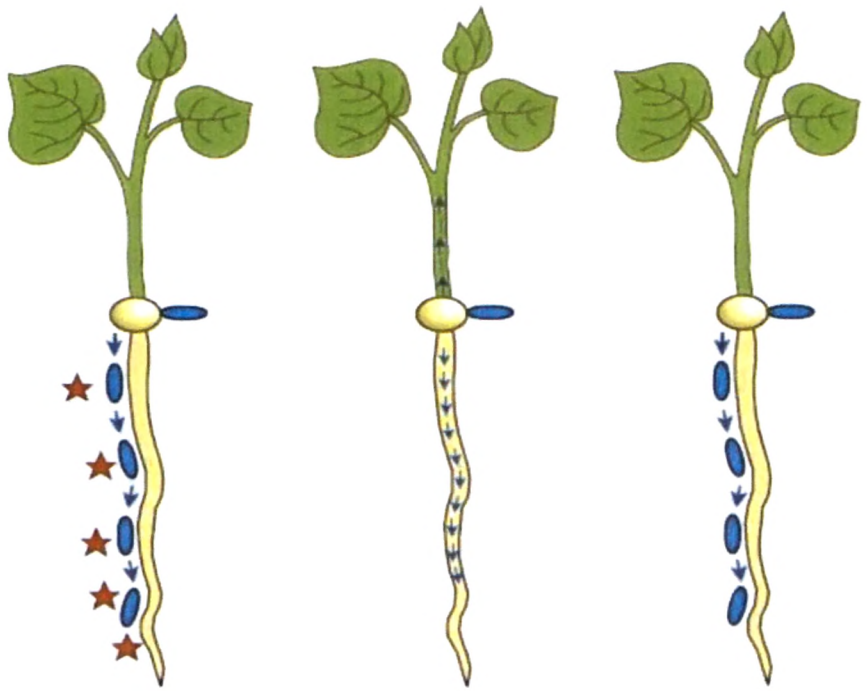
Chapter 5



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

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, expression 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.

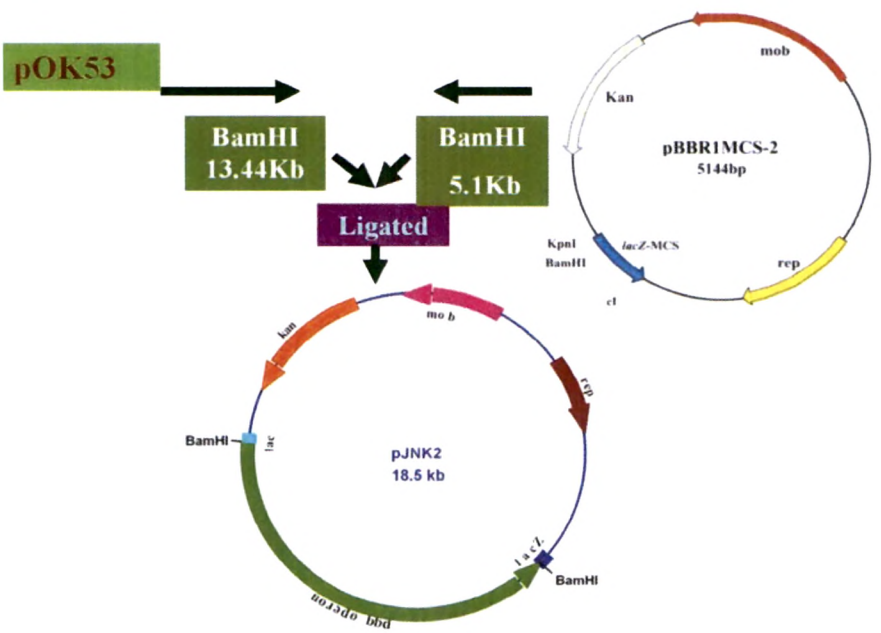


Fig. 5.1: Schematic representation of construction pJNK2 stable vector containing *pqq* gene cluster (13.4Kb) of *P. fluorescens* B16 under *lac* promoter.

