pOK53 Tet^r were transformed by electroporation in *H. seropedicae* Z67 described by Unge et al. (1998).

4.3: Results:

4.3.1: Subcloning of *pqqE* gene of *E. herbicola* in broad host range vector pBBR1MCS2 km^r from pMGC898.

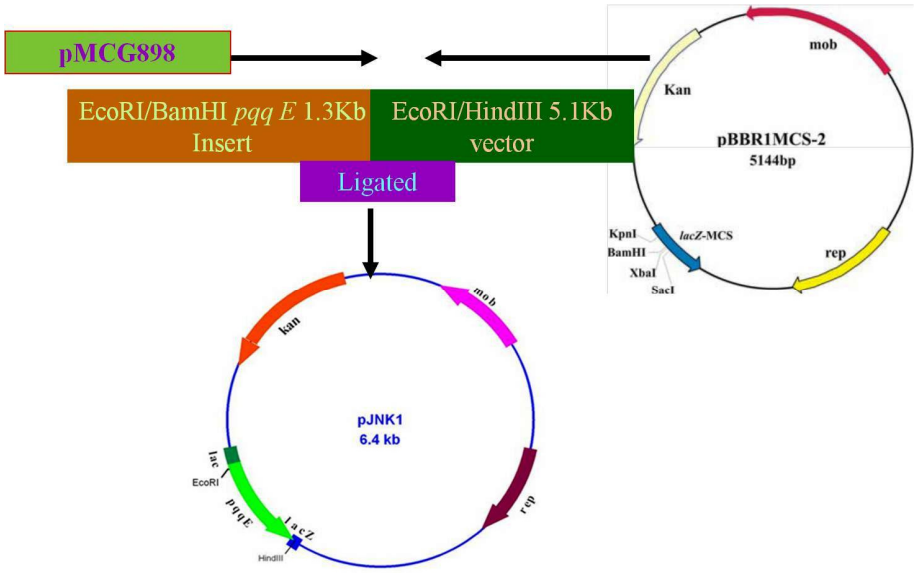


Fig. 4.1: Schematic representation of construction of *H. seropedicae* Z67 stable vectors containing *pqqE* gene under *lac* promoter.

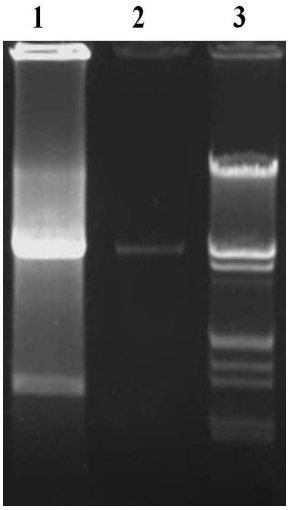


Fig. 4.2: Lane. 1- pJNK1 digested by EcoRI/BamHI, Lane. 2- pBBR1MCS2 digested by BamHI. Lane. 3- Marker EcoRI/HindIII

Fig. 4.2: Restriction digestion pattern for pJNK1.

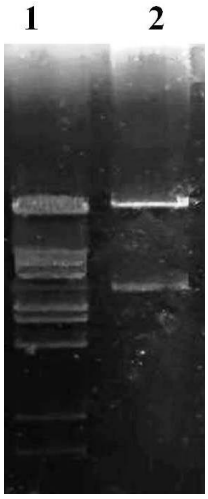


Fig. 4.3: Lane 1- Marker Lambda DNA *BstEII*. Lane 2- pSS2 plasmid digested by EcoRI/BamHI

Fig. 4.3: Restriction digestion pattern for pSS2.

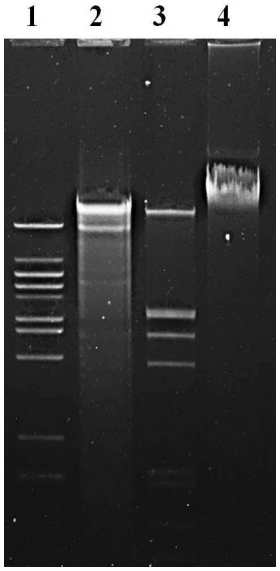


Fig. 4.4: Lane 1- *BstEII* marker, Lane 2- Release of 14 Kb *pqq* cluster digested with BamHI. Lane 3- Marker EcoRI/ Hind III. Lane 4 - Plasmid pOK53

Fig. 4.4: Restriction digestion pattern for pOK53

4.3.2: Growth and MPS ability of *H. seropedicae* Z67 transformant of *pqq* genes.

Hs Z67 transformant containing *E. herbicola pqqE* (pJNK1), *A. calcoaceticus pqq* gene cluster (pSS2) and *P. fluorescens* B16 *pqq* gene cluster (pOK53) plasmid were obtained by electroporation (Eppendorf electroporator). *Hs* transformants containing pJNK1 plasmid showed poor P solubilization on Pikovaskya's agar, HEPES buffer methyl red plates while transformants containing pSS2 and pOK53 plasmids showed very good acidification on 50 mM glucose, 50mM xylose and a mixture of 25mM glucose and 25mM xylose (**Fig. 4.5**).

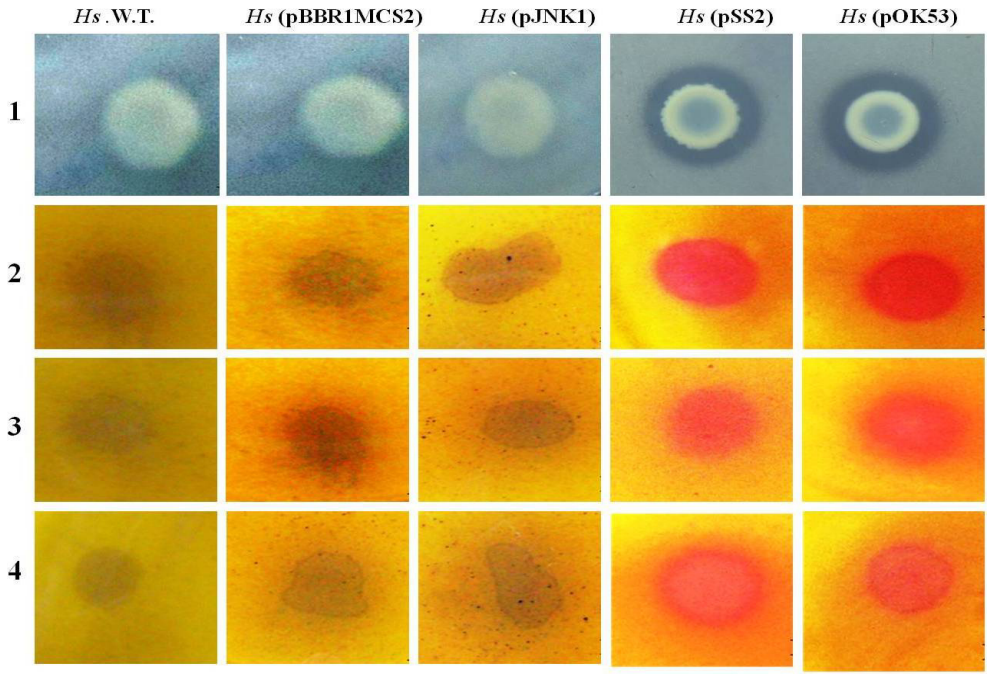


Fig. 4.5: MPS phenotype. 1-Pikovskaya's medium; **2**- 50 mM HEPES 50mM glucose rock phosphate medium; **3**- 50 mM HEPES 50mM xylose rock phosphate medium and **4**- 50 mM HEPES 25mM glucose and 25 mM xylose rock phosphate medium.

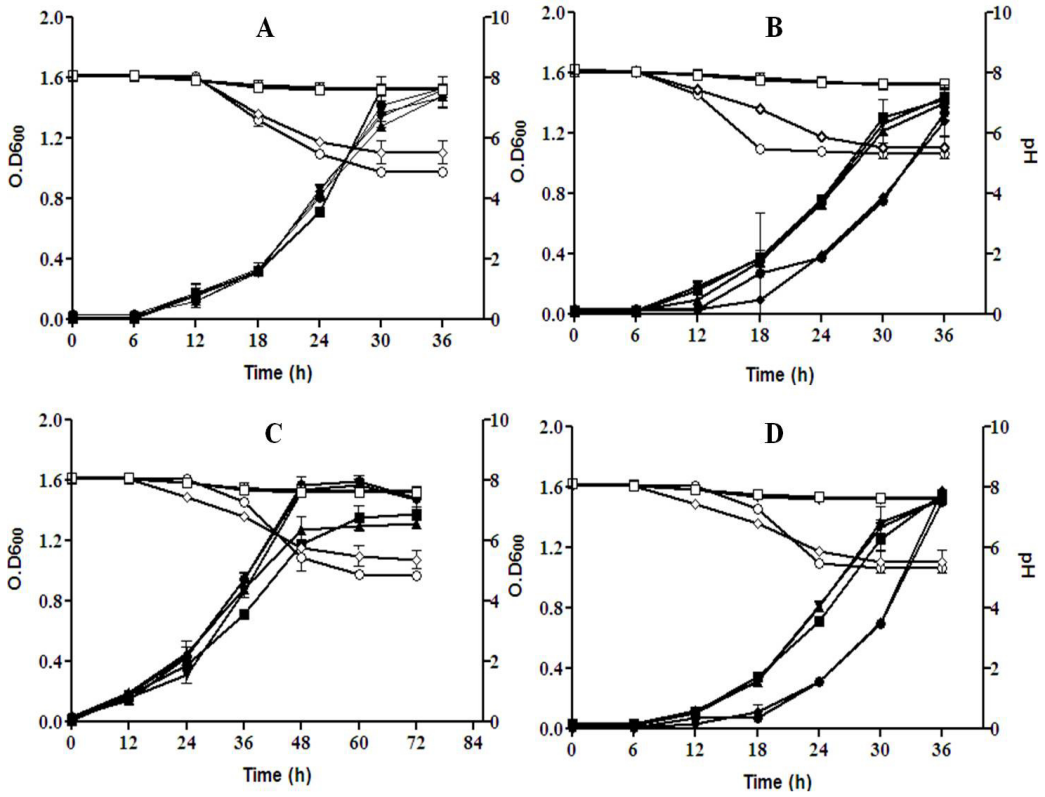


Fig. 4.6: Growth and pH profile. Extracellular pH (□, Δ, ▽, ◊, ○) and growth profile on 50mM HEPES rock phosphate medium (■,▲,▼,◆,●) of *H. seropedicae* Z67 over expressing *pqq* gene clusters, □, ■ *Hs* (wild type); Δ, ▲, *Hs* (pBBR1MCS2); ▽,▼, *Hs* (pJNK1) ; ◊, ◆, *Hs* (pSS2), & ● ,○, *Hs* (pOK53). A,B,D –Aerobic condition on 50 mM glucose and C - Nitrogen free medium 50 mM glucose; B- Glucose and Xylose Mixture of 25 mM each. D- 50 mM Xylose. OD600 and pH values at each time point are represented as the mean ± SD of six independent observations.

Growth rates of *Hs* Z6 and the transformants (pJNK1, pSS2 and pOK53) was similar on HRP medium (**Table 4.2**). Total glucose depletion of *Hs* Z67 transformants (pSS2 and pOK53) was ~4.4 and ~ 2.4 folds higher as against native strain and *Hs* Z6 (pJNK1), respectively, on HRP medium. On the other hand, the specific glucose utilization rate of *Hs* Z6 transformants (pSS2 and pOK53) was ~2.7 and ~ 1.3 folds higher than wild type and *Hs* (pJNK1), respectively, on HRP medium**.** However, **g**lucose consumption was almost similar in all *Hs* Z67 transformants when compared with wild type. In contrast, biomass yield of *Hs* Z67 transformants (pSS2 and pOK53) was decreased by ~ 2.4 and ~ 2.8 folds on HRP medium when compared

with wild type. *Hs* (pJNK1) showed ~2 fold less biomass yield as compared with native strain**.**

Bacterial Strain	Growth rate μ (h^{-1})[a]	Total glucose depleted (mM)[b]	Glucose consumed (mM)[b]	Biomass yield $Y_{dcw/Glc}$ [a] (g /g)	Sp. glucose utilization rate Q_{Glc} [a] ($g.g\ dcw^{-1}.h^{-1}$)
H s	0.34 ±0.04	10.200± 0.91	10.200± 0.91	0.068±0.005	0.40 ±0.03
Hs (pBBR1MCS2)	0.20 ±0.003	11.66 ±0.61^{ns}	11.66±0.61 ^{ns}	0.054±0.006	0.51±0.06
Hs (pJNK1)	0.21±0.02 ^{ns}	18.70 ± 1.04*	14.61±1.05 ^{ns}	0.034±0.001*	0.81±0.02*
Hs (pSS2)	0.22±0.008 ^{ns}	44.56 ±0.58***	12.35±0.90 ^{ns}	0.024±0.001**	1.13±0.08***
Hs (pOK53)	0.24±0.008 ^{ns}	45.16 ±0.30***	11.48±0.61 ^{ns}	0.028±0.001**	1.19 ± 0.06***

Table 4.2: Physiological attributes and metabolic record of *H. seropedicae* Z67 transformants grown on HRP minimal medium with 50mM glucose as C source under aerobic condition.

Growth rate and glucose depletion rate were determined using mid-log phase cultures. Parameters compared HRP minimal medium for each transformant. The results are expressed as Mean ± SEM of readings from 3 independent observations., ***P < 0.001, **,P < 0.01, * P < 0.05 and ns=non-significant (as compared wild type culture controls) ; [a] Determined from mid log phase of each experiment. [b] Determined at the time of pH drop (36 h).

When inoculated in HRP minimal medium, similar pattern of absence of pH change was found in the transformants containing pJNK1 but the transformants containing pSS2 and pOK53 plasmids showed good growth and a drop in pH up to 5.0 within 36 h in the presence of glucose, xylose and mixture of xylose and glucose and solubilized RP (Fig. 4.6). *Hs* native culture and transformant of pJNK1 plasmid did not show acidification and solubilize RP while *Hs* transformants containing pSS2 and pOK53 released P up to ~168.07 µM, ~127.96 µM, and ~155.07µM on 50 mM glucose, 50 mM xylose and, 25 mM glucose and 25 mM xylose, respectively (**Table 4.2**). In micro aerobic condition similar results were obtained (**Table 4.3**).

Table 4.3: Physiological attributes and metabolic record of *H. seropedicae* Z67 transformants grown on N free under microaerobic condition HRP minimal medium with 50mM glucose as C source.

Bacterial Strain	Growth rate μ (h^{-1})[a]	Biomass yield $Y_{dcw/Glc}$ [a] (g /g)	Sp. glucose utilization rate Q_{Glc} [a] (g.g $dcw^{-1}.h^{-1}$)	Nitrogen Free HRP medium [b]
Hs	0.073 ± 0.030	0.011 ± 0.001	0.62 ± 0.11	UD
Hs (pBBR1MCS2)	0.098 ± 0.004*	0.011 ± 0.001^{ns}	0.66 ± 0.08^{ns}	UD
Hs (pJNK1)	0.13±0.017***	0.0072 ± 0.0008***	0.98 ± 0.11*	UD
Hs (pSS2)	0.12 ± 0.022***	0.0042 ± .0003***	1.41 ± 0.09***	130.19 ± 6.2***
Hs (pOK53)	0.13 ± 0.041***	0.0054 ± 0.0005***	1.28 ± 0.14***	172.04 ± 9.1***

Parameters compared HRP minimal medium for each transformant. The results are expressed as Mean ± SEM of readings from 3 independent observations, ***P < 0.001, ** P < 0.01, * P < 0.05 and ns=non-significant (as compared wild type culture controls) ; [a] Determined from mid log phase of each experiment. [b] Determined at the time of pH drop (72 h).

Under N free HRP medium, growth rates of *Hs* Z67 transformants (pJNK1, pSS2 and pOK53) were higher by ~1.5 fold when compared with wild type culture (**Table 4.3**). On the other hand, the specific glucose utilization rate of *Hs* Z67 transformants (pSS2 and pOK53) was ~2 folds higher than wild type and *Hs* (pJNK1), respectively, on N free HRP medium**.** In contrast, biomass yields of *Hs* Z67 transformants (pSS2 and pOK53) was decreased by ~ 2 folds on N free HRP medium when compared with wild type. *Hs* (pJNK1) showed ~2.5 fold less biomass yield as compared with native strain**.**

Bacterial transformants of *H. seropedicae* Z67 when inoculated in N free HRP minimal medium, no change in pH was found in the transformants containing pJNK1

but the transformants containing pSS2 and pOK53 plasmids showed good growth and a drop in pH upto 5.0 within 72 h in the presence of glucose and solubilized RP (**Fig. 4.6**). *Hs* native culture and transformant of pJNK1 plasmid did not show acidification and did not solubilize RP, whereas *Hs* transformants containing pSS2 and pOK53 released P up to ~130.19 µM and ~172.04 µM, respectively, on 50 mM glucose (**Table 4.3**). Extent of P release in microaerobic condition is similar to that of aerobic conditions.

4.3.3: GDH activity and gluconic acid secretion in *H. seropedicae* Z67 transformant of *pqq* genes.

