Chapter 5

Genomic integration of *A. calcoaceticus pqq* cluster and *P. putida gad* operon with *vgb*, *egfp* in *B. japonicum* USDA110, *M. loti* MAFF303099 and *S.fredii* NGR234

5.1 Introduction

Presence of plasmids in *Azotobacter vinelandii*, *Azospirillum brasilense*, and *Pseudomonas putida* GR12-2 showed impaired growth, phosphate solubilization, nitrogen fixation, siderophore production, and indole acetic acid biosynthesis, under various experimental conditions (Glick 1995). Diverse effects were found in physiology, cellular metabolism, alteration in ATP biosynthesis as well as perturbations in host DNA replication, transcription, and translation in *E. coli* due to presence of plasmid (Rozkov et al. 2004; Wang et al. 2006; Chou 2007; Ow et al. 2006; 2009). *P. fluorescens* WCS365 and *P. putida* GR12-2 containing plasmid showed reduced stability and competitiveness under rhizospheric conditions, respectively (Simons et al. 1996; Schmidt-Eisenlohr et al., 2003). Additionally, presence of antibiotic marker and plasmid instability impose problem to use genetically modified organism for field studies (Neubauer et al, 2003; De Gelder, 2007). Previous reports from our laboratory showed overexpression of genes in plasmids exerted greater metabolic alterations on *P. fluorescens* ATCC 13525 metabolism despite of low copy number (Buch et al., 2010). Similarly, presence of plasmids adversely affected growth and gluconic acid secretion of phosphate solubilizing *E. asburiae* PSI3 under phosphorus limited condition (Sharma et al., 2011). Thus to overcome the drawbacks of plasmid mediated genetic manipulation, incorporation of genes into the genome becomes an attractive strategy (Sibley and Raleigh, 2012).

Genomic integration by additional copies of *nif*A and *dct*ABD in *Rhizobium meliloti* increased, root colonization, nodule occupancy and alfalfa yield in the field condition (Bosworth et al., 1994). *B. cepacia* G4 TOM- (toluene *o*-monooxygenase) was stably integrated in chromosome of *Rhizobium* strains (ATCC 10320 and ATCC 35645), engineered bacteria degraded trichloroethylene and were competitive against the unengineered hosts in wheat and barley rhizospheres (Shim et al., 2000). *Sphinomonas* CAR-1.9-dioxygenase gene constitutively integrated in *R. tropici*, degraded 48 % dioxin within first 3 days in field condition (Saiki et al., 2003). Genetic manipulation of *R. leguminosarum* bv. *trifolii* containing multiple copies of *ros*R and *pss*A genes responsible for EPS production, resulted in increased growth rate, EPS production and number of nodules (Janczarek et al., 2009). ACC deaminase

gene integrated in to *M. loti* expressing ACC deaminase only in nodules, expressed the gene constitutively and showed more number of nodules and competitiveness compared to wild type on *L. japonicus* (Conforte et al., 2010).

Majority of genetic manipulations in *Rhizobium* species are done to enhance its nodulation competitiveness, nitrogen fixation ability, biocontrol activity , with stand biotic-abiotic stresses and rhizoremediation (Rengel, 2002; Archana et al., 2010; Mabrouk and Belhadj 2010; Singh et al., 2011). There are no reports of genetic manipulation of *Rhizobium* species for mineral phosphate solubilization. Our studies show that genetic modification by heterologus overexpression of *A. calcoaceticus pqq* gene cluster along with *Pseudomonas putida gad* operon in *B. japonicum*, *M. loti* and *S. fredii* NGR234 expressed in pUCPM18 Gm^r plasmid under *Plac* promoter secreted, ~ 20 mM GA and ~15 mM 2KGA, 2.5 fold increase in P release compared to pJNK5 transformants was observed. Higher 2KGA secretion increased MPS efficiency of *Rhizobium* transformants. It would be interesting to study genomic integration of *A. calcoaceticus pqq* gene cluster , *Pseudomonas putida gad* operon , *Vitreoscilla hemoglobin* (*vgb*) and *egfp* using Tn7 based integration system at *att* site in *B. japonicum, M. loti* and *S. fredii* strains and characterize the biochemical, growth and MPS abilities of the integrants.

Single copy tagging of bacteria without, any deleterious effects at neutral and unique site is carried out by using mini-Tn7 transposon system. The Tn7 transposon was originally discovered by Barth et al. (1976) on the plasmid R483 (IncIα) as an element carrying the resistance genes trimethoprim (TmR) and streptomycin/ spectinomycin (SmR/ SpR), which could be transposed to other replicons. These genes are flanked by the ends of the transposon, named the left (Tn7L) and the right (Tn7R) end (Lichtenstein and Brenner, 1982; Rogers et al., 1986). Tn7 inserts into specific location named the attTn7 site with high efficiency and unique orientation in *E. coli*, located downstream of the coding region of enzyme glucosamine synthetase - *glm*S gene and thereby does not disrupt the gene (Gringauz, et al., 1988; Peters et al., 2001). Glucosamine synthetase is required for cell wall synthesis and it is conserved among many bacteria and, therefore Tn7 is likely to have the same specific insertion site in many different bacteria (Volger et al., 1989). The transposon genes require Trans functioning *tnsABCD* for specific insertion into the attTn7 site. Thus, sequences

located in the 3"end of the coding region of *glm*S are recognised by transposase proteins directing the actual insertion into the attTn7 site, down-stream of the *glmS* gene. However, if this site is unavailable the transposon can insert into other sites with low frequency.