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

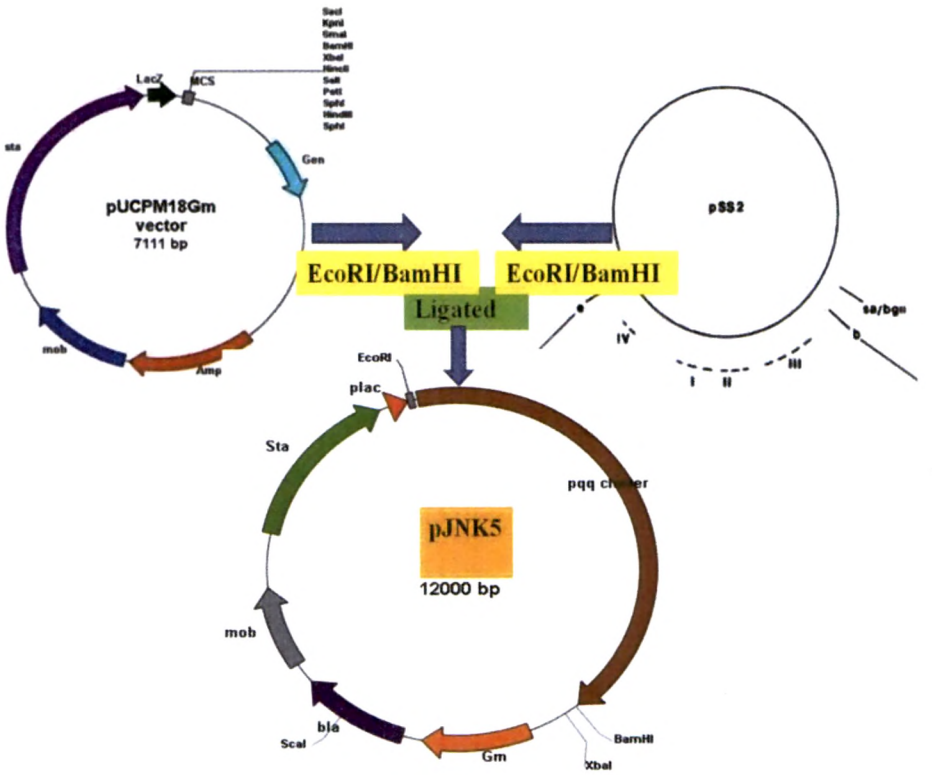


Fig. 5.2: Schematic representation of construction pJNK5 stable vector containing *pqq* gene cluster (5.1Kb) of *A. calcoaceticus* under *lac* promoter.

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

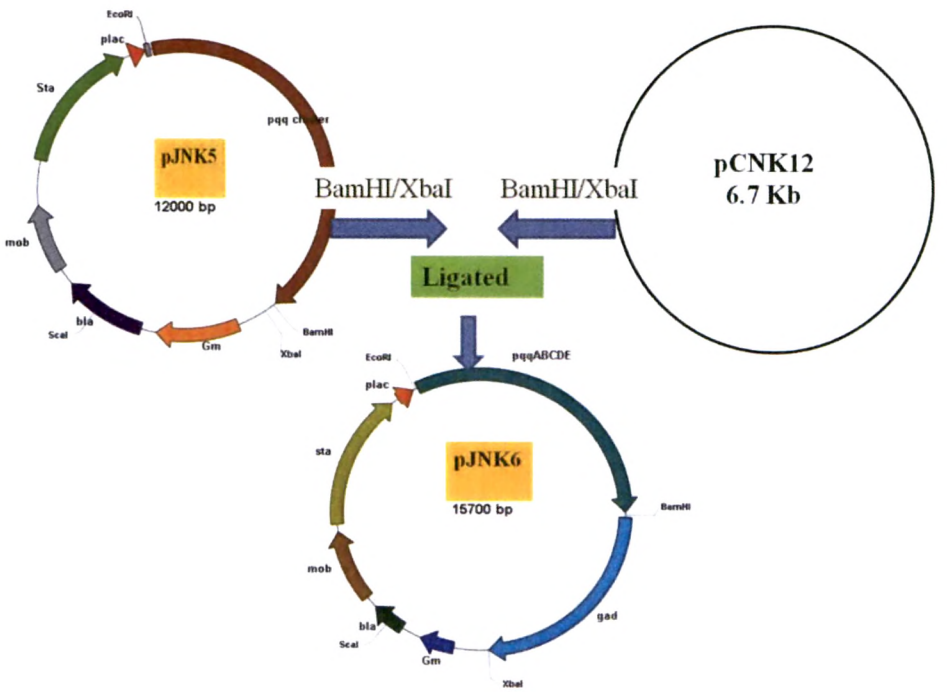


Fig. 5.3: Schematic representation of construction of pJNK6 stable vectors containing *P. putida* KT 2440 *gad* operon under *lac* promoter.