Native culture did not show any GDH activity but *Hs* transformants pJNK1 on M9 medium showed GDH activity 14.98 U with glucose, 12.47 U with xylose and 15.89 U on mixture of glucose xylose. In HRP medium, the activity was 19.14 U with glucose, 14.46 U with xylose and 19.14U with mixture of glucose and xylose (**Table 4.4**). *Hs* pSS2 and *Hs* pOK53 showed ~5.5 to ~6 fold increase in GDH activity with glucose, xylose and mixture of glucose xylose in M9 medium whereas *Hs* pSS2 and *Hs* pOK53 on HRP medium GDH activity was enhanced by ~12 to ~13 fold when compared with pJNK1. Gluconic acid secretion was not found in *Hs* native culture and vector transformant. Gluconic acid production was ~4.26 mM with *Hs* pJNK1 transformants while *Hs* pSS2 and pOK53 transformants secreted 32.0 mM and 33.46 mM gluconic acid, respectively (**Fig. 4.7**). Yield of gluconic acid by *Hs* Z67 transformants (pSS2 and pOK53) was ~ 3 fold more than *Hs* Z67 (pJNK1) on HRP medium.

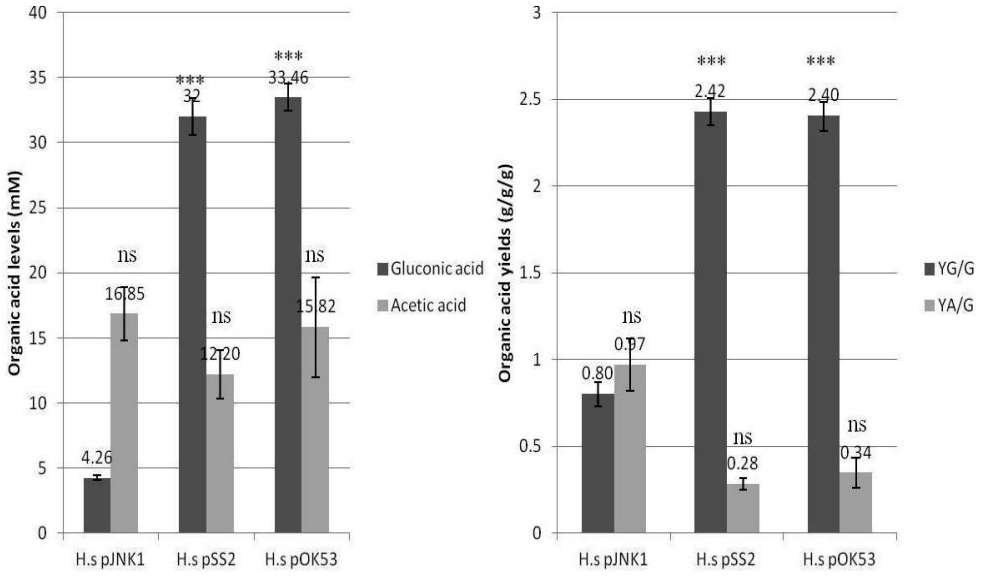


Fig. 4.7: Organic acid levels and yield. Parameters compared HRP minimal medium for each transformant. The results are expressed as Mean ± SEM of readings from 3 independent observations, ***P < 0.001, ** P < 0.01, * P < 0.05 and ns=non-significant (as compared wild type culture controls).

Table 4.4: GDH activity gluconic acid secretion and mps activity of *H. seropedicae* Z67 transformants.

***H. seropedicae* transformants**	**GDH activity (U)**						**Phosphate released. Pi (µM)**		
	M9 minimal medium			**HEPES rock phosphate medium**			**HEPES rock phosphate medium**		
	Glucose	Xylose	Glucose + Xylose	Glucose	Xylose	Glucose + Xylose	Glucose	Xylose	Glucose + Xylose
Hs	UD	UD	UD	UD	UD	UD	UD	UD	UD
pBBR1MCS2	UD	UD	UD	UD	UD	UD	UD	UD	UD
pJNK1	14.98±2.6	12.47 ± 3.3	15.89 ± 1.5	19.14 ± 2.8	14.46 ± 2.53	19.14 ± 2.2	UD	UD	UD
pSS2	87.50±6.9	80.70±10.4	82.43 ± 5.4	240.93 ± 2.8	215.24 ±15.2	179.20 ± 2.4	125.47 ±15.1	106.64 ± 6.4	119.20 ± 16.4
pOK53	86.34±7.0	79.32 ± 7.1	88.34 ± 18.4	228.86 ± 7.1	224.36 ± 0.9	197.99 ± 3.1	168.07 ±14.5	127.96 ± 17	155.07 ± 7.3

UD – Undetectable

4.3.4: Secretion of PQQ by *H. seropedicae* Z67 transformants.

Hs Z67 wild type did not produce PQQ in M9 minimal medium. *Hs* Z67 (pJNK1) secreted 0.011 µM PQQ while *Hs* Z67 transformants (pSS2) and (pOK53) showed ~ 100 and ~ 200 fold increase in PQQ secretion, respectively. On the other hand, two phosphate solubilizing strains, *E. asburiae* PSI3 and *P. aeruginosa* P4 produced 4.52 µM and 1.4 µM, respectively, in M9 minimal medium**.**

Table 4.5: Amount of PQQ secretion in native and transformant bacteria.

Host	Nature of the genes incorporated	Amount of PQQ in the medium	Ref.
E. coli K12	*A. calcoaceticus pqq* gene cluster	Very Low	Goosen et al., 1989
E. coli	*Klebsiella pneumoniae* pqq gene cluster	0.18 µM	Meulenberg et al., 1995
E. coli (JM109, BL21)	*G. oxydans* M5 *pqqABCDE* gene cluster	6 µM*	Yang et al., 2010
Methylovorus sp. MP688		45 µM*	Ge et al., 2013
P. aeruginosa ATCC 17933	-	0.455 µM*	Gliese et al., 2010
H. seropedicae Z67	*Erwinia herbicola pqqE*	0.011 ± 0.002 µM	This study
H. seropedicae Z67	*Acinetobacter calcoaceticus pqq* gene cluster	1.10 ± 0.64 µM	This study
H. seropedicae Z67	*Pseudomonas fluorescens* B161 *pqq* gene cluster	2.55 ± 0.10 µM	This study
E. asburiae PSI3	-	4.52 ± 0.84 µM	This study
P. aeruginosa P4	-	1.41 ± 0.12 µM	This study

*Calculated from amount of PQQ given in µg/ml.

4.3.5: Influence of phosphorous levels on IAA, EPS and biofilm production.

Hs transformants (pSS2 and pOK53) showed increase in EPS production up to (~28 µg/ml) while wild type culture, vector control and (pJNK1) produced ~15 µg/ml (**Fig. 4.8. A**). Under N fixing conditions, the EPS production was similar to that under aerobic N sufficient condition (**Fig. 4.8. B**). Biofilm formation also showed similar pattern of *Hs* transformants (pSS2 and pOK53) had enhanced biofilm formation by ~2 fold in both N-sufficient aerobic and N-free microaerobic conditions when compared with wild type culture (**Fig. 4.8. C&D**). On the other hand, IAA production was enhanced in *Hs* transformants (pSS2 and pOK53) to ~1.6 fold (~35 µg/ml) in aerobic conditions than that of wild type culture, vector control and pJNK1 transformant (~22 µg/ml) in aerobic condition (**Fig. 4.8. E**). All *Hs* transformants showed 50% decrease in IAA under N free microaerobic conditions as compared to aerobic conditions (**Fig. 4.8. F**).

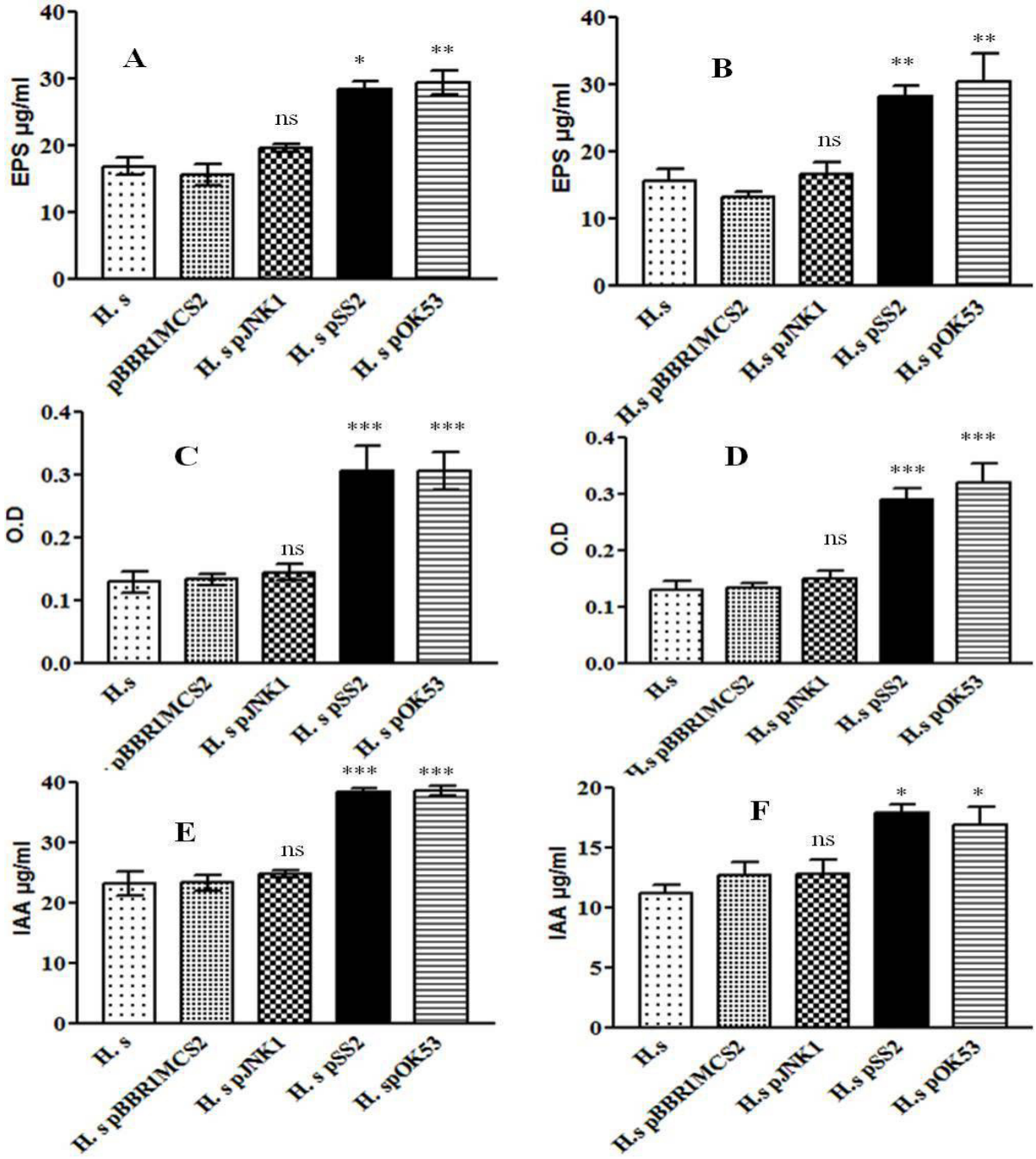


Fig. 4.8: Effect of *Hs* transformants on plant growth promoting factors.

The results are expressed as Mean ± SEM of readings from 3 independent observations, ***P < 0.001, ** P < 0.01, * P < 0.05 and ns=non-significant (as compared wild type culture controls) ; **A, C** and **E** under aerobic **, B, D** and **F** under microaerobic condition.

4.4: Discussion

Phosphate solubilizing rhizobacteria produce chiefly gluconic acid by direct oxidation pathway, gluconic acid production is mediated by periplasmic Glucose dehydrogenase (GDH) which requires PQQ as a redox cofactor for direct oxidation of glucose to gluconic acid (Goldstein, 1995). Although many rhizobacteria *viz* fluorescent pseudomonads, *Klebsiella*, *Gluconobacter*, *Azotobacter* possess PQQ biosynthesis genes, only a few strains secrete high amount of gluconic acid. Exogenous supplementation of PQQ has been shown to secrete gluconic acid (Babu-Khan et al., 1995). Additionally, overexpression of *pqq* biosynthesis gene(s) in *Pseudomonas sp., Burkholderia Azospirilluim brasileinse* and *E. coli* resulted in gluconic acid secretion. Thus, amount of PQQ but not apoprotein limits the gluconic acid secretion.

PQQ biosynthesis in bacteria is not clearly understood but a putative pathway suggests a set of modifications on an oligopeptide involving glutamate and tyrosine residues (Puehringer et al., 2008). Analysis of PQQ biosynthesis genes from a variety of bacteria showed that *pqqABCDE* genes are conserved and presence of additional genes is variable. A single *pqqE* and PQQ transporter gene incorporation in *E. coli, P.cepacia* and *Azospirillum brasiliense* resulted in gluconic acid secretion leading toi MPS ability (Goldstein and Liu, 1987; Liu et al. 1992; Vikram et al., 2007). In contrast, over expression of *pqqE* gene in *Hs* Z67 could not confer MPS phenotype and gluconic acid secretion was limited ~4 mM, Reasons for these variations in *pqqE* overexpression is not clear.

Incorporation entire *pqq* gene cluster of *A. calcoaceticus* in *E. coli* produced very low amount of PQQ but high amount in *A. lwoffi* **(**Goosen et al., 1992**).** Additionally, incorporation of *pqq* gene clusters of *Deinococcus radiodurans* and *Serratia marcescens* resulted in gluconic acid secretion and MPS phenotype (Apte et al., 2003; Kim et al., 2006). *H. seropedicae* Z67 transformants of both *pqq* gene clusters

of *A. calcoaceticus* and *P. fluorescens* B16 resulted in good amount of PQQ secretion. However, the amount of PQQ level, GDH activity, gluconic secretion and MPS phenotype were better with *Hseropedicae* Z67 containing *P. fluorescens* B16 *pqq* gene cluster as compared to that of *A. calcoaceticus.* *A. calcoaceticus* encodes *pqq* genes in 5.1 kb region containing III, II, I, V and IV which are similar to *K. pneumonia pqqE, pqqD, pqqC, pqqB* and *pqqA*. Two additional genes *pqqR* and *pqqL* are found in this gene cluster. On the other hand, *P. fluorescens* B16 *pqq* gene cluster is of 13.4 kb in size and contains *pqqABCDEFHIJKM*. Incorporation of *A. calcoaceticus* gene cluster in *Hs* Z67 has produced 1.10 µM of PQQ while *P. fluorescens* B16 *pqq* gene cluster has produced 2.4 µM which is ~2 folds more (Table 5). This may be due to the differences in the extent of *pqq* gene expression or to the presence of additional proteins. Both pSS2 (25Kb), which is derived from pRK290 and pOK53 plasmid (33Kb), a derivative of pLAFR3, are low copy plasmids (Ditta et al., 1980; Staskawicz et al., 1987) low copy number both the clusters are under their native promoter, low copy number of plasmid might play major role in low production in PQQ production in organism. Interestingly, overexpression of *G. oxydans pqqABCDE* in *E. coli* has produced 6 µM PQQ under pET28a-P_{T7}-pqq expression vector (Yang et al., 2010). Thus expression of minimal set of genes appears to be sufficient for high levels of PQQ biosynthesis. Nevertheless, it will be interesting to understand the functions of *pqqHIJKM* of *P. fluorescens* B16 and *pqqR* and *pqqL* genes of *A. calcoaceticus* in PQQ biosynthesis.

PQQ biosynthesis by overexpression of both gene clusters is less than some of the natural phosphate solubilizing rhizobacteria. *E. asburiae* PSI3 and *P. aeruginosa* P4 have been isolated from the rhizosphere of pigeon pea and mung bean secreted PQQ at 4.5 µM and 1.4 µM, levels, respectively. This suggests that PQQ secretion could be further improved for better MPS ability. It would be interesting to understand the basis for high PQQ secretion in such natural isolates.

The present results demonstrated the overexpression of *pqq* gene clusters of *A. calcoaceticous* and *P. fluorescens* B16 in *H. seropedicae* Z67 resulted in rock

phosphate solubilization on glucose, xylose and mixture of both sugars. When both the GDH substrates were supplied 25 mM of each of these sugar sufficient while 50 mM was necessary individually. Similarly, *E. asburiae* PSI3 showed MPS phenotype at 15 mM concentration of a mixture of seven GDH substrates while independently 75 mM amount of sugar was required (Sharma et al., 2005). The amount of the sugars was further reduced to 12 mM when *E. asburiae* PSI3 was incorporated with *Pseudomonas putida* KT 2440 gluconate dehydrogenase (*gad*) operon secreting 2-ketogluconic acid (Kumar et al., 2013). Earlier, it has been suggested that an organism that is capable of using a variety of C sources for its bioenergetics could have an advantage over competitors that use limited C sources in natural environments (Goodwin and Anthony 1998). In rhizospheric soils, xylose is one of the predominant monosaccharide of plant root exudates (Roy et al., 2002; Kumar et al., 2006).