Gene complementation, gene expression analysis, strain construction, and reporter gene-tagging of *Pseudomonas aeruginosa* and *Yersinia pestis*, particularly in biofilm and animal model studies were done using mini-Tn7 transposon. Tn7-based delivery system was used to integrate genes encoding for different fluorescent protein and luciferase protein along with promoter into the chromosomes of *Erwinia chrysanthemi, P. fluorescens, P. syringae, P. putida* and many gram negative bacteria by te (Koch et al., 2001, Lambersten et al., 2004; Hee et al.,2008). Thus mini Tn7 transposition is a powerful technique for the single-copy integration or excision of a gene of interest at chromosomal level to carry out protein functional studies, gene expression analysis, insertional random mutagenesis, or gene-tagging of bacteria in living organisms.

Function of *Vitreoscilla hemoglobin* (VHb) is to enable the organism to survive in oxygen-limited environments by acting as an oxygen storage-trap or to facilitate oxygen diffusion to terminal oxidases under hypoxic conditions (Wakabayashi et al., 1986). Several cloning experiments showed that the presence and expression of VHb can improve not only cell growth but also productivity of recombinant protein (Khosravi et al, 1990; Geckil et al, 2004; Chung et al., 2009). Cell-free extracts of *A. calcoaceticus* showed low levels of GDH activity when grown on acetate-glucose mixture in oxygen-limited condition, activity got restored with introduction of oxygen (Van Schei etal., 1988). Genetically modified *Rhizobium* with *vgb* overexpression has enhanced the growth of *Rhizobium* and also increased nitrogen content in bean plants (Ramirez et al., 1999). In soil and nodules, amount of oxygen plays an important role for growth, nitrogenase activity, colonization etc.

The present study describes the genomic integration of *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon , *V. hemoglobin* (*vgb*) and *egfp* using Tn7 based integration system at *att* site in *B. japonicum, M. loti* and *S. fredii* strains and compares its effect to plasmid based expression on growth amd MPS ability.

5.2 Experimental design

5.2.1: Bacterial strains used in this study

Plasmid/Strains	Characteristics	Source or Reference
pGRG36	Intergration vector (Tn7)	Gregory et al., 2006
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r	Hester et al., 2000
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) USA
pJNK6	pUCPM18Gm^r with *pqq* gene cluster of *A. calcoaceticus* and *gad* operon of *P. putida* KT2440 under *plac*; Ap^r, Gm^r	Wagh, 2013
pJIPgv	pGRG36 with *pqq* gene cluster of *Acinetobacter calcoaceticus* and *gad* operon of *P. putida* KT2440 under *plac*; Ap^r, *vgb* gene and *egf* gene Ap^r	This study
Bj intPgv	Genomic integrant of *B. japonicum* containing *lac-pqq, gad, vgb, egfp* Ap^r	This study
Ml intPgv	Genomic integrant of *M. loti* containing *lac-pqq, gad, vgb, egfp* Ap^r	This study
Sf intPgv	Genomic integrant of *S. fredii* containing *lac-pqq, gad, vgb, egfp* Ap^r	This study

Table 5.1: Bacterial strains used in this study. The details of the plasmids and the concentration of the antibiotics used are given in the **Table 21** and **2.2**.

5.2.2: Cloning of *pqq- gad* operon in integration vector.

Construction of artificial *pqq gad* operon containing constitutive *lac* promoter, of *A. calcoaceticus* and *gad* operon of *P. putida* KT2440, *vhb* gene and *gfp* was done as per the following strategy. XT-20 polymerase was used for PCR amplification (Bangalore Genei, India) from plasmid pJNK6. Sequence of forward (*lac*) rimerTCCGAAATGTGAAATACGAAGGCCGAGCATACAACACACAGGA

GGACGCATG GAATTCCACCATAAGTTTTGAC3` and reverse primer of *gad* operon 5' AGT CGC TTCGCTGAA ATGTAGTGG3' was used to amplify *pqq- gad* operon. Plasmid pGRG36-mini-Tn7-Amp-*egfp* was digested with *Sma*I, gel purified

and ligated with 9 kb amplicon containing artificial *pqq- gad* operon was ligated with pGRG36 containing *vgb* and *egfp* **(Fig. 5.1).** Clone was confirmed by restriction digestion and PCR amplification. Resultant construct of 17.9 kb, named as pJIPgv consisted of artificial *pqq- gad* operon.