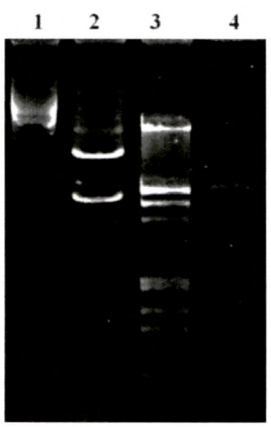


Fig. 5.4: Lane 1- pJNK2 plasmid undigested, Lane 2- pBBR1MCS2 digested by BamHI. Lane 3- Marker EcoRI/HindIII

Fig. 5.4: Restriction digestion pattern for pJNK2.

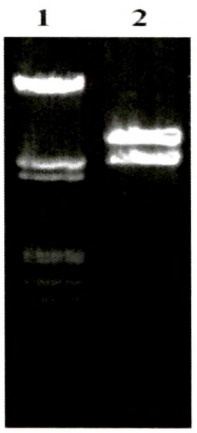


Fig. 5.5: Lane 1- Marker EcoRI/HindIII. Lane 2- pJNK5 digested by EcoRI/BamHI

Fig. 5.5: Restriction digestion pattern for pJNK5.

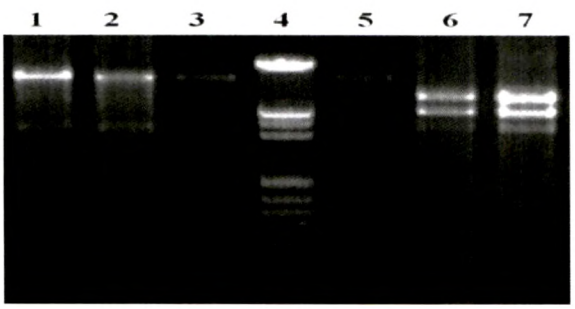


Fig. 5.6: Lane 1, 2, 3 and 5- pJNK6 digested by BamHI/XbaI

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).**

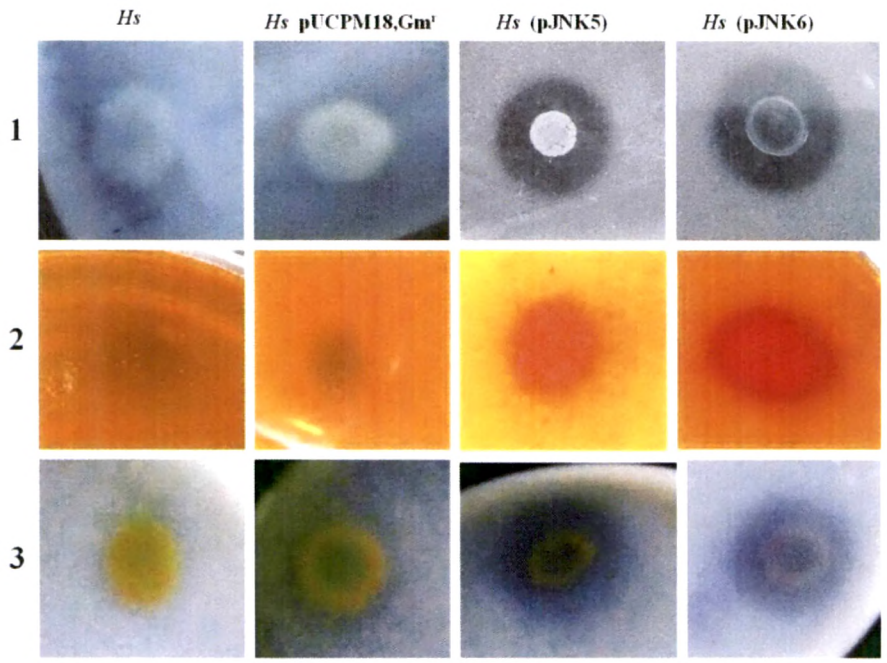


Fig. 5.7: MPS and KS phenotype of *H. seropedicae* Z67 transformants.

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.