H. seropedicae Z67 transformants also showed MPS ability under microaerobic nitrogen fixing conditions. Similar results were observed in *A. brasielnse pqqE* transformant which solubilized tricalcium phosphate on N free medium (Vikram et al., 2007). Additionally, incorporation of *pqq* genes improved the biomass and specific glucose utilization of *H. seropedicae* Z67 in both aerobic and microaerobic conditions in HRP medium.

Significant contribution in plant growth promotion is shown by plant growth-promoting bacteria by producing factors (EPS, Biofilm and IAA) responsible for plant growth promotion in adverse conditions (Vicente et al., 2012). *Herbaspirillum* is kmown to produces IAA (Radwan et al., 2002). PQQ secretion also improved IAA production in *H. seropedicae* Z67. Similarly, exopolysaccharide production and biofilm formation was better in *Hs* Z67 transformants in HRP medium. *Hs* Z67 has been shown to be present in the mucilaginous and a fibrillar material in maize and sorghum root surfaces (Gyaneshwar et al. 2002; Roncato-Maccari et al. 2003b). EPS plays an important role during the colonization of the protoxylem vessels of sorghum and sugarcane by *H. seropedicae* Z67 and *H. rubrisubalbicans* (James et al., 1997; Olivares

et al. 1997; James and Olivares 1998). Biofilm formation is required for the attachment in plant tissues (Baldotto et al., 2011: Santacecilia et al., 2012). Microbial growth and biofilms production is kmown to be increased by phosphorous (Lehtola et al. 2002; Chu et al. 2005; Fang et al., 2009). The production of EPS is directly correlated with biofilm formation as EPS can protect the cells External addition of phosphorous in water also enhanced biofilm formation (Mohamed et al., 1998). EPS productivity was increased in *Bacillus cereus* GU812900 by increasing nitrogen and phosphate (Bragadeeswaran et al., 2011). *Hs* Z67 transformants containing *pqq* gene clusters could also have better colonization. In conclusion, incorporation of *pqq* gene cluster could significantly enhance the plant growth promoting ability of *Hs* Z67 by MPS phenotype, better colonization, and IAA secretion.

Chapter 5

5.1: Introduction.

Phosphorous, Nitrogen and Potassium are the essential macronutrients required for growth and development of plants. In field experiments, *Penicillium bilaii* and *Bacillus megatherium* are known to be most effective phosphate solubilizing microorganisms (PSMs) (Asea et al., 1988; Kucey, 1988). PSM-plant inoculations increased the crop yield by 10–15% (Tandon, 1987). PSMs increase the availability of phosphates for plant growth and development by secreting organic acids such as citric, oxalic, gluconic, 2-ketogluconic, lactic, malic, succinic, tartaric and acetic for mineral phosphate solubilizing (MPS) ability (Gyansehwar et al., 2002; Khan et al., 2009; Richardson 2011; Archana et al., 2012). Solubilization of mineral P from soils depends on the nature and amount of the acid which varies from 10 to 100 mM from strong to weak organic acid (Gyaneshwar et al., 1998).

Phosphate solubilizing rhizobacteria produce chiefly gluconic acid employing direct oxidation pathway mediated by pyrroloquinoline quinone (PQQ) dependent periplasmic glucose dehydrogenase (GDH) (Goldstein, 1995). Although many rhizobacteria *viz* fluorescent pseudomonads, *Klebsiella*, *Gluconobacter*, *Azotobacter* possess PQQ biosynthesis genes, only a few strains secrete high amount of gluconic acid. Exogenous supplementation of PQQ has been shown to enhance gluconic acid secretion (Babu-Khan et al., 1995). Additionally, overexpression of *pqq* biosynthesis gene(s) in *Pseudomonas sp., Burkholderia, Azospirilluim brasileinse* and *E. coli* resulted in gluconic acid secretion (Goldstein and Liu, 1987; Liu et al. 1992; Vikram et al., 2007). Although PQQ biosynthesis involves multiple genes, incorporation of single *pqqE* and PQQ transporter gene in *E. coli, P. cepacia* and *A. brasiliense* was sufficient for gluconic acid secretion leading to MPS ability. Incorporation of *pqq* gene clusters of *Deinococcus radiodurans* and *Serratia marcescens* resulted in gluconic acid secretion and MPS phenotype in *E. coli* (Apte et al., 2003; Kim et al., 2006).

E. coli, Azospirillum, Rhizobium and Herbaspirillum do not encode PQQ biosynthesis genes in their genome but possess glucose dehydrogenase (GDH) apoprotein (Pedrosa et al., 2011). Incorporation of pqq gene clusters in *E. coli* resulted in PQQ biosynthesis and active GDH enzyme (Goosen et al., 1989; Meulenberg et al., 1990; Khairnar et al., 2003; Yang et al., 2010). Interestingly, **e**xpression of *Erwinia herbicola pqqE* gene alone in *E. coli* HB101 and *Azospirillum* resulted in active GDH enzyme and secreted gluconic acid (Liu et al., 1992; Vikram et al, 2007). Similarly, expression of *Rahnella aquatilis pqqED* genes also resulted in gluconic acid secretion in *E. coli* (Kim et al., 1998).

2-Ketogluconic acid is much stronger than gluconic acid for phosphate solubilization simultaneously; it can chelate calcium in soils (Moghimi and Tate, 1978; Moghimi et al., 1978). Many bacteria are known to secrete 2-ketogluconic acid (Webley and Duff 1965; Yum et al., 1997; Walker et al., 2003; Saichana et al., 2008; Vyas and Gulati 2009; Gulati et al., 2010; Park et al., 2010). *Pseudomonas, K. pneumoniae, S. marcescens,* and *E. cypripedii* ATCC 29267 possess GADH enzyme (Matsushita and Ameyama, 1982; Yum et al., 1997; Toyama et al., 2007; Saichana et al., 2008). Conversion of gluconic acid in 2-ketogluconic acid in the periplasm by FAD- dependent gluconate dehydrogenase (GADH) enzyme encoded by *gad* operon (Saichana et al., 2008; Toyama et al., 2007 and Yum et al., 1997). *E. cypripedii* ATCC 29267 *gad* operon overexpressed in *E. coli*, enabled secretion of high amount of 2-ketogluconic acid (Yum et al., 1997). Overexpression of *Pseudomonas putida* KT 2440 *gad* operon in *E. asburiae* PSI3 resulted in conversion of gluconic acid in 2 ketogluconic acid with enhanced P solubilization phenotype (Kumar et al., 2013).

Many microorganisms including *Bacillus mucilaginosus* are known to solubilize rock K such as micas, illite, orthoclases and feldspar by secreting organic acids (Groudev, 1987; Malinovskaya et al., 1990; Friedrich et al., 1991; Ullman et al., 1996; Bennett et al., 1998; Sheng and Huang, 2002). Organic acids such as oxalic, citric and gluconic acids secreted by many microorganisms including *Thiobacillus, Clostridium* and *Bacillus* promote dissolution of potassium from albite, quartz and koalinite by (Vainberg et al.,

1980; Berthelin, 1983; Freidrich et al., 1991; Hazen et al., 1991; Vandevivere et al., 1994; Barker et al., 1998). *Bacillus edaphicus* K solubiliser showed significant increase in N, P and K content of wheat plants (Sheng and He 2006). PSB and KSB enhanced P and K content in cucumber (Han et al. 2006).

Baldani et al. (1986) isolated *Herbaspirillum* as nitrogen-fixing bacteria, which is associated with the roots of rice (*Oryza sativa*), maize (*Zea mays*) and sorghum (*Sorghum bicolor*). *Herbaspirillum* colonizes vascular tissues of economically important plants; it is found in roots and stems of sugarcane, sorghum, and rice, and found to be able to fix nitrogen (Pimentel et al., 1991; Ureta et al., 1995). *H. seropedicae* colonizes graminaceous plants and significantly improves plant growth (Baldani et al., 1992; Olivares et al., 1996). *Herbaspirillum* spp. is an endophyte and contributes 40 - 60% of nitrogen from biological nitrogen fixation (Urquiaga et al., 1992; Boddey et al., 1995; James and Olivares, 1997; Yoneyama et al., 1997). It also promotes plant growth by phytohormone production which results in higher yields (Baldani et al., 1995; Bastian et al., 1998).

5.1.1: Rational of study

In present study, we demonstrated the effect of over expression of *Acenitobacter calcoaceticus pqq* gene cluster and *Pseudomonas putida* KT2440 *gad* operon in *H. seropedicae* Z67 on 2-Ketogluconic acid secretion and its effect on mineral phosphate and potassium solubilizing ability.

5.2: EXPERIMENTAL DESIGN

5.2.1: Bacterial strains used in this study

Table 5.1: Bacterial strains used in this study

Plasmid/Strains	Characteristics	Source or Reference
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r (100µg/ml)	Hester et al., 2000
pSS2	25Kb plasmid contains 5.1-kb of *pqq* gene cluster of *A. calcoaceticus* Tet^r (40µg/ml)	Goosen et al., 1989
pCNK12	pJET2.1 with *gad* operon. Amp^r (100µg/ml)	Chanchal et al., 2013
E. coli DH10B	Host strain for routine DNA manipulation experiments and plasmid maintenance. $Strp^r$ (50µg/ml)	Invitrogen,USA
pJNK2	pBBR1MCS2, Km^r (50 µg/ml) with 13.4 Kb *pqq* gene cluster of *P. fluorescens* B16.	This study
pJNK5	pUCPM18, Gm^r (20µg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus*.	This study
pJNK6	pJNK5, Gm^r (20µg/ml) with *gad* operon 3.8 Kb of *P. putida* KT 2440	This study
Hs	Nitrogen fixing rice endophyte, Nal^r (10µg/ml)	Baldani et al., 1986
Hs (pUCPM18)Gm^r	*H. seropedicae* Z67 Nal^r (10µg/ml) with (pUCPM18)Gm^r (20µg/ml)	This study
Hs (pJNK2)	*H. seropedicae* Z67 Nal^r (10µg/ml) with pJNK2, Km^r (50µg/ml)	This study
Hs (pJNK5)	*H. seropedicae* Z67 Nal^r (10µg/ml) with pJNK5Gm^r (20µg/ml)	This study
Hs (pJNK6)	*H. seropedicae* Z67 Nal^r (20µg/ml) with pJNK6Gm^r (20µg/ml)	This study

5.2.2: Bacterial strains, plasmids, Media, and culture conditions.

Bacterial strains and plasmids used for this work have been listed in **Table 1.** DNA manipulation and molecular biology experiments were performed using *E. coli* DH10B as the host strain using standard protocols (Sambrook and Russell, 2001). Plasmid

bearing derivatives of *E. coli* DH10B were routinely grown at 37^0C on Luria Bertani broth (L.B) and maintained on Luria Bertani Agar (L.A) plate with 40 µg ml^{-1} streptomycin. *H. seropedicae* Z67 was routinely grown at 30^0C on Luria Bertani (L.B) and maintained on semisolid JNFb medium pH 5.8 plates containing 10 µg ml^{-1}Nalidixic acid at 30°C (Baldani et al., 1986). For nitrogen fixation, *H. seropedicae* Z67 JNFb medium was devoid of a nitrogen source.

5.2.3: DNA manipulation

Plasmid pOK53 containing *pqq* gene cluster of *Pseudomonas fluorescens* B16 was obtained as a generous gift from Dr Ingyu Hwanag, Department of Biochemistry, Seoul University, Korea. Plasmid pOK53 was digested with BamHI and the 13.4 Kb fragment was purified by using Invitrogen gel purification Kit. Broad host range vector pBBR1MCS2Km^r was digested with same restriction endonucleases (RE). The 13.4 Kb fragment of *pqq* gene cluster was ligated with pBBR1MCS2Km^r which resulted in pJNK2 plasmid. Ligation mixture was transformed in *E. coli* DH10B $Strp^r$; screening was done on the basis antibiotic resistance. Plasmid was isolated and confirmed by restriction enodnuclease digestion analysis.

Plasmid pSS2 containing *pqq* gene cluster of *A. calcoaceticus* was obtained as a generous gift from Dr. Goosen, Molecular Genetcis, University of Leiden, Netherland. Plasmid pSS2 was digested with *Eco*R1/ BamHI and the 5.1 Kb insert was purified by using Invitrogen gel purification Kit. Broad host range vector pUCPM18 Gm^r was digested with same restriction endonucleases (RE). The 5.1 Kb fragment of *pqq* gene cluster was ligated with pUCPM18 Gm^r which resulted in pJNK5 plasmid. Ligation mixture was transformed in *E. coli* DH10B $Strp^r$; screening was done on the basis antibiotic resistance. Plasmid was isolated and confirmed by restriction endonuclease digestion analysis.

Plasmid pCNK12 containing *gad* operon of *P. putida* KT 2440 was digested by BamH/XbaI. 3.8 Kb fragment was purified by using invitrogen gel purification Kit. Plasmid pJNK5 containing *pqq* gene cluster of *A. calcoaceticus* was digested with same enzymes. The 3.8 Kb fragment containing *gad* operon was ligated with pJNK5 Gm^r which resulted in pJNK6 plasmid. Ligation mixture was transformed in *E. coli* DH10B $Strp^r$; screening was done on the basis of antibiotic resistance. Plasmid was isolated and

confirmed by restriction endonuclease digested samples by running on 0.8% agarose gel. Further, plasmids pUCPM18Gm^r as vector control, pJNK5, pJNK6 were transformed by electroporation in *H. seropedicae* Z67 as described by Unge et al. (1998).

5.3: Results:

5.3.1: Subcloning of *pqq* gene of *P. fluorescens* B16 in broad host range vector pBBR1MCS2Km^r from pOK53.

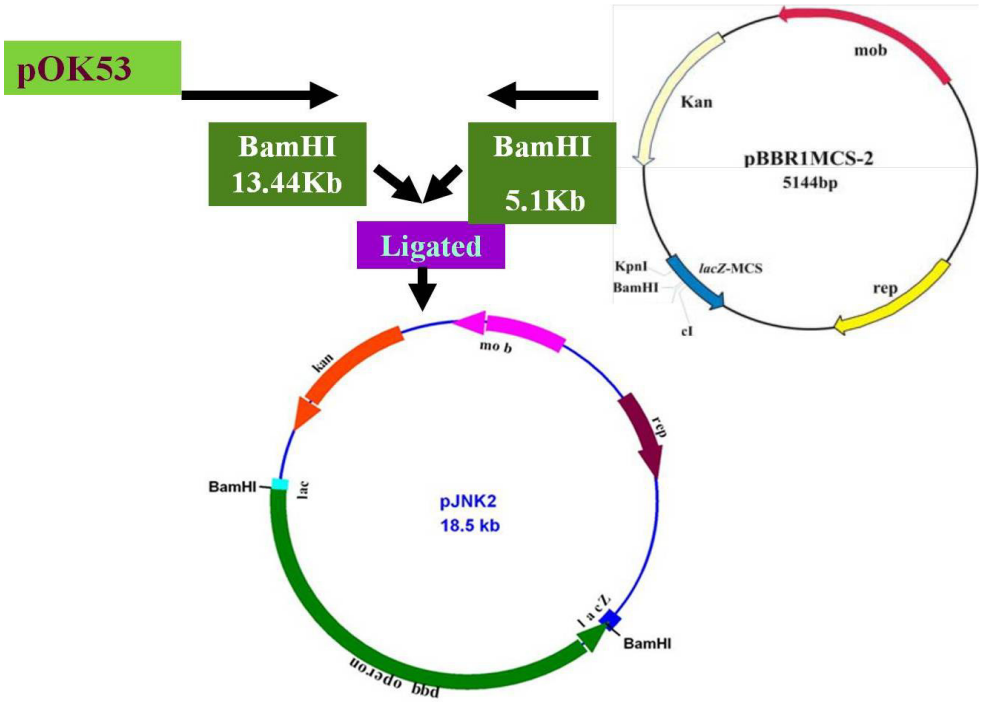


Fig. 5.1: Schematic representation of construction pJNK2 stable vector containing *pqq* gene cluster (13.4Kb) of *P. fluorescens* B16 under *lac* promoter.

5.3.2: Subcloning of *pqq* gene of *A. calcoaceticus* in broad host range vector pUCPM18Gm^r from pSS2.