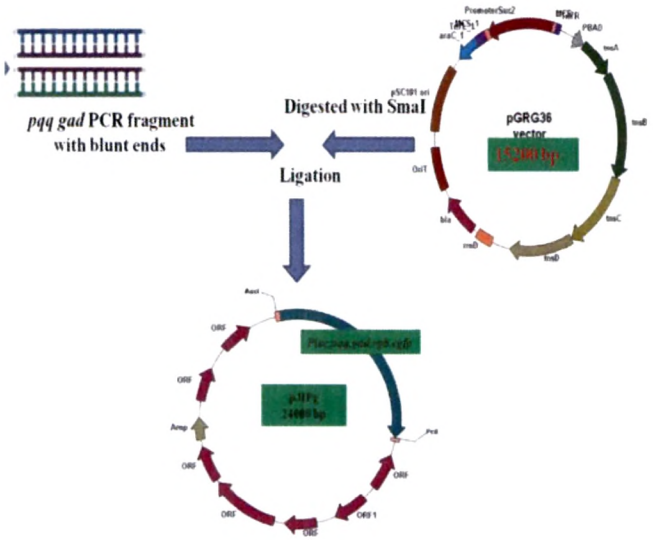

Fig. 5.1: Strategy used for cloning of *pqq-gad* gene cluster in pGRG36 containing *vhb*, *egfp* resulted in pJIPgv.

5.3: Results.

5.3.1: Construction of Genome integrants of *B. japonicum M. loti* and *S. fredii*

PCR amplification of *pqq* gene cluster along with *gad* operon under constitutive *lac* promoter was done from pJNK6 plasmid containing *pqq-gad* by using specific primers as mentioned above. This PCR amplicon was used for ligation with integration vector (**Fig. 5.2).** For further confirmation, the recombinant plasmid pJIPgv plasmid containing *pqq* gene cluster and *gad* operon under constitutive *lac* promoter was confirmed by restriction enzyme digestion. pJIPgv was digested with PvuII and pGRG36*vhb, egfp* plasmid digested with PvuII **(Fig. 5.3).**

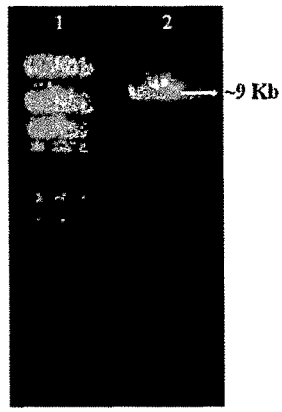

Fig. 5.2: PCR amplification of *pqq, gad* with constitutive *lac* promoter

Lane1- Marker **BstEII Digest**, Lane2- **PCR** amplification with constitutive *lac* promoter

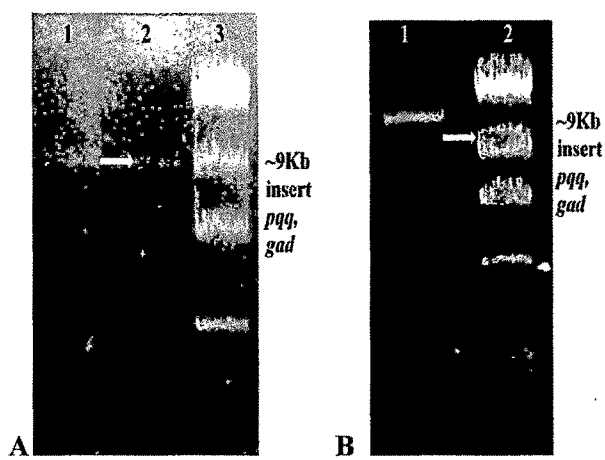

Fig.5.3: Restriction enzyme digestion pattern of pJIPgv plasmid containing *pqq* gene cluster and *gad* operon under *lac* promoter. (**A-B**) Lane1- pJIPgv digested with PvuII, Lane2- control plasmid pGRG36*vgb*, *egfp* digested with PvuII, Lane3 - λ DNA- Hind III Molecular weight marker.

B. japonicum, *M. loti* and *S. fredii* integrants were confirmed based on PCR amplification of *pqq-gad* from genomic DNA (**Fig. 5.4).**

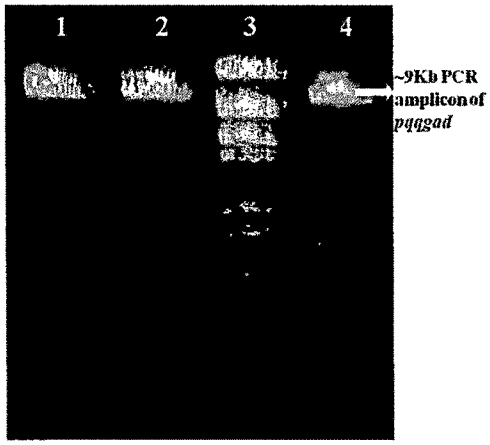

Fig. 5.4: Confirmation of Genome integrants by PCR amplification of *pqq* and *gad* from *B. japonicum, M. loti* and *S. fredii* integrants: Lane1,2 and 4 - PCR amplicon of *pqq,gad* from genomic DNA of *B. japonicum, M. loti* and *S. fredii* integrants.. Lane 3- λ DNA BstEII Molecular weight marker

5.3.2: GDH and GADH activity of *B. japonicum, M. loti* and *S. fredii* integrants.