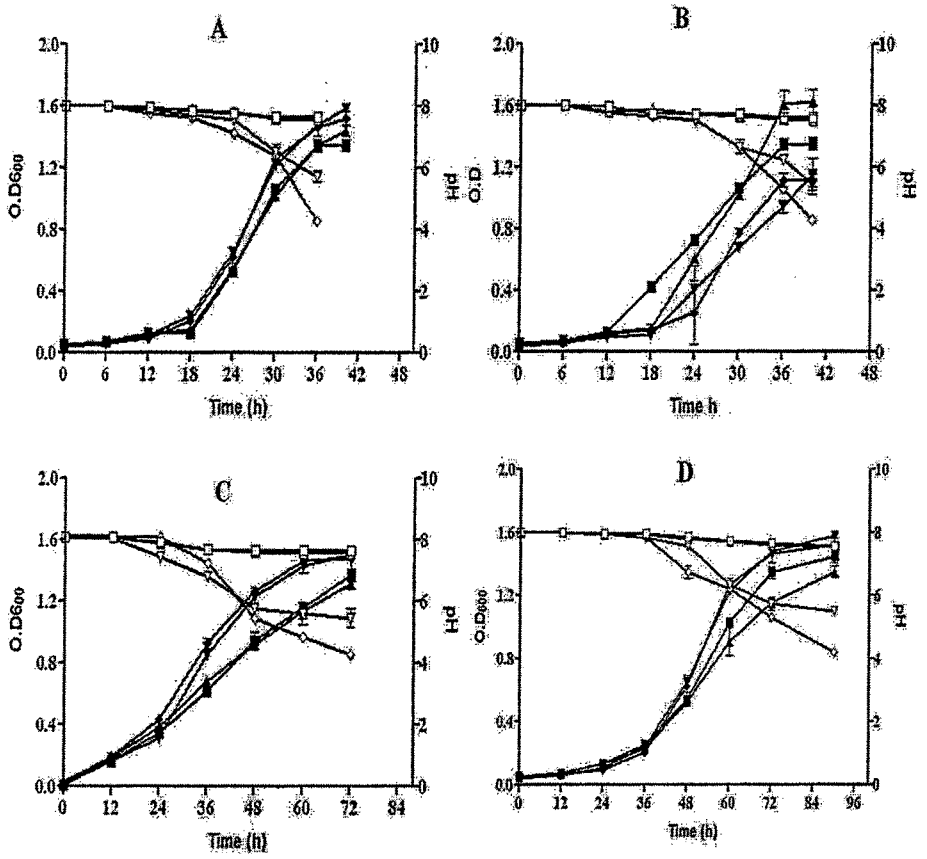


Fig. 5.8: Effect of *pqq* gene cluster and *gad* operon overexpression on growth pattern and pH profile of *H. seropedicae* Z67. (A, B - Aerobic condition and C, D- Microaerobic condition) and growth profile on50 mM glucose, 100mM HEPES rock phosphate medium. Extracellular pH (□, Δ, ▽, ◊) (■,▲,▼,◆) of *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).

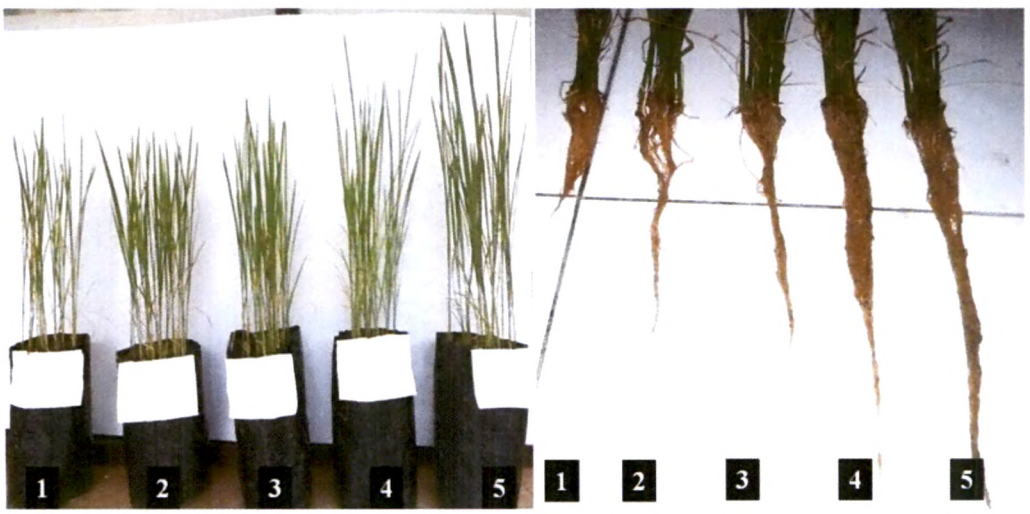


Fig. 5.9: Effect of *H. seropedicae* Z67 on rice plants. 1- Control, 2- *Hs* native, 3-*Hs* (pUCPM18Gm^r), 4-*Hs* (pJNK5), 5-*Hs* (pJNK6)

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.