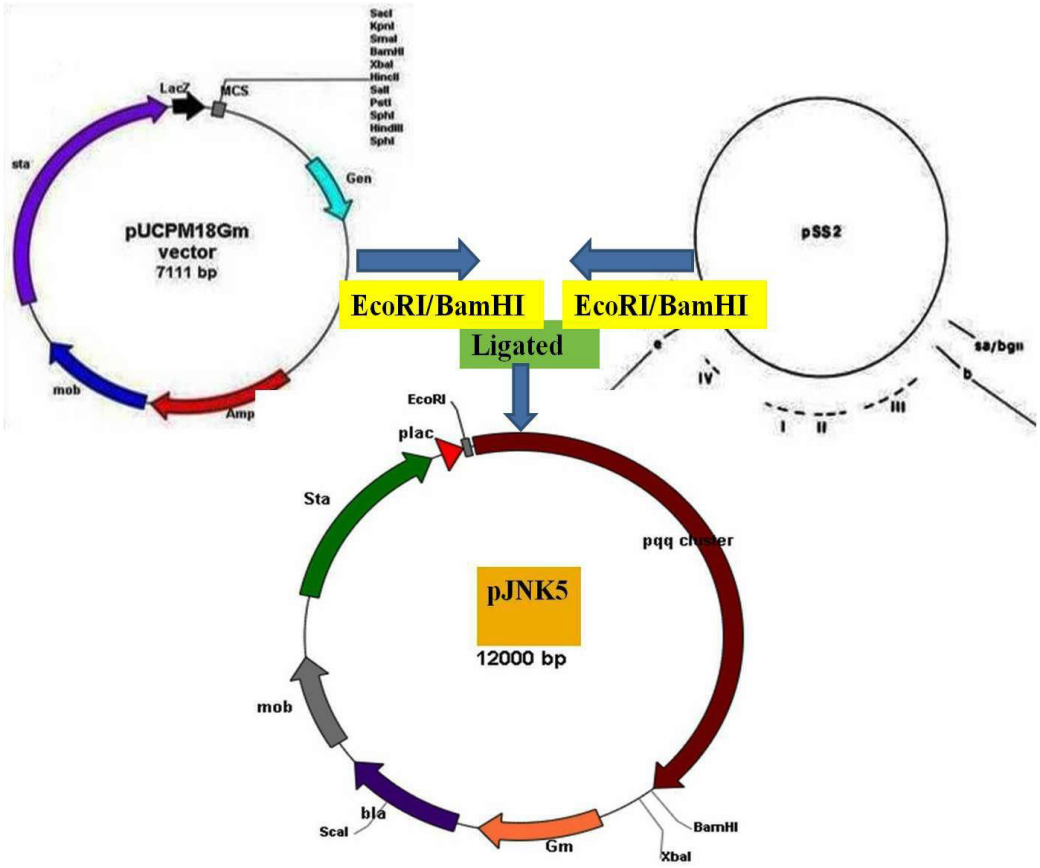


Fig. 5.2: Schematic representation of construction pJNK5 stable vector containing *pqq* gene cluster (5.1Kb) of *A. calcoaceticus* under *lac* promoter.

5.3.3: Subcloning of *gad* operon of *Pseudomonas putida* KT 2440 in broad host range vector pJNK5Gm^r from pCNK12

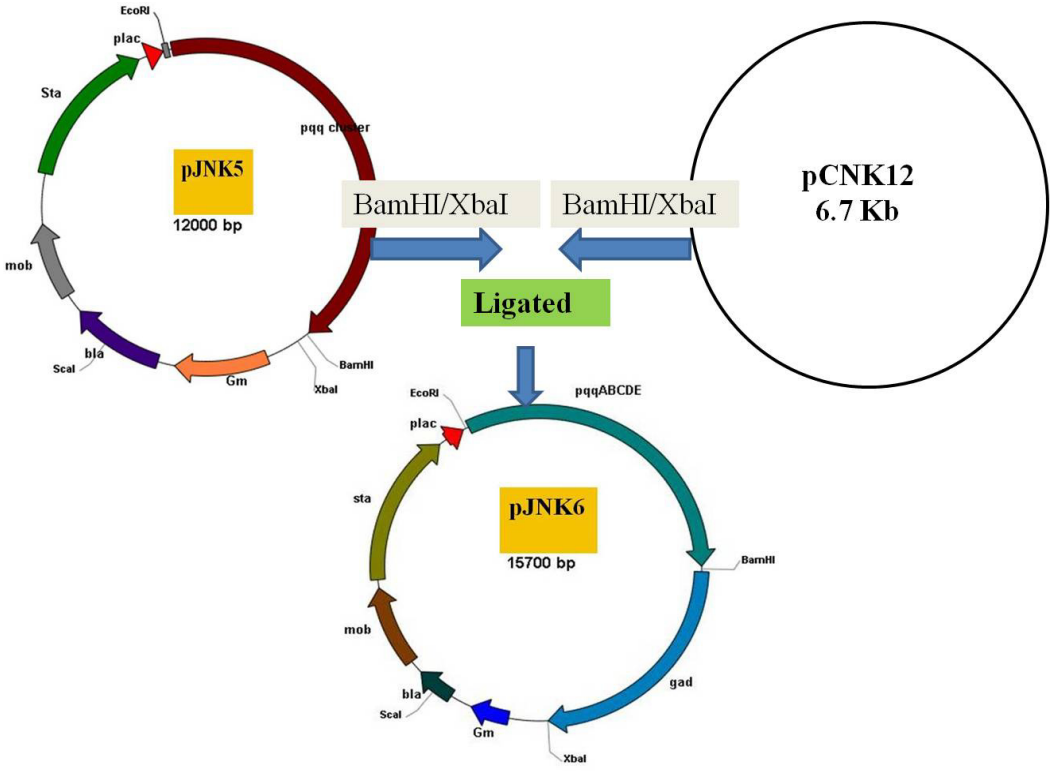


Fig. 5.3: Schematic representation of construction of pJNK6 stable vectors containing *P. putida* KT 2440 *gad* operon under *lac* promoter.

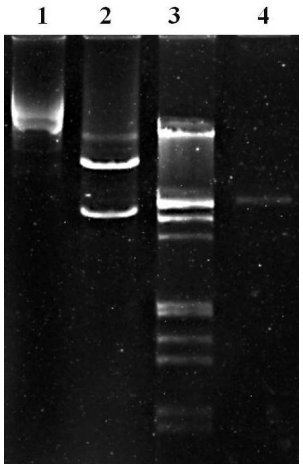


Fig. 5.4: Lane 1- pJNK2 plasmid undigested, Lane 2- pBBR1MCS2 digested by BamHI. Lane 3- Marker EcoRI/HindIII

Fig. 5.4: Restriction digestion pattern for pJNK2.

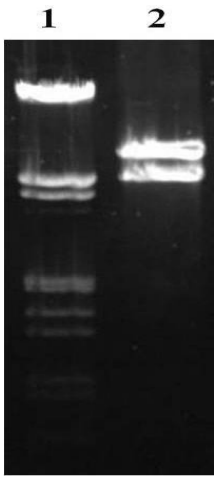


Fig. 5.5: Lane 1- Marker EcoRI/HindIII. Lane 2- pJNK5 digested by EcoRI/BamHI

Fig. 5.5: Restriction digestion pattern for pJNK5.

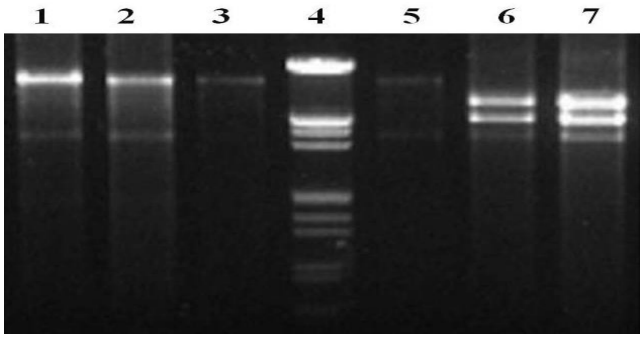


Fig. 5.6: Lane 1, 2, 3 and 5- pJNK6 digested by BamHI/XbaI
Lane 4- Marker EcoRI/HindIII
Lane 6- pJNK6 digested by EcoRI/ BamHI/ XbaI

Fig. 5.6: Restriction digestion pattern for pJNK6.

5.3.4: Growth and MPS ability of *H. seropedicae* Z67 transformants of pJNK5 and pJNK6 plasmids

5.1 Kb fragment of *A. calcoaceticus* containing *pqq* gene cluster Plasmid pSS2 was digested by restriction enzymes (RE) EcoRI and BamHI to obtain and cloned into pUCPM18 Gm^r resulting in pJNK5 plasmid. Similarly, plasmid pCNK12 was digested with restriction enzymes (RE) BamHI and XbaI to generate plasmid pJNK6. *Hs* (pJNK5) and *Hs* (pJNK6) showed good P solubilization on Pikovaskya's agar and on 100 mM HEPES buffer methyl red plates with very good acidification on 50 mM glucose and Aleksandrov feldspar agar plate for potassium **(Fig. 5.7).**

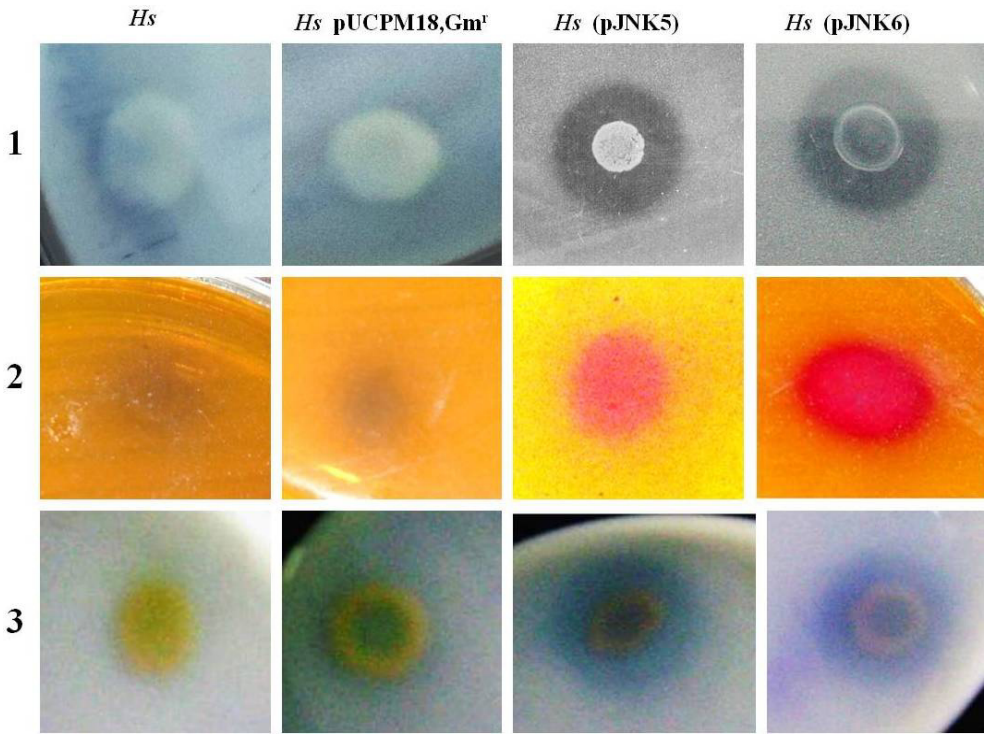


Fig. 5.7: MPS and KS phenotype of *H. seropedicae* Z67 transformants.

1- Pikovaskya's medium, 2- HRP medium with 50 mM glucose with 100 mM buffer of pH.8. , 3- Aleksandrov feldspar agar plate for potassium

Growth rates of *H. seropedicae* Z67 and the transformants *Hs* (pUCPM18), *Hs* (pJNK5) and *Hs* (pJNK6) was similar on HRP medium **(Table 5.2).** Total glucose depletion of *Hs* (pJNK5) and *Hs* (pJNK6) was ~2.8 folds higher as against native strain and *Hs* (pUCPM18), respectively, on HRP medium. On the other hand, the specific glucose utilization rate of *Hs* (pJNK5) and *Hs* (pJNK6) was ~1.1 and ~ 2 folds higher than wild type and *Hs* (pUCPM18), respectively, on HRP medium**.** However, glucose consumption was almost similar in all *H. seropedicae* Z67 transformants when compared with wild type. In contrast, biomass yield of *Hs* (pJNK6) was decreased by ~ 2 folds on HRP medium when compared with wild type. *Hs* (pJNK5) showed similar biomass yield as compared with native strain**.** When inoculated in HRP minimal medium, pH change was not found in *Hs* (pUCPM18) but *Hs* (pJNK5) and *Hs* (pJNK6) showed good growth and a drop in pH up to ~5.0 and ~4.6, respectively, within 40 h in the presence of glucose

(Fig. 5.8). *Hs* (pJNK5) and *Hs* (pJNK6) released P up to ~239.66 µM, ~457.66 µM, respectively, on 50 mM glucose **(Table 5.4).**

Bacterial Strain	Growth rate µ (h^{-1})[a]	Total glucose depleted (mM)[b]	Glucose consumed (mM)[b]	Biomass yield Ydcw/Glc[a] (g/g)	Sp. glucose utilization rate QGlc[a] (g.g $dcw^{-1}.h^{-1}$)
Hs	0.16±0.003	12.20±0.80	10.56±0.41	0.042±0.004	0.65±0.06
Hs (pUCPM18)	0.15±0.01	12.33± 0.75	10.60±0.17	0.042±0.01	0.67±0.16
Hs (pJNK5)	0.149±0.01	34.46±0.85 ***	11.89±0.59	0.041± 0.01	0.77±0.28
Hs (pJNK6)	0.16±0.001	34.16±2.38 ***	14.53±2.16	0.021±0.000 ***	1.31±0.017 ***

Table 5.2: Physiological attributes and metabolic record of *H. seropedicae* Z67 transformants grown on HRP minimal medium with 50mM glucose as C source under aerobic condition. The results are expressed as Mean ± SEM of readings from 3 independent observations. *** P<0.001, ns=non-significant (as compared with wild type culture control) ; [a]Determined from mid log phase of each experiment. [b]Determined at the time of pH drop (36 h).

Under N free HRP medium, growth rates of *Hs* (pUCPM18), *Hs* (pJNK5) and *Hs* (pJNK6) were similar as compared with wild type culture **(Table 5.3)**. On the other hand, the specific glucose utilization rate of *Hs* (pJNK6) was ~2 folds higher than wild type, *Hs* (pUCPM18), and *Hs* (pJNK5) respectively, on N free HRP medium**.** In contrast, biomass yield of *Hs* (pJNK6) was decreased by ~ 2 folds on N free HRP medium when compared with wild type, *Hs* (pUCPM18) and *Hs* (pJNK4) showed similar pattern of biomass yield as compared with native strain**.** Additionally, no change in pH was found in *Hs* (pUCPM18) but *Hs* (pJNK5) and *Hs* (pJNK6) showed good growth and decreased the pH upto ~5.0 and ~ 4.2 within 72 h in the presence of glucose and solubilized RP (**Fig. 5.8**). Native culture and *Hs* (pUCPM18) neither showed acidification nor solubilized RP whereas *Hs* (pJNK5) and *Hs* (pJNK6) released P up to ~195.56 µM and ~440.76 µM,

respectively, on 50 mM glucose (**Table 5.3**). Extent of P release in microaerobic condition is similar to that of aerobic conditions.

Bacterial Strain	**Growth rate μ (h^{-1})[a]**	**Biomass yield $Y_{dcw/Glc}$[a] (g /g)**	**Sp. glucose depletion rate Q_{Glc}[a] (g.g $dcw^{-1}.h^{-1}$)**	**Phosphate released P (μM) [b]**	**Potassium released, K (μM) [b,c]**
Hs	0.084±0.001	0.021±0.002	0.32±0.03	UD	UD
Hs (pUCPM18)	0.076±0.00	0.021±0.005	0.33±0.08	UD	UD
Hs (pJNK5)	0.074±0.00	0.020±0.007	0.36±0.11	195.56±15.00	63.66±4.79
Hs (pJNK6)	0.083±0.00***	0.010±0.000***	0.65±0.00***	440.76±23.61***	198.18±10.78

Table 5.3: Physiological attributes and metabolic record of *H. seropedicae* Z67 transformants grown under microaerobic condition on HRP minimal medium with 50mM glucose as C source. The results are expressed as Mean±SEM of readings from 3 independent observations. *** P<0.001, ns=non-significant (as compared to wild type culture controls) ; [a]Determined from mid log phase of each experiment; [b]Determined at the time of pH drop (72 h); [c]HRP medium containing 1 mg/ml feldspar.