In *B. japonicum M. loti* and *S. fredii* integrants GADH activity was found to be ~193.06 U, ~200.10 U and ~162.9 U, respectively, which is 24, 34 and 18 fold increase compared to native strain (**Fig.**). In integrants, GDH activity was found to be 129.83 U, 80.23 U and 147.03 U, respectively, with a fold increase of 10, 5 and 10 compared to native strains, respectively. GADH and GDH activity decreased by 1.5 - 2.0 fold compared to pJNK6 *Rhizobium* transformants.

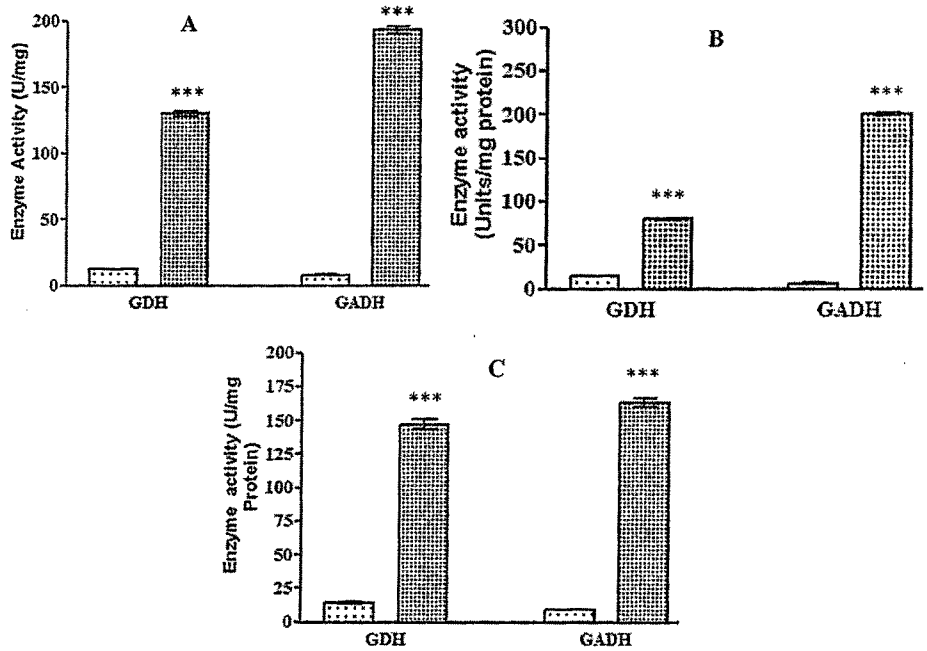

Fig. 5.5: GDH and GADH activity of *Rhizobium* integrants of *pqq* gene cluster and *gad* operon, *vgb* gene. (A) *Bj* intPgv **(B** *Ml* intPgv **(C)** *Sf* intPgv. Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

5.3.3: Growth and MPS ability of *B. japonicum, M. loti* and *S. fredii* integrant on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

B. japonicum M. loti and *S. fredii* integrants showed good phenotype compared to native and control both on PVK and TRP plates after 3 days of incubation at 30°C. Integrant showed compared to pJNK6 transformant decreased MPS phenotype on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate, while on PVK plates integrant showed stronger Ca-P solubilization **(Fig. ; Table 5.2).**