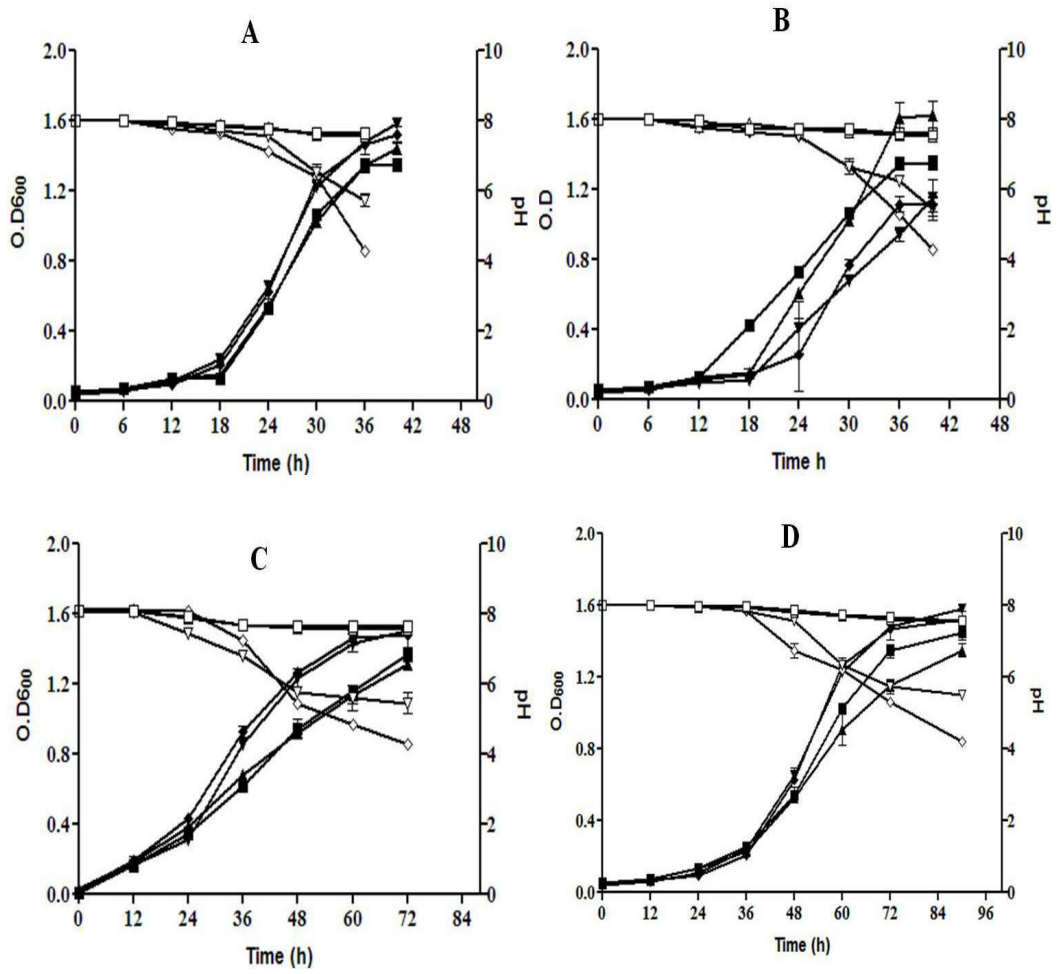


Fig. 5.8: Effect of *pqq* gene cluster and *gad* operon overexpression on growth pattern and pH profile of *H. seropedicae* Z67. (A, B - Aerobic condition and C, D- Microaerobic condition) and growth profile on50 mM glucose, 100mM HEPES rock phosphate medium. Extracellular pH (□, Δ, ▽, ◊) (■,▲,▼,◆) of *H. seropedicae* Z67 over expressing *pqq* gene clusters, □, ■ ,*Hs* (wild type); Δ, ▲, *Hs* (pUCPM18Gm^r); ▽,▼, *Hs* (pJNK5) ; ◊, ◆, *Hs* (pJNK6), B & D- HRP medium contains additionally feldspar 1m/ml OD600 and pH values at each time point are represented as the mean ± SD of six independent observations.

5.3.5: Potassium solubilizing ability of *H. seropedicae* Z67 transformants.

Hs (pJNK5) *Hs* (pJNK6) showed K solubilization, on Aleksandrov agar plate (**Fig. 5.7).** *H. seropdicae* Z6 and the transformants *Hs* (pUCPM18), *Hs* (pJNK5) and *Hs*

(pJNK6) inoculated in HRPF minimal medium supplemented with feldspar, no pH change was found in *Hs* (pUCPM18) but *Hs* (pJNK5) and *Hs* (pJNK6) showed good growth and a drop in pH up to ~5.0 and ~4.6, respectively, within 40 h in the presence of glucose **(Fig. 5.8)**. *Hs* (pJNK5) and *Hs* (pJNK6) also released K up to ~76.66 µM, ~222.66 µM, on 50 mM glucose **(Table 5.4).**

Under N free HRPF medium, *Hs* (pUCPM18), *Hs* (pJNK5) and *Hs* (pJNK6) were able to grow but no change in pH was found in *Hs* native and *Hs* (pUCPM18) whereas *Hs* (pJNK5) and *Hs* (pJNK6) showed good growth and decreased the pH upto ~5.0 and ~ 4.2, respectively, within 72 h in the presence of glucose and solubilized K **(Fig. 5.8).** Native culture and *Hs* (pUCPM18) neither showed acidification nor solubilized feldspar while *Hs* (pJNK5) and *Hs* (pJNK6) released K up to ~63.66 µM and ~198.18 µM, respectively, on 50 mM glucose **(Table 5.3).** Extent of K release in microaerobic condition is similar to that of aerobic conditions.

5.3.6: Activities of GDH and GADH enzymes and organic acid secretion in *H. seropedicae* Z67 transformants.

Hs native and *Hs* (pUCPM18) did not showed any GDH activity in HRP medium while *Hs* (pJNK5) and *Hs* (pJNK6) showed ~221.5 U and ~234 U of GDH activity, respectively **(Table 5.4).** On the other hand, GADH activity was undetectable in *Hs* native, *Hs* (pUCPM18) and *Hs* (pJNK5) whereas ~ 414 U of GADH activity was found in *Hs* (pJNK6) when compared with *Hs* native strain.

Bacterial Strain	Enzyme activity (U) HRP medium		Amount of organic acid secreted		P release (µM)[b]	K release (µM)[bc]	PQQ secreted in minimal medium (µM)
	GDH	GADH	Gluconic acid (mM)	2-keto-gluconic acid (mM)			
Hs	UD	UD	UD	UD	UD	UD	UD
Hs (pUCPM18)	UD	UD	UD	UD	UD	UD	UD
Hs (pJNK5)	221.66±10.69	UD	23.47±3.93	UD	239.66±8.73	76.66±5.033	1.15±0.11
Hs (pJNK6)	234.33±8.08	414.00±9.84	3.79±0.27	15.83±0.42	457.66±22.05	222.66±19.08	1.17±0.06

Table 5.4: Effect of *H. seropedicae* Z67 transformants on the enzyme activity, organic acid secretion and mineral phosphate and potassium solubilization in HRP medium containing 50mM glucose as the carbon source under aerobic condition. The results are expressed as Mean ± SEM of readings from 3 independent observations. *** P<0. 001, ns=non-significant as compared to wild type culture controls) ; UD- undetectable. [b]Determined at the time of pH drop (36 h). [c]HRP medium containing 1 mg/ml feldspar.

5.3.7: Effect of *Hs* (pJNK5) and *Hs* (pJNK6) on the nutrient status of rice plants

Rice plant shoot length, root length, fresh weight, dry weight, N, P and K content, in rice plants were monitored after 30 days after inoculation **(Table 5.5 and Table 5.6).** The shoot length of *Hs* native strain and *Hs* (pUCPM18) inoculated rice plants showed ~1.3 fold increase when compared with control plants without any culture, while *Hs* (pJNK5) and *Hs* (pJNK6) showed ~1.7 and ~2 folds increase when compared with native strain. Root length was increased in both *Hs* and *Hs* (pUPM18) by ~2 folds when compared with control plants, while *Hs* (pJNK5) and *Hs* (pJNK6) showed ~1.7 and ~2 folds increase in root length as compared with *Hs* plants. Fresh weight of plants inoculated with *Hs* and *Hs* (pUCPM18) increased by ~2 folds when compared with control set, while *Hs* (pJNK5) and *Hs* (pJNK6) showed significant increase in fresh weight by ~1.6 and ~2 folds when compared with *Hs* treated plants. Total chlorophyll content was found similar in control, *Hs* and *Hs* (pUCPM18) of ~1.5 fold increase when compared with control plants, while both *Hs* (pJNK5) and *Hs* (pJNK6) increased by ~1.4 and ~1.6 folds when compared with native strain.

Hs (pJNK5) and *Hs* (pJNK6) inoculated with rice plants showed presence of PQQ in rice plants. Control plants did not show any presence of PQQ. Plants inoculated with *Hs* (pJNK5) and *Hs* (pJNK6) showed 0.726 and 0.728 ng of PQQ respectively **(Table 5.5).** Nitrogen content in plants leaves was significantly increased with *Hs* and *Hs* (pUCPM18) by ~1.3 folds as compared with control plants while N levels in *Hs* (pJNK5) and *Hs* (pJNK6) showed ~1.5 and ~2.16 folds increase, respectively, when compared *Hs* plants. N levels in roots in *Hs* and *Hs* (pUCPM18) showed ~2 folds increase against control, while *Hs* (pJNK5) and *Hs* (pJNK6) showed ~1.7 and ~2.2 folds increase when compared with *Hs*. Phosphorus content in leaves and roots in both *Hs* (pJNK5) and *Hs* (pJNK6) by ~ 2.2 and ~ 3 folds when compared with *Hs* plants, respectively, K content of leaves and roots was increased in *Hs* (pJNK5) and *Hs* (pJNK6) by almost ~1.3 and 1.6 folds when compared against *Hs* **(Table 5.6)**.

Table 5.5: Effects of *H. seropedicae* Z67 transformants on rice plant fresh weight, dry weight, plant height leaf chlorophyll content at 30 DAI.

***H. seropedicae* Z67 transformants**	**Shoot length (cm)**	**Shoot/Root ratio**	**Root length/Plant (cm)**	**Root/Shoot ratio**	**Total plant fresh weight /Plant (mg)**	**Total plant Dry weight /Plant (mg)**	**Total leaf chlorophyll content (mg/g fresh weight)**	**PQQ levels in plants (ng/g of fresh weight /plant)**
Control	21.66±2.51	1.91±048	11.66±2.08	0.54±0.16	109.66±2.51	13.66±0.57	2.86±0.17	UD
Hs	29.66±1.52	1.38±0.39	22.33±4.72	0.75±0.19	210.66±10.01	16.33±0.57	3.31±0.27	UD
Hs (pUCPM18)	29.66±2.51	1.27±0.21	23.66±3.21	0.80±0.129	204.33±6.02	15.33±1.52	3.02±0.18	UD
Hs (pJNK5)	50.33±2.51***	1.27±0.10***	39.66±2.08***	0.78±0.06	340.66±4.04	37.66±1.52***	4.70±0.35***	0.726±0.03
Hs (pJNK6)	63.00±2.0***	1.11±0.13***	57.00±8.18***	0.90±0.11***	423.33±10.01	46.66±1.52***	5.55±0.79***	0.728±0.05

The results are expressed as Mean±SEM of readings from 6 plants of treatments.

*** P<0.001, ns=non-significant (as compared with wild type culture inoculant and controls plants).

Table 5.6: N, P and K status of rice plants after 30 DAI with *H. seropedicae* Z67 transformants.

***H. seropedicae* transformants**	**N content in leaves (%)**	**N content in roots (%)**	**P content in leaves (%)**	**P content in roots (%)**	**K content in leaves (%)**	**K content in roots (%)**
Control	0.88±0.04	0.44±0.02	0.039±0.002	0.025±0.002	1.04±0.05	0.390±0.020
Hs	1.22±0.03***	0.94±0.03***	0.055±0.003***	0.042±0.004***	1.33±0.09***	0.95±0.03***
Hs (pUCPM18Gm^r)	1.27±0.03	0.91±0.02	0.052±0.26	0.042±0.004	1.34±0.04	0.91±0.03
Hs (pJNK5)	1.83±0.19***	1.58±0.04***	0.30±0.002***	0.094±0.003***	1.77±0.13***	1.07±0.06***
Hs (pJNK6)	2.64±0.07***	2.03±0.06***	0.40±0.01***	0.13±7 0.02***	2.25±0.4***	1.48±0.02***

The results are expressed as Mean±SEM of readings from 6 plants of treatments.
*** P<0.001, ns=non-significant (as compared with wild type culture inoculant and controls plants).

Fig. 5.9: Effect of *H. seropedicae* Z67 on rice plants. 1- Control, 2- *Hs* native, 3-*Hs* (pUCPM18Gm^r), 4-*Hs* (pJNK5), 5-*Hs* (pJNK6).

5.4: Discussion

Phosphate solubilizing rhizobacteria chiefly secrete gluconic acid by direct oxidation pathway mediated by periplasmic Glucose dehydrogenase (GDH) which requires PQQ as a redox cofactor (Babu-Khan et al., 1995; Goldstein, 1995). Although many rhizobacteria *viz* fluorescent pseudomonads, *Klebsiella*, *Gluconobacter*, *Azotobacter* possess PQQ biosynthesis genes, only a few strains secrete high amount of gluconic acid. Exogenous supplementation of PQQ has been shown to secrete gluconic acid (Babu-Khan et al., 1995). Additionally, overexpression of *pqq* biosynthesis gene(s) in *Pseudomonas sp., Burkholderia, Azospirilluim brasileinse* and *E. coli* resulted in gluconic acid secretion. Thus, amount of PQQ but not apoprotein limits the gluconic acid secretion. (Babu-Khan et al., 1995; Krishnaraj and Goldstein 2001; Vikram et al., 2007). Our results demonstrate that overexpression of *A. calcoaceticus pqq* gene cluster in *H. seropedicae* Z67 enhances gluconic acid secretion and solubilization of mineral rock phosphate. Similar results were shown upon overexpression of *pqq* gene clusters of *Deinococcus radiodurans, Enterobacter intermedium* and *Serratia marcescens* (Krishnaraj and Goldstein 2001; Apte et al., 2003; Kim et al., 2003).

Gluconic acid in the periplasm gets converted to 2-ketogluconic acid which is stronger than gluconic acid. In present study, overexpression of *A. calcoaceticus pqq* gene cluster along with *P. putida* KT2440 *gad* operon in *H. seropedicae* Z67 led to 15.83 mM 2-ketogluconic acid along with 3.79 mM gluconic acid. These results are similar to that of *E. asburiae* PSI3 possessing high level of PQQ biosynthesis in which overexpression of *P. putida* KT2440 *gad* operon alone was sufficient to secreted 11.63 mM 2-ketogluconic along with 21.65 mM gluconic acid (Kumar et al., 2013).

Hs (pJNK5) and *Hs* (pJNK6) has enhanced GDH. On other hand GADH activity of *Hs* (pJNK6) was ~414 U, in HRP minimal medium which is almost similar to *E. asburiae* PSI3 *gad* transformant reported ~438 U of GADH activity in TRP minimal medium (Kumar et al., 2013). Overexpression of *gad* in *E. coli* has enhanced GADH activity by ~2 folds (Yum et al., 1997). *Hs* (pJNK6) transformant released ~ 457.66 µM P from 100 mM HEPES buffer in 50 mM glucose demonstrating improvement in MPS ability which correlated with secretion of ~3.79 mM and ~15.83 GA and 2 ketogluconic mM, respectively. In contrast, *E. cypripedii* ATCC 29267 *gad* operon when cloned and transformed in *E. coli* could secrete ~ 13mM 2KGA in LB containing 100mM glucose (Yum et al., 1997). Interestingly, 10 mM 2KG is sufficient for the release of P from alkaline soils (Moghimi et al., 1978). The amount of each of the seven sugars was reduced to 12 mM when *E. asburiae* PSI3 was incorporated with *P. putida* KT 2440 *gad* operon secreting 2-ketogluconic acid which could reflect the potential of the transformant in the rhizosphere containing a variety of sugars secreted by the roots (Kumar et al., 2013). It will be interesting to determine such property of the *H. seropedicae* transformant.

Similar to mineral phosphates, organic acids such as oxalic, citric, malic, succinic, tartaric and acetic secreted by bacteria solubilize inorganic potassium (Vainberg et al., 1980; Berthelin, 1983; Friedrich et al., 1991; Hazen et al., 1991; Vandevivere et al., 1994; Ullman et al., 1996; Barker et al., 1997; Barker et al., 1998; Gyaneshwar et al., 2002; Kumar et al., 2013; Liu et al., 2006; Shrivastava et al., 2006; Sheng and He, 2006). *Hs* transformants (pJNK5) and (pJNK6) also solubilized potassium from minerals. Earlier studies reported phosphate and potassium solubilization from minerals by *Pseudomonas*

isolates secreting 2-ketogluconic acid (Duff et al., 1963). Although *Bacillus* sp. also showed phosphate and potassium solubilization, the nature of the organic acid is not clear (Hu et al., 2006). It is unlikely that it is due to 2-ketogluconic acid as direct oxidative pathway is not reported in *Bacillus* sp..

Incorporation entire *pqq* gene cluster of *A. calcoaceticus* in *E. coli* produced very low amount of PQQ but high amount in *A. lwoffi* (Goosen et al., 1992). Interestingly, *Hs* (pJNK5) and *Hs* (pJNK6) has produced ~1.15 and ~1.17 µM of PQQ in medium after over expression, increase in PQQ secretion is in agreement with overexpression of *G. oxydans pqq* cluster in *E. coli* has produced 6 µM PQQ (Yang et al., 2010). Presence of PQQ and GAD enzyme resulted in GDH activity, 2-ketogluconic acid secretion and MPS ability in *Hs* (pJNK6). *Hs* transformants also showed MPS ability under microaerobic nitrogen fixing conditions. Similar results were observed in *A. brasielnse pqqE* transformant which solubilized tricalcium phosphate on N free medium (Vikram et al., 2007). Additionally, incorporation of *pqq* genes has maintained the biomass and specific glucose utilization of *Hs* Z67 in both aerobic and microaerobic conditions in HRP medium.