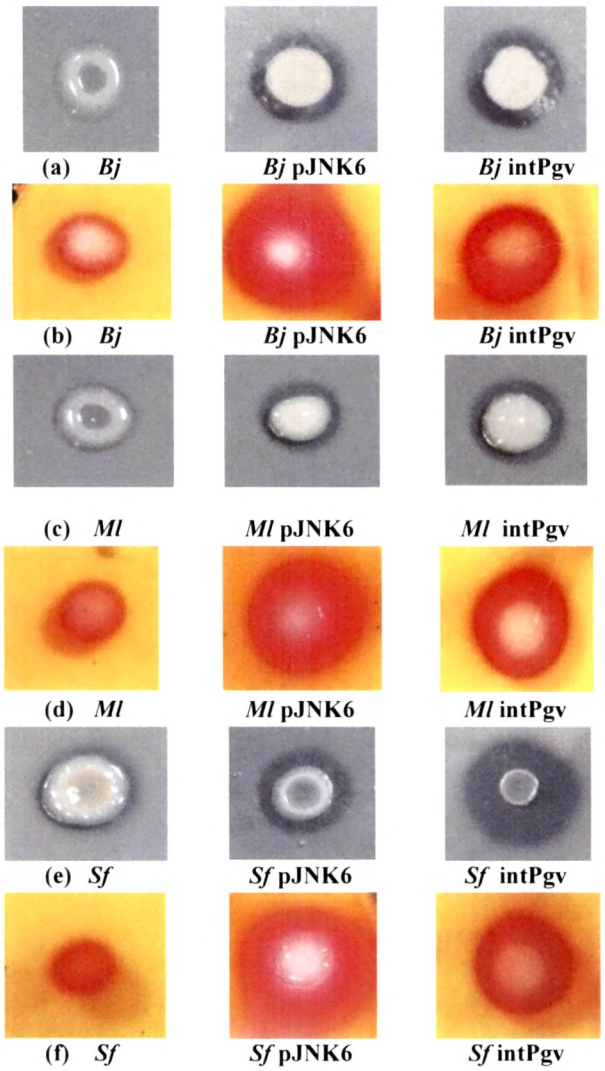

Fig. 5.6: MPS phenotype of *B. japonicum M. loti* and *S. fredii* integrants (a), (b) Zone of clearance formed by *Rhizobium* integrants on Pikovskaya's agar and (c), (d) zone of acidification Tris rock phosphate agar containing 50 mM glucose and 50 mM Tris Cl buffer pH 8.0. The results were noted after an incubation of 3 days at 30 °C. Media composition and other experimental details are as described in Sections 2.2.4 and 2.7.

Rhizobium Strains	Diameter of zone of clearance (mm)	Diameter of colony (mm)	Phosphate Solubilizing Index
Bj	12.17 ± 0.29	11.17 ± 0.29	1.09
Bj pJNK6	14.17± 0.29	9.50 ± 0.50	1.55
Bj intPgv	14.50± 0.50	8.17 ± 0.29	1.77
Ml	12.83 ± 0.29	11.50 ± 0.50	1.09
Ml pJNK6	12.83 ± 0.29	8.83 ± 0.29	1.45
Ml intPgv	14.50 ± 0.50	9.17 ± 0.29	1.58
Sf	12.17 ± 0.29	10.50 ± 0.50	1.20
Sf pJNK6	14.50 ± 0.50	10.17 ± 0.29	1.42
Sf intPgv	20. 83 ± 0.29	6.50 ± 0.50	3.20

Table 5.2: P solubilization index on Pikovskyas agar of *B. japonicum, M. loti* and *S. fredii* integrants during 3 days of growth on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate. *Bj, Ml* and *Sf* : wild type strain; and *Bj* intPgv, *Ml* intPgv and *Sf* intPgv: *B. japonicum, M. loti* and *S. fredii* integrants. The results were noted after an incubation of 3 days at 30 °C and are given as mean ± S.D. of three independent observations as compared to native *Bj, Ml* and *Sf.*

5.3.4: Growth pattern and pH profile of *B. japonicum, M. loti* and *S. fredii* integrants on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

The growth of *Bj* intPgv, *Ml* intPgv and *Sf* intPgv on 50 mM Tris-Cl buffer pH 8 and 50mM glucose reached to a maximum of 1.8 O.D. within 12 h in integrants compared to 20 h of the native *B. japonicum, M. loti* and *S. fredii* strains. pH of the medium dropped to 6.8, 6.8 and 5.7 within 20 h in the native *B. japonicum, M. loti* and *S. fredii* while pH drop to 4.1, 5.1 and 4.2 was seen within 12 h in *B. japonicum, M. loti* and *S. fredii* integrants, respectively (**Fig. 5.7 A,B,C**).

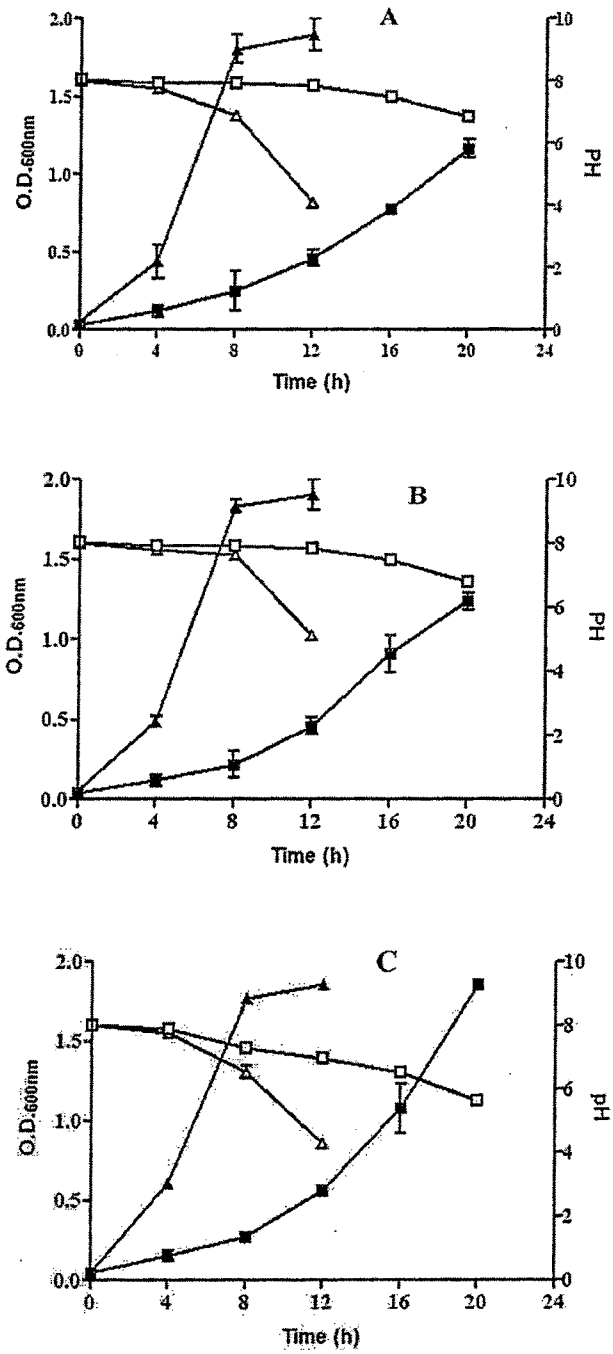

Fig. 5.7: Extracellular pH (□, Δ,) and growth profile on glucose 50 mM, Tris-Cl 50 mM rock phosphate medium (■, ▲,) of *B. jap*, *M. loti* and *S. fredii* integrants containing *pqq* gene cluster and *gad* operon, *vgb*, *egfp*. □, ■, *B. jap*, *M. loti* and *Sf* (wild type); Δ, ▲, *B. jap*, *M. loti* and *Sf* intPgv. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

5.3.5: Physiological effect of genomic integration on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

In presence of 50 mM glucose, genomic integration showed ~1.73, ~1.72 and ~2.2 fold increase in growth profile by *Bj* intPgv, *Ml* intPgv and *Sf* intPgv, respectively. The total glucose utilization rate at the time of pH drop remained unaffected and the total amount of glucose consumed at the time of pH drop showed ~1.15, ~1.10 and ~1.4 fold decrease in *Bj* intPgv, *Ml* intPgv and *Sf* intPgv respectively, and Specific Glucose utilization Rate Q_{Glc} (g.g $dcw^{-1}.h^{-1}$) decreased ~1.6, ~2.1 and ~1.9 fold. Increase in enzyme activity improved the biomass yield by ~1.5, ~2.1 and ~2.1 fold in the integrants of *B. japonicum, M. loti* and *S. fredii,* respectively, compared to native *B. japonicum, M. loti* and *S. fredii* (**Table 5.3**).

***Rhizobium* Strains**	**Specific Growth Rate $K(h^{-1})$ [a]**	**Total Glucose Utilized (mM) [b]**	**Glucose Consumed (mM) [b]**	**Biomass Yield $Y_{dcw/Glc}$ (g/g) [a]**	**Specific Glucose Utilization Rate Q_{Glc} ($g.g\ dcw^{-1}.h^{-1}$) [a]**
Bj	0.186 ±0.03	46.20 ±0.2	38.23 ±1.33	1.78 ±0.14	0.14 ±0.01
Bj intPgv,	0.417 ±0.01	49.37 ±0.12	20.80 ±0.63	5.18 ±0.81	0.05 ± 0.001
Ml	0.221 ±0.03	45.91 ±0.64	37.07 ±0.55	1.36 ±0.26	0.19 ±0.04
Ml intPgv	0.471 ±0.02	49.60 ±0.17	24.47 ±0.37	3.13 ±0.07	0.08 ± 0.001
Sf	0.260 ±0.02	46.10 ±0.42	37.17 ±0.55	1.85 ±0.1	0.15 ±0.02
Sf intPgv	0.383 ±0.01	49.57 ±0.06	18.57 ±0.35	2.99 ± 0.1	0.06 ± 0.001

Table 5.3: Physiological variables and metabolic data of *B. japonicum, M. loti* and *S. fredii* integrants grown on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate. The results are expressed as Mean ± S.E.M of 6-10 independent observations. *a* Biomass yield $Y_{dcw/Glc}$, specific growth rate (k) and specific glucose utilization rate (Q_{Glc}) were determined from mid log phase of each experiment. *b* Total glucose utilized and glucose consumed were determined at the time of pH drop. The difference between total glucose utilized and glucose consumed is as explained in Section 2.9.3. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

5.3.6: P solubilization and organic acid by *B. japonicum, M. loti* and *S. fredii* integrants in 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

There was significant increase in release of P by ~8.28, ~6.79 and ~7.86 fold by *Bj* intPgv, *Ml* intPgv and *Sf* intPgv, respectively, compared to wild type when grown on 50 mM Tris-Cl Buffer pH 8 containing 50 mM glucose containing Rock Phosphate 1mg/ml (**Fig. 5.9**).