Significant contribution in plant growth promotion is shown by plant growth-promoting bacteria (Rodriguez and Fraga 1999; Richardson 2001). Many phosphate solubilising bacteria such as *Pseudomonas aeruginosa* ATCC 17933, *Klebsiella pneumoniae*, *Pseudomonas fluorescens* B161 and *Gluconobacter,* possess PQQ (Gliese et al., 2010; Meulenberg et al., 1995; Choi et al., 2008; Yang et al., 2010). Plant inoculation studies demonstrated the positive effect of *Hs* transformants on growth of rice plants. PSB are known to enhanced yield in rice and maize (Tiwari *et al.,* 1989; Pal, 1999). Inoculation of rice with *Hs* transformants *Hs* (pJNK5) and *Hs* (pJNK6) resulted in significant increased all growth parameters in rice including improved N and P status . Similar results of increase in leaves, roots and P status were observed in plants inoculated with PSM (Igual et al., 2001; Chen et al., 2006; Vyas and Gulati, 2009; Gulati et al., 2010). High amount of GA (up to ~ 87mM) is secreted by *Acinetobacter rhizosphaerae*

strain, enhanced maize plant growth when tricalcium phosphate was supplemented as compared to the plants supplemented with super phosphate (Gulati et al., 2010).

Increase in the P uptake and crop yield was found in wheat and sorghum when inoculated with *Rhizobium* with phosphate solubilizing bacteria (Belimov et al., 1995; Algawadi and Gaur, 1992; Rojas et al. 2001; Galal, 2003; Matias et al. 2009). *Rhizobium* sp. having PSM property increased P status and stimulated soybean growth (Qin et al., 2011). PSM stimulated plant growth and uptake of N, P, K and attributed in biological nitrogen fixation (Kannapiran et al., 2011). Nitrogen fixing *Azotobacter* and phosphate solublising *Bacillus megaterium* co-inoculation has enhanced N, P status of *Tectona grandis* and *Chukrasia tubularis* (Aditya et al., 2009). Co inoculation of of phosphate solubilizing bacteria and nitrogen fixing bacteria has stimulated plant growth (Lugtenberg and Kamilova 2009) Co-inoculation of *Bacillus* a potent PSM and nitrogen fixing *Rhizobium* sp has enhanced mung bean growth and P levels (Qureshi et al., 2011).

Significant increase in uptake of N, P and K status is cumulative effect of organic acid secretion for phosphate solubilization and nitrogen fixing ability of bacteria. Consortium of nitrogen fixer *Azotobacter*, phosphate solubilizer *Burkhorderia* and potassium solubilizer *Bacillus* has enhanced the N, P, K status and growth of vegetables and corn (Leaungvutiviroj et al., 2010)

Phosphate solubilizing endophytic bacteria increases in rhizospheric population which is restricted by biotic and abiotic factors. Rhizospheric bacteria do not get protection from biotic and abiotic stresses but endophytic bacteria could be protected due to its ability to colonize in side vascular tissues of plants (Duff et al., 1989; Sa and Israel, 1991). Endophytes are known to fix nitrogen from 30 up to 80 kg N/ha/year (Bieleski, 1973). Nitrogen fixation is limited by ATP, which correlates direct importance of soluble phosphate for the generation of ATP, the amount of energy essential for the complete nitrogen fixation is around 40 mol of ATP is required per mole of N_2. (Boddey et al. 1995; Hallmann et al. 1997; 1999; Fuentes et al. 1999; Kuklinsky et al. 2004; Shah and Emerich, 2006).

Our present study determines the effect of *Herbaspirillum seropedicae* Z67 containing phosphate solubilizing, potassium solubilizing and Nitrogen fixing ability on rice plants which could positively regulate the plant growth promotion.

Chapter 6

6.1: Introduction

Microorganisms colonize at rhizosphere and rhizoplane and use the nutrients secreted by plants (Hiltner, 1904). Some rhizobacteria promote plant growth and control plant pathogens. These are known as plant growth promoting rhizobacteria (PGPR). Atmospheric nitrogen can also be fixed by rhizobacteria, which is consequently used by the plants for growth and development. Rhizobacteria also show synergistic inhibition of plant pathogens (Schippers, 1992). PGPR include *Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia, Bacillus, Paenibacillus*, and some are members of the *Enterobacteriaceae*. There are various mechanisms involved in growth promotion such as nitrogen fixation, production of auxins, gibberellins, cytokinins, ethylene, solubilization of phosphorous, antibiotics production, lytic enzymes which helps to increases in root permeability, for the colonization which reduces competition for the available nutrients and root sites, which could enhance the uptake of essential plant nutrients etc. (Weyens et al., 2009). Plant diseases caused by plant pathogens such as fungi, bacteria, viruses, nematodes etc are controlled by PGPR (Saharan and Nehra, 2011). Blast and leaf blight diseases in rice (Oryza sativa) are caused by *Xanthomonas oryzae and Magnaporthe oryzae*, which are most destructive diseases (Haruhiko et al., 2013, Liu et al., 2013). *Rhizoctonia solani* causes sheath blight disease in rice and results in low crop yield (Sundravadana et al., 2007). Chemicals used to control this disease have not been very effective (Noda et al., 1999).

Heavy metal toxicity in soil poses a major challenge for sustainability of crop growth and yield (Gill et al., 2011). Cadmium (Cd) is one of the heavy metal which causes extremely high toxicity to plants (Mattioni et al., 1976; Adriano, 1986). Cd toxicity causes chlorosis and reduces plant growth (Choudhury and Panda, 2004). Cd toxicity to plants is mediated by generating reactive oxygen species (ROS) causing cell death (Davies and Cury, 1991; Molassiotis *et al*., 2006). Salinity and Cd toxicity is known to significantly reduce plant productivity of wheat, barley and rice (Balestrasse et al., 2004). Drought, salinity and cadmium stresses involve reactive oxygen species.

Organic acids produced by rhizobacteria can efficiently bind with heavy metals and release P from mineral phosphates for plant growth (Gyansehwar et al, 2002; Khan et al., 2009; Archana et al., 2012). Organic acids have metal chelating ability which leads to decrease oxidative stress against cadmium (Kavita et al., 2008). Plant growth promoting bacteria inoculation to crop seeds alleviated salt stress (Dodd and Perez-Alfocea, 2012). Rhizobacteria possess several traits that can alter heavy metals stress through the release of chelating substances, acidification and changes the redox potential (Leong, 1986; Smith and Read, 1997; McGrath et al., 2001; Whiting et al., 2001; Lasat, 2002). Bacterial glucose dehydrogenases (GDH) require pyrroloquinoline quinone (PQQ) as a redox cofactor for activity which determines phosphate solubilization (Goosen et al., 1989; Meulenberg et al., 1992; Goldstien 1985). PQQ is known to promote plant growth and reduce oxidative stress which could be attributed to its very high catalytic redoxing cycling efficiency of ~ 20,000 cycles (Choi et al., 2008; Rucker et al., 2009). Additionally, PQQ is involved in production of antimicrobial compounds (Schnider et al., 1995; Han et al., 2008; de Werra et al., 2009; Guo et al., 2009).

H. seropedicae Z67 is an endophytic nitrogen-fixing bacterium which colonizes roots of economically important crops such as rice, sugarcane, and wheat (Pedrosa et al., 2001; da Silva et al., 2003; Monteiro et al., 2003). *Herbaspirillum* sp. has been isolated from the interior of roots, stem, and leaves of many Graminaceous plants (Baldani et al., 1992; DSbereiner et al., 1994; Olivares et al., 1996). *Herbaspirillum* spp. has the pectinolytic and cellulolytic enzymes that are essential for the cell wall degradation of plant during infection (Elbeltagy et al., 2001; Gyaneshwar et al., 2002). *H. seropedicae* Z67 increased biomass and N content of rice plants equivalent to the treatment with 40 kg of N/ha (Pereira and Baldani 1995). Thus the present study determines the potential of *H. seropedicae* transformants secreting PQQ in protecting the rice seedlings against the Cd^{+2} exposure.

6.2: EXPERIMENTAL DESIGN

6.2.1: Bacterial strains used in this study

Table 6.1 : Bacterial strains used in this study

Plasmid/Strains	Characteristics	Source or Reference
Hs (pOK53)	35Kb plasmid contains 13.4-kb of *pqq* gene cluster of *P. fluorescens* B16 Tet^r (40µg/ml)	This study
Hs (pSS2)	25Kb plasmid contains 5.1-kb of *pqq* gene cluster of *A. calcoaceticus* Tet^r (40µg/ml)	This study
E. coli DH10B	Host strain for routine DNA manipulation experiments and plasmid maintenance. $Strp^r$ (50µg/ml)	Invitrogen,USA
E. coli DH10B	pUC18*gfp* Amp^r (100 µg/ml)	This study
pJNK2	pBBR1MCS2, Km^r (50 µg/ml) with 13.4 Kb *pqq* gene cluster of *Pseudomonas fluorescens* B16.	This study
pJNK5	pUCPM18, Gm^r (20µg/ml) with 5.1 Kb *pqq* gene cluster of *Acinetobacter calcoaceticus*.	This study
pJNK6	pJNK5, Gm^r (20µg/ml) with *gad* operon 3.8 Kb of *Pseudomonas putida* KT 2440	This study
Hs	Nitrogen fixing rice endophyte , Nal^r(10µg/ml)	Baldani et al., 1986
Hs (pUCPM18)Gm^r	*H. seropedicae* Z67 Nal^r (10µg/ml) with (pUCPM18)Gm^r (20µg/ml)	This study
Hs (pJNK1)	*H. seropedicae* Z67 Nal^r(10µg/ml) with pBBR1MCS2, Km^r (50µg/ml)	This study
Hs (pJNK5)	*H. seropedicae* Z67 Nal^r(10µg/ml) with pJNK5Gm^r (20µg/ml)	This study
Hs (pJNK6)	*H. seropedicae* Z67 Nal^r(20µg/ml) with pJNK6Gm^r (20µg/ml)	This study
Enterobacter asburiae PSI3		Gyaneshwar et al., 1998

6.2.2: Bacterial strains, plasmids, Media, and culture conditions.

Bacterial strains and plasmids used for this work have been listed in Table **1.** DNA manipulation and molecular biology experiments were performed using *E. coli* DH10B as the host strain using standard protocols (Sambrook and Russell, 2001).

Plasmid bearing derivatives of *E. coli* DH10B were routinely grown at 37^0C on Luria Bertani broth (L.B) and maintained on Luria Bertani Agar (L.A) plate with 40 μg ml^{-1} streptomycin. *H. seropedicae* Z67 was routinely grown at 30^0C on Luria Bertani (L.B) and maintained on semisolid JNFb medium pH 5.8 plates containing 10 μg ml^{-1}Nalidixic acid at 30°C (Baldani et al., 1986). For nitrogen fixation, *H. seropedicae* Z67 JNFb medium was devoid of a nitrogen source. Further, plasmids pUCPM18Gm^r as vector control, pJNK1, pSS2, pOK53, pJNK5 and pJNK6 were transformed by electroporation in *H. seropedicae* Z67 as described by Unge et al. (1998). Fungal and bacterial pathogens were maintained on potato dextrose agar (PDA) plate.

6.2.3: Antibacterial and antifungal activity of *Hs* transformants

Antibacterial and antifungal agar spot assay test was carried out by using method described by Murugalatha and Mohankumar (2011). Fresh culture of *Hs* Z67 transformants and obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30ºC was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed thrice with sterile normal saline and used to determine antibacterial and antifungal activity against pathogenic bacteria and fungus, pathogens used in this study are (i) Pathogenic bacteria – *Serratia, Shingella, Salmonela typhimurium* and *Xhanthomonas oryzae* (rice plant pathogen), and (ii) Pathogenic fungi - *Fusorium oxosporium, Botrytis cinerea, Rhizoctonia solani* (rice plant pathogen) and *Magnaporthe gresia* (rice plant pathogen). The fungal pathogens *Fusorium oxosporium* and *Botrytis* were generous gift from Dr Arun Arya, Department of Botany, M. S. Univerity of Baroda, Baroda while *Magnaporthe gresia* and *Xhanthomonas oryzae* were obtained from Indian Type Culture Collection, New Delhi. All pathogenic bacteria were placed on potato dextrose agar (PDA) plate for 5 min to get dry, *Hs* transformants saline washed culture was spotted on in uniform concentration on plates, and plates were kept for drying and placed at 30^o C for 24 h. In case of antifungal activity, fungus was placed at center of PDA plate and allowed to grow on plate for 36 h, saline washed *Hs* transformant culture was spotted on plates, plates were kept for drying and placed at 30^o C for 3-4 days.

6.3: Results:

6.3.1: PQQ secretion by *H. seropedicae* Z67 tranformants

The pOK53 plasmid containing *pqq* gene cluster of *P. fluorescens* B16, pSS2 plasmid containing *pqq* gene cluster of *A. calcoaceticus* and *pqq E* gene of *E. herbicola* were transformed in *H. seropedicae* by electroporation. *H. seropedicae* Z67 tranformants *Hs* (pOK53) and *Hs* (pSS2) had shown fluorescence property which was equivalent to *E. coli* containing pUC18*gfp* (**Fig 6.1**). Fluorescence was seen on normal Luria agar plate as well as on JNFb medium agar plate. *H. seropedicace* Z67 and pBBR1MCS2Km^r transformant did not show any fluorescence. On the other hand, fluorescence was also not seen in *pqqE* transformant.

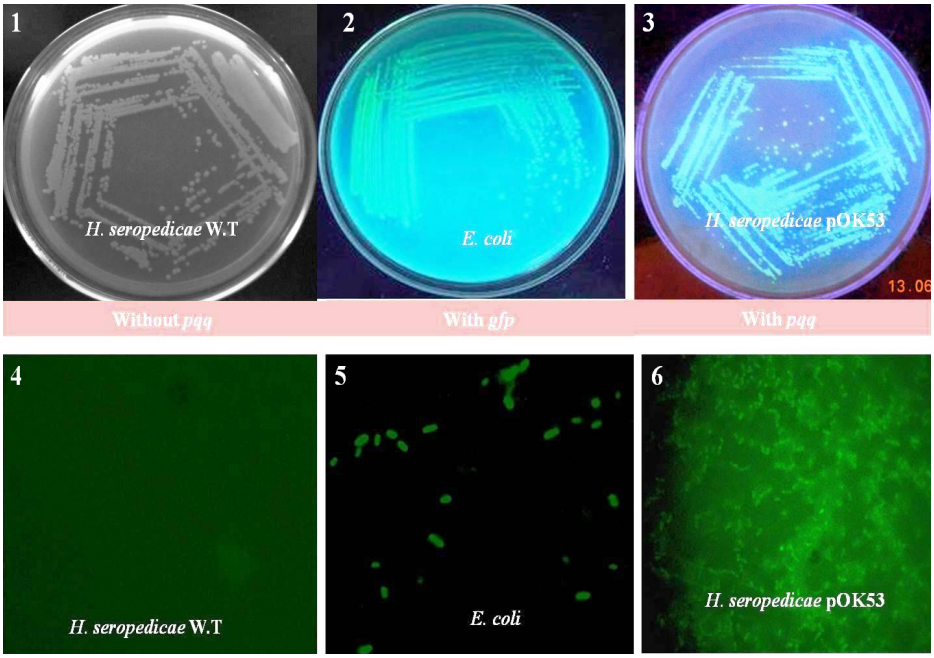


Fig. 6.1: PQQ fluorescence of *E. coli* and *H. seropedicae* Z67 transformants containing *pqq* genes. 1, 2 and 3- culture plates under UV light, 4, 5 and 6 cells under fluorescence microscopy. 1, 4- *Hs* native strain. 2, 5- *E. coli* with pUC18*gfp*. 3, 6- *Hs* (pOK53).

6.3.2: Antibacterial activity of *H. seropedicae* transformants secreting PQQ.

H. seropedicae Z67 harboring *pqq* gene clusters were used for antimicrobial activity against pathogenic bacteria. *H. seropedicae Z67* containing *pqq* gene clusters transformants have shown antimicrobial activity against *Serratia, Shingella, S. typhimurium* and *X. oryzae,* while *H. seropedicae* containing *pqqE* has not shown zone of inhibition against any pathogenic bacteria (**Fig. 6.2**). *E. asburiae* PSI3 has shown antibacterial activity against *X. oryzae*.