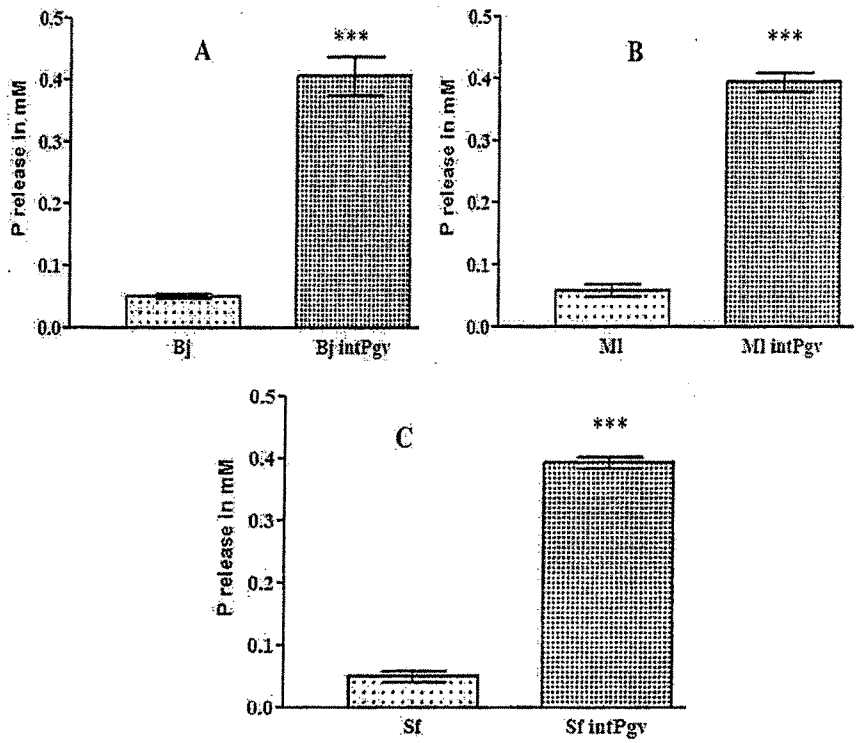

Fig. 5.8: P Solubilization by *Rhizobium* integrants.(A)- ▭, *Bj*, ▭, *Bj.* intPgv) **(B)-** ▭, *Ml*, ▭, *Ml.* intPgv and **(C)** ▭, *Sf*, ▭, *Sf.* intPgv; on **50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.** Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

Extracellular medium of integrants of *B. japonicum, M. loti* and *S. fredii* had 3.32, 2.81 and 3.90 folds higher amounts of 2-KGA, respectively, whereas levels of GA acid were 3.28, 3.07 and 2.64 folds higher compared to native and controls (**Table 5.4**). *Bj* intPgv, *Ml* intPgv and *Sf* intPgv also secreted ~3.4 µM , ~3.64 µM and ~4.26 µM PQQ, there is ~2 fold decrease compared to pJNK6 transformants (**Fig. 5.9**).

Rhizobium Strain	GA mM	2-KGA mM	Specific Yield GA $Y_{C/G}$	Specific Yield 2-KGA $Y_{C/G}$
Bj	6.26 ± 0.23	2.37 ± 0.47	1.39 ± 0.12	0.49 ± 0.09
Bj intPgv	20.69 ± .27***	7.87 ± .20***	1.04 ± 0.06	0.40 ± 0.12
Ml	6.53 ± 0.18	2.60 ± 0.20	1.01 ± 0.21	0.40 ± 0.1
Ml intPgv	18.40 ± 0. 4***	6.73 ± .25***	0.88 ± 0.02	0.31 ± 0.03
Sf	6.760 ± 0.10	2.20 ± 0.15	1.59 ± 0.05	0.54 ± 0.10
Sf intPgv	22.41 ± .42***	8.6 ± 0.23***	1.22 ± 0.03	0.46 ± 0.02

Table 5.4: Organic acid secretion and organic acid yield of *B. japonicum*, *M. loti* and *S. fredii pqq* integrant grown on TRP medium 100mM Tris-Cl buffer pH 8 and 50mM Glucose containing Rock Phosphate 1mg/ml. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

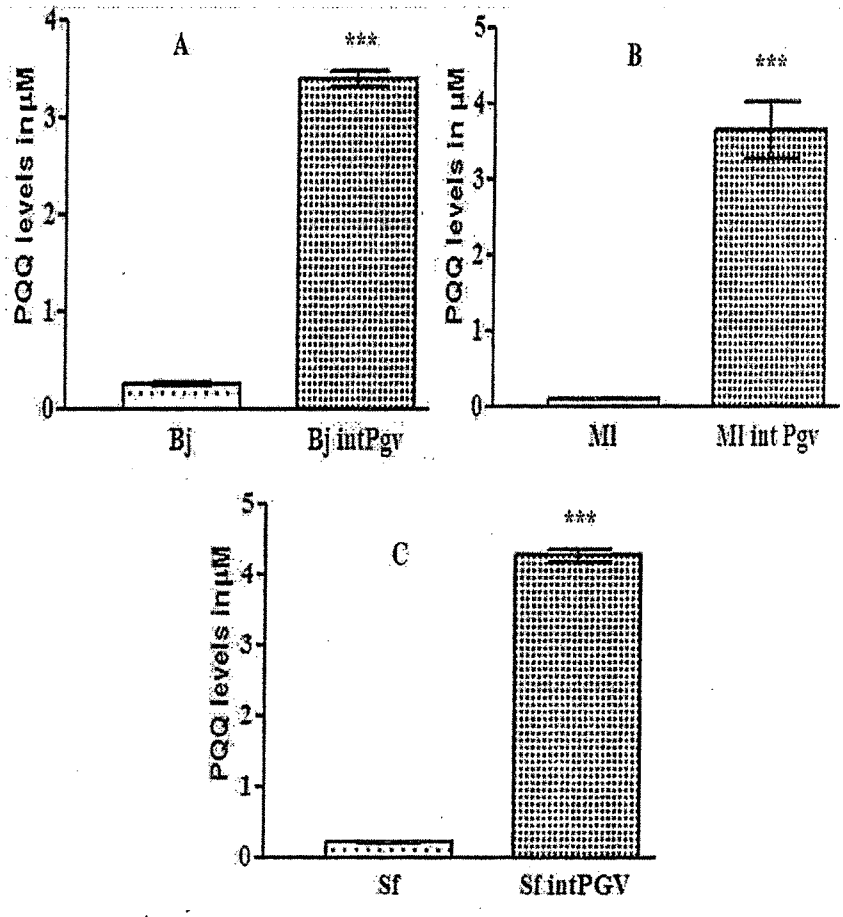

Fig. 5.9: PQQ secreted by *Rhizobium* integrants. **(A)-**▭, *Bj*,▭, *Bj*. intPgv) **(B)-**▭, *Ml*, ▭, *Ml*. intPgv and **(C)**▭, *Sf*, ▭, *Sf*. intPgv; on **50 mM glucose in M9 medium** Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

5.4: DISCUSSION

High-level expression of a target gene may be achieved by employing a high copy number vector. However, gene expression systems based on high copy number vectors have a number of drawbacks, such as , instability of recombinant plasmids, loss of the desired traits, formation of insoluble protein aggregates , decreased growth rate, metabolic perturbations (Han. 2004). Hence, genetic manipulations need to be directed towards chromosomal integration as it would lead not only to increased stability but also decrease the metabolic load caused by the presence of the plasmids or to nullify the adverse effect on the host metabolism. The present study describes the genomic integration of *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon, *V. hemoglobin* (*vgb*) and *egfp* using Tn7 based integration system at *att* site in *B. japonicum, M. loti* and *S. fredii* strains and compares its effect to plasmid based expression on growth amd MPS ability.

In our study, *pqq* cluster under constitutive *lac* promoter and *gad* operon with its own promoter were integrated in to genome of *B. japonicum, M. loti* and *S. fredii* NGR 234. GADH and GDH activities in M9 medium were decreased by ~ 2 and ~ 1.5 fold, respectively compared to plasmid overexpressed strain. It appears that higher level of overexpression of genes by more copy number of plasmid is more favourable for growth and P solubilization in laboratory condition than single copy on chromosome.

Integrants showed increased PSI on PVK plates compared to MPS phenotype on TRP medium containing 50 mM Tris pH 8.0 while corresponding plasmid transformants showed phenotype even on 100 mM Tris pH 8.0 medium. Hence integrants by releasing ~ 21 mM GA and ~ 7 mM 2-KGA, can solubilize Ca-P efficiently compared to pJNK6 transformants. This shows that multiple copies of pUCPM18 does not have any effect on metabolic load in *Rhizobium* strains.

Specific growth rate, biomass yield increased in genomic integrant of *Rhizobium* strains, which was contrary to *E. asburaie* PSI3 results where growth and gluconic acid secretion decreased (Sharma et al., 2011).

Activity of *A .calcoaceticus* GDH in micro-aerobic condition on glucose-acetate mixture was lost due to loss of PQQ activity, which was restored by addition

of oxygen (Van Schei et al., 1988). In this study along with *pqq* cluster and *gad* operon, *vgb* is integrated in *Rhizobium* genome to improve the recombinant protein production and better survival of host under microaerobic conditions (Frey and Kallio, 2003). Effect of *vgb* on growth, GA and 2-KGA secretion and MPS ability in microaerobic condition is remains to be investigated.

Genetic modification either by plasmid or chromosomal integration resulted in conferring MPS and PGPR attributes to *Rhizobium* strains. This is a novel study as till now, role of P in BNF and effect on growth and yield of plants were studied by co innoculation of *Rhizobium* species with PSMs. It would be interesting to see effect of P solubilizer - Nitrogen fixer *Rhizobium* on plant growth.