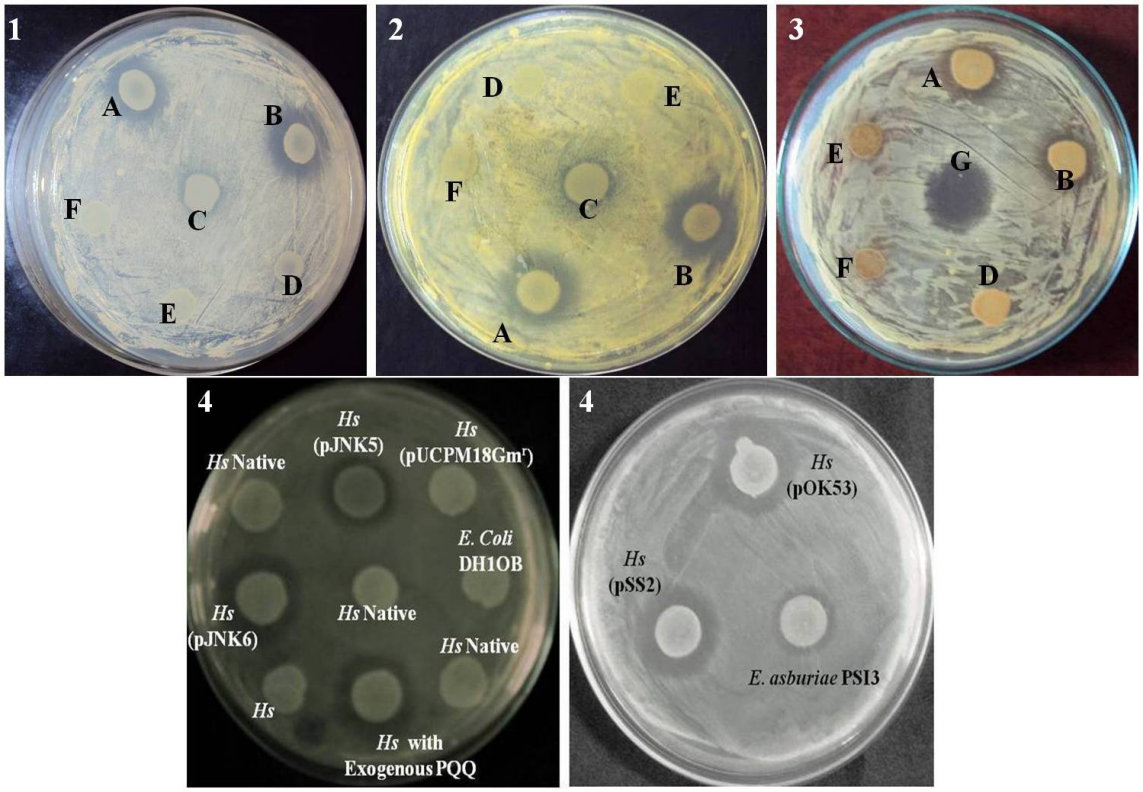


Fig. 6.2: Antibacterial activity of *H. seropedicae* Z67 containing *pqq* genes. 1- *Serratia*; 2- *Shingella* ; 3- *Salmonela typhimurium* and 4 - *Xhanthomonas oryzae*. A- *Hs* (pOK53), B- *Hs* (pSS2), C- supernatant of culture A, D- *Hs* native, E- *Hs* (pJNK1), F- *Hs* (pBBR1MCS2Km^r), G- Concentrated extract from culture A.

6.3.3: Antifungal activity

H. seropedicae Z67 containing *pqq* gene clusters showed antifungal activity against *F. oxosporium, B. cinerea, R. solani* and *M. gresia* (**Fig. 6.3**). In contrast to

antibacterial activity, *pqqE* has shown zone of inhibition against pathogenic fungi. *H. seropedicae Z67* native culture was unable to show antifungal activity.

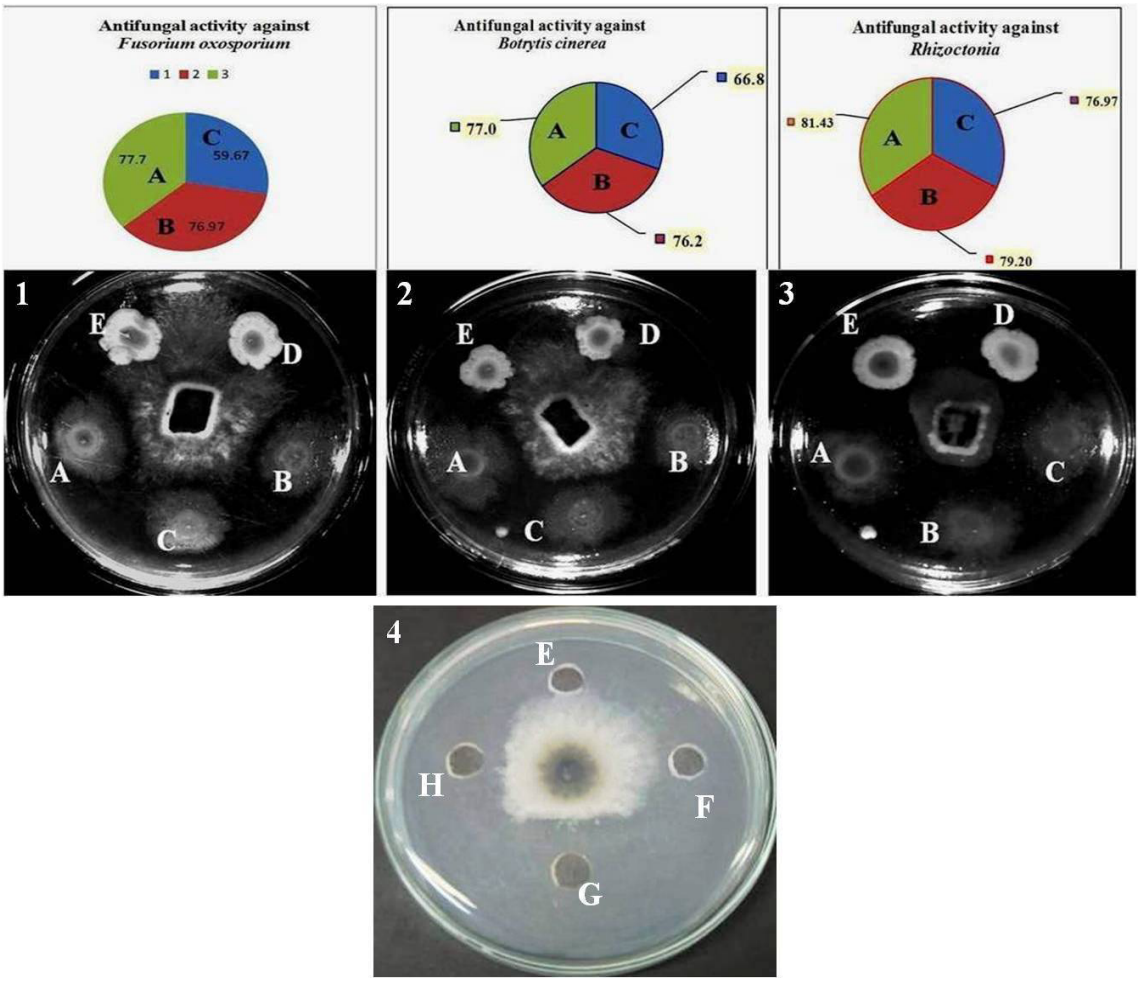


Fig. 6.3: Antifungal activity of *H. seropedicae* Z67 containing *pqq* gene cluster. 1- *Fusorium oxosporium*, 2- *Botrytis cenerea*, 3- *Rhizoctonia solani*, 4- *Magnaporthe gresia* A- *Hs* (pOK53), B- *Hs* (pSS2), C- *Hs* (pJNK1), D- *Hs* (pBBR1MCS2Kan^r), E- *Hs* native , F- *Hs* (pUVPM18Gm^r), G- *Hs* (pJNK5) and H- *Hs* (pJNK6).

6.3.4: Effect of *H. seropedicae* Z67 transformants *Hs* (pJNK5) and *Hs* (pJNK6) on cadmium tolerance.

Hs native and *Hs* (pUCPM18) did not secrete PQQ while *Hs* (pJNK5) and *Hs* (pJNK6) secreted ~1.15 µM and ~1.17 µM PQQ, respectively, in M9 minimal medium. Since PQQ is known to confer tolerance to Cd, *Hs* transformants were monitored for

minimum inhibitory and tolerance levels. *Hs* native and *Hs* (pUCPM18) were able to tolerate 40 µM of $CdCl_2$ minimum inhibitory concentration (MIC). However, *Hs* (pJNK5) and *Hs* (pJNK6) was able to grow and tolerate up to 100 µM $CdCl_2$**.**

6.3.5: *H. seropedicae* Z67 *pqq* transformants (pJNK5) and (pJNK6) effect on growth of rice seedling against cadmium stress.

Under hydroponic condition, rice seedling treated with 10 µM cadmium showed growth inhibition on root and shoot length up to ~80% and ~70% by respectively **(Fig. 6.4)**. *Hs* native and *Hs* (pUCPM18Gm^r) reduced inhibition ~35% when compared with independent Cd treated seedlings, while *Hs* (pJNK5) and *Hs* (pJNK6) showed growth promotion of rice seedlings by reducing stress up to ~55% and ~65%, respectively. On the other hand, PQQ treated seedlings with 10 µM cadmium had root and shoot length of rice similar to that of untreated seedlings **(Fig. 6.5).**

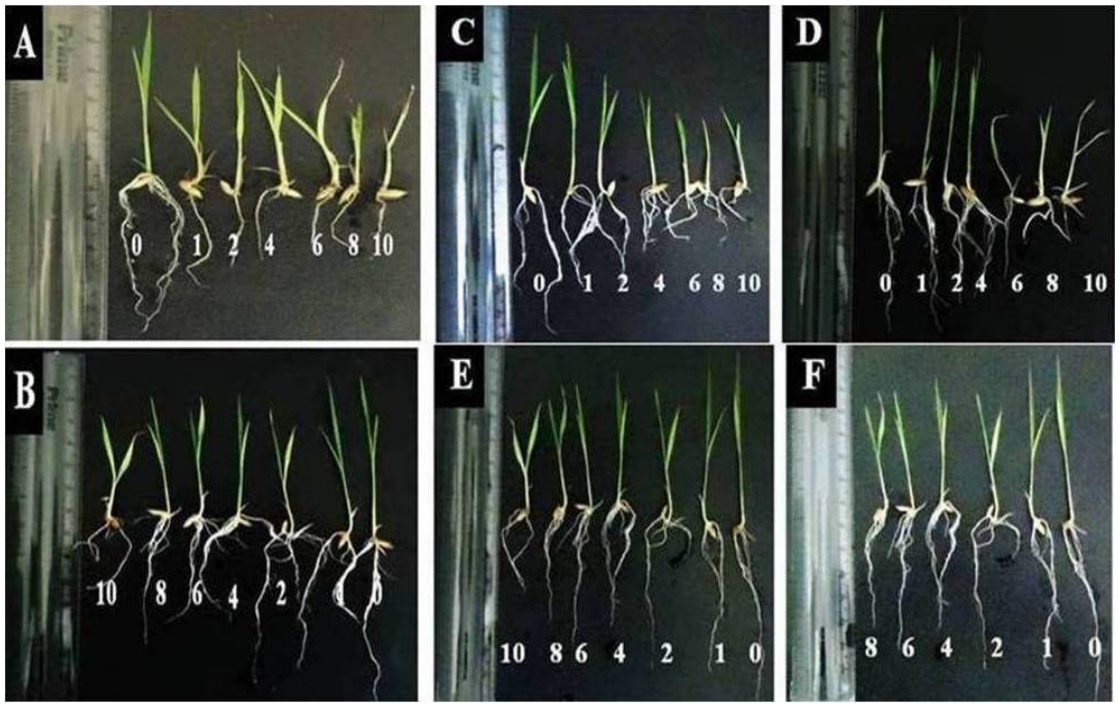


Fig. 6.4 : Effect of *Hs* transformants on rice seedling treated with cadmium. A-$CdCl_2$, B- $CdCl_2$ with PQQ, C- *Hs* with $CdCl_2$, D- *Hs* (pUCPM18Gm^r) with $CdCl_2$, E- *Hs* (pJNK5) with $CdCl_2$, F- *Hs* (pJNK6) with $CdCl_2$; Concentration used ($CdCl_2$- 10 µM, PQQ- 100 nM).

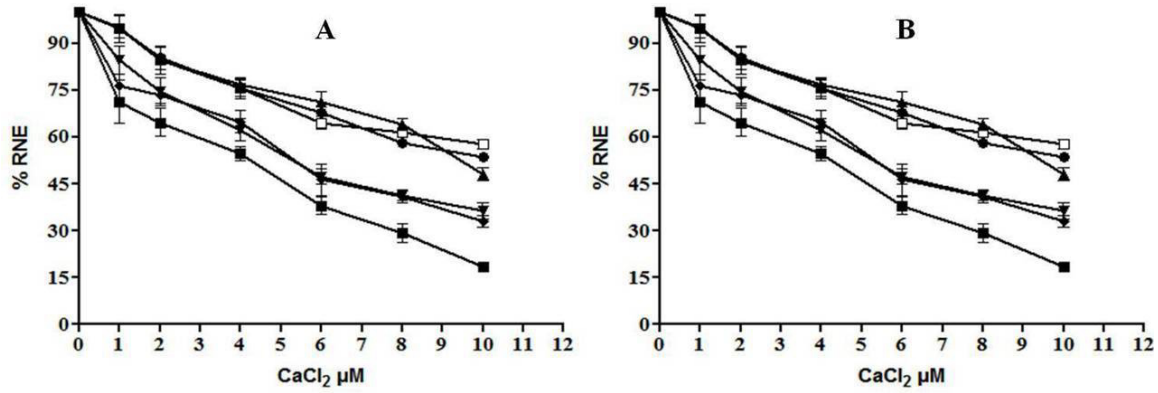


Fig.6.5: Relative net elongation (%) of root and shoot of rice seedling (RNE). (A, B- $CaCl_2$ treated, A-% RNE of root; B-% RNE of shoot C- % RNE of root and shoot, NaCl treated). **(A, B)** ■- $CaCl_2$; ▲- Exogenous PQQ; ▼- *Hs* (wild type); ◆- *Hs* (pUCPM18Gm^r); ●- *Hs* (pJNK5) ; □- *Hs* (pJNK6).

6.3.6: Effect *H. seropedicae* transformants (pJNK5) and (pJNK6) on the antioxidant enzyme activity of rice seedlings exposed to cadmium.

Cadmium treated rice seedlings exhibited a significant change in the activities of CAT and SOD. CAT activity of control seedlings was 18 U and ~17 U while seedling treated with 10 µM cadmium showed ~3.4 folds increase in activity when compared with control plants without cadmium. Hs native and Hs (pUCPM18Gm^r) showed similar ~44 U of CAT and SOD activity. On the other hand Hs (pJNK5) and Hs (pJNK6) significantly reduced CAT and SOD activities by ~3 folds **(Fig. 6. 6)**. Similarly, PQQ treated plants showed significant decrease in CAT and SOD activity by ~ 3 folds in presence of 10 µM cadmium.

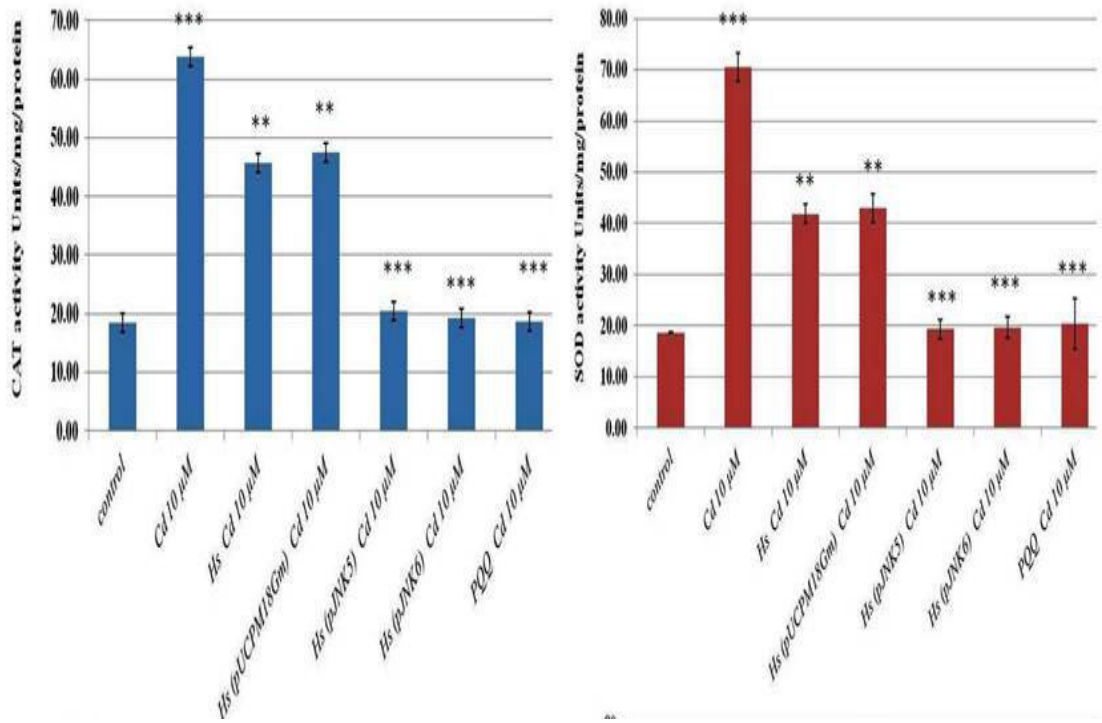


Fig. 6.6: SOD and CAT enzyme activities of rice seedlings inoculated with *H. seropedicae* Z67 transformants.

6.4: Discussion

Mineral phosphate solubilization by microorganisms is mediated by organic acids (Archana et al., 2012). Phosphate solubilising bacteria produce PQQ as cofactor for GDH which catalyzes gluconic acid production. In present study our results demonstrated the overexpression of *pqq* gene cluster and *gad* operon in *H. seropedicae* Z67 enhanced phosphate solubilization and secreted PQQ in medium. Plant inoculation studies showed that the transformants transported PQQ to plant tissues (C**hapter 5**). Present study demonstrated that *H. seropedicae* Z67 containing *pqq* gene cluster has shown fluorescence property equivalent to fluorescence of *E. coli* containing *egfp* gene on pUC18 which is a high copy plasmid. PQQ is known to show good fluorescence (Duine and Olsthoorn., 1998). Hence, overexpression of *pqq* genes in PGPR could also help in monitoring the presence of the bacteria in plant tissues and in the rhizosphere.

Hs containing *pqq* gene clusters has shown antibacterial and antifungal activity against plant pathogens including rice pathogens *X. oryzae* and M. *gresia*. However, PQQ alone does not show any antibacterial and antifungal activity. Hs (pJNK6) showed lesser antibacterial and antifungal activity as compared with Hs (pJNK5). This is contrast to earlier studies which showed mutation in *pqq* genes increased the biosynthesis of biocontrol compound (pyoluteorin) (Schnider et al., 1995). On the other hand, over expression of *pqq* genes of *S. marcescens* in *Escherichia coli* has inhibited fungal growth of *M. gresia* (Kim et al., 2006). In *Rahnella aquatilis,* mutation in *gdh* or *pqq* resulted in loss of antibacterial compound formation (Guo et al., 2009). An antibacterial compound ABS has also been purified from this bacteria (Chen et al., 2009). Mutation in *pqqA* and *pqqB* genes of *Enterobacter intermedium* has lost antifungal activity against *M. gresia* (Han et al., 2008). PQQ–GDH are involved in production of antipathogen compounds (James and Gutterson 1986; Schnider et al. 1995; Han et al. 2008; de Werra et al. 2009; Guo et al. 2009; and Ahmed and Shahab, 2010). Gluconic acid is also known to directly inhibit pathogenic fungi (Kaur et al., 2006). Antibacterial and antifungal activities of *H. seropedicae* Z67 transformants could be mediated by compounds which require gluconic acid as ABS compound of *R. aquatilis* contains uncharacterized sugar moiety (Chen et al., 2009). This also supported by the fact that Hs (pJNK6) showed lesser antibacterial activity as compared to Hs(pJNK5) which secrete 3mM and 22mM gluconic acid, respectively.

Cd significantly inhibits the growth of roots and earlier studies showed that chelation of Cd by organic acids including gluconic acid decrease the oxidative damage (Kavita et al., 2008). *Enterobacter asburiae* PSI3 secreting high levels of gluconic acid, decreased oxidative damage and improved the root growth of mung bean seedlings in presence of Cd. Cd caused oxidative damage of rice seedlings by altering the activities of CAT and SOD leading to increased generation of ROS as reported in many other studies (Mittler et al., 2004; Fariduddin et al., 2009; Verma et al., 2011). Our result showed that overexpression of *pqq* gene cluster and gad operon in *H. seropedicae* Z67 has reduced stress of rice seedlings and could tolerate 100 µM Cd while uninoculated rice plants could only tolerate up to 10 µM Cd. *H. seropedicae* Z67 transformants (pJNK5) and

(pJNK6) showed similar extent of Cd tolerance but secrete gluconic and and 2-ketogluconic acids at different levels suggest that the tolerance could be mediated by PQQ. This is supported by the fact PQQ is very efficient antioxidant with a redox cycling of 20,000 (Rucker, 2009). PQQ as antioxidant plays an important role in DNA repair and reduces oxidative stress (Misra et al., 2004; Misra et al., 2010; Choi et al., 2008). PQQ has role in protection of cells against γ radiation, which protects cells from oxidative stress (Khairnar et al., 2003). In mammals, PQQ has shown to protect mitochondria from oxidative stress (Nukada et al., 2003). PQQ neutralizes reactive species by scavenging reactive oxygen species (ROS) (Klinman and Mu, 1994; McIntire, 1998; Morris et al., 1994; Misra et al., 2004). Additionally, PQQ supplementation to mung bean seedlings resulted in growth promotion (Shahab and Ahmed, 2011). Thus, *H. seropedicae* Z67 transformants secreting strong 2-ketogluconic acid along with PQQ can confer multiple benefits to rice plants by improving phosphate solubilization, growth promotion of PQQ, tolerance to Cd exposure and protection against bacterial as well as fungal pathogens.

ROS scavenging resulted in decrease of CAT and SOD activity whereas Cd treated rice seedlings showed increase in SOD and CAT which are important antioxidant enzymes for ROS scavenging (Miller et al., 2008). Similarly, increase in SOD and CAT activity during stress condition have been reported (Scebba et al., 2006; Mobin and Khan, 2007; Zhang et al., 2009). Our results demonstrated that PQQ has normalized CAT and SOD activity and protected rice seedling from Cd when compared with control plants, it indicates the PQQ showed antioxidant property against Cd toxicity. Thus, *H. seropedicae* Z67 secreting PQQ and 2-ketogluconic acid could benefit rice plants in multiple ways by improving the P status, N status and Cd tolerance which in turn significantly enhance the efficacy of the bacteria in field conditions.

Summary

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

Organic acids secreted by PSMs play a major role in P solubilization which chelate mineral ions and drop pH. Most of the PSMs secrete gluconic acid or 2-ketogluconic acid in the periplasm of Gram negative bacteria *via* direct oxidation pathway. In direct oxidation pathway glucose gets converted into gluconic acid by glucose dehydrogenase (GDH) while gluconate converted into 2-ketogluconic acid by gluconate dehydrogenase (GADH) enzymes. However, in plant rhizosphere where PSBs colonize, glucose might not available in sufficient amount to solubilize bound P. Root exudates are major carbon and energy sources for rhizobacteria and are known to contain high amount of sucrose and fructose along with small molecular weight organic acids. PSMs ability to use multiple substrates for organic acid production will be more effective in field conditions. Most of the rhizobacteria produce gluconic acid is required in high

amount but 2-ketogluconic acid is stronger acid than gluconic acid. Most of the PSMs do not possess GAD required for the conversion of gluconic acid into 2-ketogluconic acid. Rhizobacteria having the ability to use multiple carbon sources along with 2-ketogluconic acid secretion may perform better in field condition.

Herbaspirillum seropedicae Z67, nitrogen fixing endophyte, significantly promotes the growth of cereals and economically important crops. *Herbaspirillum* does not secrete organic acids; therefore this bacterium was genetically modified for the secretion of organic acids to solubilize mineral phosphate. Organic acids determine the extent of phosphate solubilization and citrate synthase (CS) activity is a key enzyme of tricarboxylic acid cycle. Plasmids pAB7, pJNK3 and pJNK4 containing *E. coli cs*, NADH insensitive *cs* (*cs**) and *cs** along with *Salmonella typhimurium* Na^+ dependent citrate transporter (*citC*) genes were constructed under constitutive *lac* promoter in broad host range plasmid pUCPM18-Km^r. The plasmid transformants of *H. seropedicae* Z67 were obtained by electroporation. *H. seropedicae* 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. Upon inoculation to rice plants (Gujarat – 17), increase of Fresh weight, Dry weight N, P and K content was observed.

H. seropedicae Z67, an endophytic diazotroph of cereals, encodes glucose dehydrogenase (GDH) apoprotein but does not possess pyrroloquinoline quinone (*pqq*) genes required for its activity. *pqqE* of *Erwinia herbicola* (pJNK1), two different *pqq* gene clusters of *Pseudomonas fluorescens* B16 (pOK53) and *Acinetobacter calcoaceticus* (pSS2) were over expressed in *H. seropedicae* Z67. Transformants (pJNK1, pSS2 and pOK53) secreted 0.011 µM, 1.1 µM and 2.55 µM PQQ, respectively. GDH activity in the *Hs* (pJNK1) was 14.98 U GDH while *Hs* (pSS2) and *Hs* (pOK53) 87.50 U and 86.34 U, respectively, and secreted *Hs* (pJNK1) 4.26 mM, *Hs* (pSS2) 32.00 mM and *Hs* (pOK53)

33.46 mM of gluconic acid. Transformants of *pqqE* (pJNK1) did not show P solubilization phenotype, while *Hs* (pSS2) and *Hs* (pOK53) transformants showed P solubilization phenotype on HEPES agar plate and released 125.47 µM and 168.07 µM P, respectively, in minimal medium containing 50 mM glucose under aerobic condition. *Hs* (pSS2) and *Hs* (pOK53) could also solubilise rock phosphate in minimal medium containing in a mixture of 25 mM glucose and 25mM xylose. Under N free HRP minimal medium, *Hs* (pSS2) and *Hs* (pOK53) released P up to 130.19 µM and 172.04 µM from rock phosphate. Additionally, *H. seropedicae* Z67 *pqq* gene cluster transformants had enhanced growth in nitrogen free medium, biofilm formation, exopolysaccharide (EPS) secretion while IAA production was inhibited. Thus, *pqq* gene cluster but not *pqqE* is necessary for phosphate solubilization and *Hs* transformants can solubilize rock phosphate even under nitrogen fixing conditions.

Pyrroloquinoline quinone (PQQ) and 2-ketogluconic acid determine mineral phosphate solubilization in bacteria. *H. seropedicae* Z67 significantly improves N status of cereals. Two plasmids containing 5.1 kb *pqq* gene cluster of *Acinetobacter calcoaceticus* alone (pJNK5) and with *Pseudomonas putida* KT2440 *gad* operon (pJNK6) were constructed in pUCPM18Gm^r under *Plac* promoter and transformed in *H. seropedicae* Z67. *Hs* (pJNK5) and *Hs* (pJNK6) secreted ~1.15 µM and ~1.17 µM PQQ in medium, had ~221.66 U and ~234.33 U GDH activity, respectively while *Hs* (pJNK6) showed ~414.00 U of GADH activity. *Hs* (pJNK5) and *Hs* (pJNK6) secreted ~23.47mM and ~3.79 mM of gluconic acid, respectively, while *Hs* (pJNK6) ~15.83 mM of 2-ketogluconic acid. Under aerobic condition, *Hs* (pJNK5) and *Hs* (pJNK6) solubilized ~239.66 µM and ~ 457.66 µM P on HEPES rock phosphate and, 76.66 µM and 222.66 µM K HRPF (feldspar), respectively, minimal medium containing 50 mM glucose. Under N free HRP and HRPF minimal medium, similar results of P and K solubilization were obtained. These transformants also showed increase in Fresh weight, Dry weight N, P, K content upon inoculation to rice plants (Gujarat – 17). Rice plants contained ~0.73 ng/g PQQ levels. Thus, these genetic modifications can significantly enhance the efficacy of *H. seropedicae* Z67 in promoting the growth of rice plants in field conditions.

The pOK53 plasmid containing *pqq* gene cluster of *P. fluorescens* B16, pSS2 plasmid containing *pqq* gene cluster of *A. calcoaceticus* and *pqq E* gene of *Erwinia herbicola* were transformed in *H. seropedicae* Z67 by electroporation,. Native *H. seropedicace* Z67 did not show any fluorescence, while *Hs* (pOK53) and *Hs* (pSS2) had showed good fluorescence equivalent to *E. coli* containing pUC18*gfp*. The fluorescence was attributed to ~1.15 µM and ~1.17 µM amount of PQQ secreted by *Hs* transformants (pJNK5) and *Hs* (pJNK6), respectively, in M9 minimal medium.

H. seropedicae Z67 does not produce any antibacterial compounds. However, *H. seropedicae* Z67 harboring *pqq* gene clusters demonstrated antimicrobial activity against pathogenic bacteria and fungi. Antifungal activity was found against *Fusorium oxosporium, Botrytis cinerea, Magnaporthe grisea* and *Rhizoctonia solani.* In contrast to antibacterial activity, *pqqE* has shown zone of inhibition against pathogenic fungi. *H. seropedicae Z67* containing *pqq* gene clusters and extract from the transformants showed antimicrobial activity against *Xanthomonas oryzae, Serratia, Shingella* and *Salmonela typhimurium* while *H. seropedicae* containing *pqqE* has not shown any zone of inhibition against any pathogenic bacteria, intrestingely wild type *Enterobacter asburiae* PSI3 is PQQ producer, inhibited Pathogenic *Xhanthomonas* growth, while mix inoculum of *H. seropedicae Z67* and *E. asburiae* PSI3 showed antibacterial activity.

PQQ is known to confer tolerance to Cd and *Hs* transformants secreting PQQ, *Hs* (pJNK5) and *Hs* (pJNK6), could grow and tolerate $CdCl_2$ up to 100 µM while native strain could tolerate only 40 µM $CdCl_2$**.** Under hydroponic condition, rice seedling treated with 10 µM Cd showed growth inhibition on root and shoot length by ~70% and ~80%, respectively, when compared with control seedlings while PQQ treated seedlings with 10 µM Cd had root and shoot length of rice similar to that of untreated seedlings. Cd treated rice seedlings exhibited a significant change in the activities of CAT and SOD. CAT activity of control seedlings was 18 U while seedling treated with 10 µM Cd. When PQQ supplemented with 10 µM Cd, SOD activity was significantly reduced by ~2.3 folds as compared to 10 µM Cd treated seedlings.

In conclusion, present study demonstrated incorporation of PQQ and 2-ketogluconic acid secretion into *H. seropedicae* Z67. Native apoprotein of glucose

dehydrogenase (GDH) enzyme present in *H. seropedicae* Z67 was functionally active and sufficient for high gluconic acid synthesis. 2-ketogluconic acid was secreted up to 15 mM which was sufficient for solubilizing rock phosphate and feldspar in buffered medium. The PQQ secreted by the transformants enabled to improve Cd tolerance to rice plants by decrease the oxidative damage. Thus, *H. seropedicae* Z67 transformants developed in this study could be useful in helping the rice plants by increasing P, N and K status, conferring Cd tolerance and protect against plant bacterial and fungal pathogens which may significantly enhance the efficacy of this bacteria in field conditions.

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Heterologous expression of pyrroloquinoline quinone (*pqq*) gene cluster confers mineral phosphate solubilization ability to *Herbaspirillum seropedicae* Z67

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Abstract Gluconic acid secretion mediated by the direct oxidation of glucose by pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) is responsible for mineral phosphate solubilization in Gram-negative bacteria. *Herbaspirillum seropedicae* Z67 (ATCC 35892) genome encodes GDH apoprotein but lacks genes for the biosynthesis of its cofactor PQQ. In this study, *pqqE* of *Erwinia herbicola* (in plasmid pJNK1) and *pqq* gene clusters of *Pseudomonas fluorescens* B16 (pOK53) and *Acinetobacter calcoaceticus* (pSS2) were over-expressed in *H. seropedicae* Z67. Transformants *Hs* (pSS2) and *Hs* (pOK53) secreted micromolar levels of PQQ and attained high GDH activity leading to secretion of 33.46 mM gluconic acid when grown on 50 mM glucose while *Hs* (pJNK1) was ineffective. *Hs* (pJNK1) failed to solubilize rock phosphate, while *Hs* (pSS2) and *Hs* (pOK53) liberated 125.47 μM and 168.07 μM P, respectively, in minimal medium containing 50 mM glucose under aerobic conditions. Moreover, under N-free minimal medium, *Hs* (pSS2) and *Hs* (pOK53) not only released significant P but also showed enhanced growth, biofilm formation, and exopolysaccharide (EPS) secretion. However, indole acetic acid (IAA) production was suppressed. Thus, the addition of the *pqq* gene cluster, but not *pqqE* alone, is sufficient for engineering phosphate solubilization in *H. seropedicae* Z67 without compromising growth under nitrogen-fixing conditions.

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Introduction

Herbaspirillum seropedicae is an agronomically important endophytic, nitrogen-fixing bacterium which colonizes roots, stem, and leaves of many graminaceous plants including economically important crops such as rice, sugarcane, and wheat (Pedrosa et al. 2001; da Silva et al. 2003; Monteiro et al. 2008). In a greenhouse experiment, *H. seropedicae* increased N content of rice plants up to 30 % (James et al. 1997; Baldani et al. 2000) and is thus considered important for increasing plant yields by providing nitrogen sufficiency. However, in field conditions, nitrogen fixation is strongly constrained by the availability of phosphorus (P; Olivera et al. 2004; Schulze et al. 2006). Co-inoculation of nitrogen-fixing bacteria with phosphate-solubilizing microorganisms (PSMs) has been shown to increase growth and N status of plants (Gull et al. 2004; Elkoca et al. 2007; Valverde et al. 2007; Kumar and Chandra 2008). Certain strains of nitrogen-fixing bacteria solubilize phosphate (Abril et al. 2007) and their inoculation has been demonstrated to increase the growth of maize and lettuce (Chabot et al. 1996). Phosphate-solubilizing, nitrogen-fixing *Mesorhizobium mediterraneum* increased growth and N uptake in chick pea and barley (Peix et al. 2001) indicating the importance of P solublization in maximizing the benefits of N-fixing bacteria.

H. seropedicae Z67 does not show mineral phosphate solubilization, and this could limit the growth benefit to plants colonized by this bacterium (Hayat et al. 2010). Most phosphate-solubilizing microorganisms release P by the secretion of low molecular weight organic acids such as citric