Chapter 2

Materials & 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** The wild type and genetically modified *E. coli* and *Rhizobium* strains (*Bradyrhizobium japonicum* USDA110; *Mesorhizobium loti* MAFF303099 and *Sinorhizobium freddi* (NGR234) are listed in **Table 2.1** *E. coli* DH10B was used for all the standard molecular biology experiments wherever required. *Rhizobium* strains used in all this study is generous gift from (Dr Poole, U.K. John Innes Center). The *pqqE* gene was gifted by Goldstein 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).

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

Plasmid/Bacterial strains	Characteristics	Source/Reference
E. coli DH10B	Used to maintain plasmids for routine use	Invitrogen, USA
pBBR1MCS-2	Broad-Host-Range vector; Km^r	Kovach et al., 1995
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r (100μg/ml)	Hester et al., 2000
pJNK1	pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	Wagh, 2013
pJNK5	pUCPM18, Gm^r (20μg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus.*	Wagh, 2013
***Rhizobium* strains**		
Bradyrhizobium japonicum USDA110	NC_004463.1	NCBI
Mesorhizobium loti **MAFF030669**	NC_002678.2	NCBI
Sinorhizobium fredii **NGR234**	NC_012587.1	NCBI
***Bj* pUCPM18**	*B. japonicum* with pUCPM18, Gm^r (control vector)	Chapter 3
***Bj* pJNK1**	*B. japonicum* with pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	Chapter 3
***Bj* pJNK5**	*B. japonicum* pUCPM18, Gm^r (20μg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus.*	Chapter 3
***Ml* pUCPM18**	*M. loti* with pUCPM18, Gm^r (control vector)	Chapter 3
***Ml* pJNK1**	*M. loti* with pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	Chapter 3
***Ml* pJNK5**	*M. loti* with pUCPM18, Gm^r (20μg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus.*	Chapter 3
***Sf* pUCPM18**	*S. fredii* with pUCPM18, Gm^r (control vector)	Chapter 3
***Sf* pJNK1**	*S. fredii* with pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	Chapter 3
***Sf* pJNK5**	*S. fredii with* pUCPM18, Gm^r (20μg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus.*	Chapter 3
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r (100μg/ml)	Hester et al., 2000
pJNK6	pJNK5, Gm^r (20μg/ml) with *gad* operon 3.8 Kb of *P. putida* KT 2440	Wagh, 2013
***Bj* pUCPM18**	*B. japonicum* with pUCPM18, Gm^r (control vector)	Chapter 4

***Bj* pJNK6**	*B. japonicum* with pJNK6Gm^r (20µg/ml)	Chapter 4
***Ml* pUCPM18**	*M. loti* with pUCPM18, Gm^r (control vector)	Chapter 4
***Ml* pJNK6**	*M. loti* with pJNK6Gm^r (20µg/ml)	Chapter 4
***Sf* pUCPM18**	*S. fredii* with pUCPM18, Gm^r (control vector)	Chapter 4
***Sf* pJNK6**	*S. fredii* with pJNK6Gm^r (20µg/ml)	Chapter 4
pGRG36	Intergration vector (Tn7)	McKenzie 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 *Acinetobacter 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	Chapter 5
Bj intPgv	Genomic integrant of *B. japonicum* containing *lac-pqq,gad, vgb, egfp* Ap^r	Chapter 5
Ml intPgv	Genomic integrant of *M. loti* containing *lac-pqq,gad, vgb, egfp* Ap^r	Chapter 5
Sf intPgv	Genomic integrant of *S. fredii* containing *lac-pqq,gad, vgb, egfp* Ap^r	Chapter 5

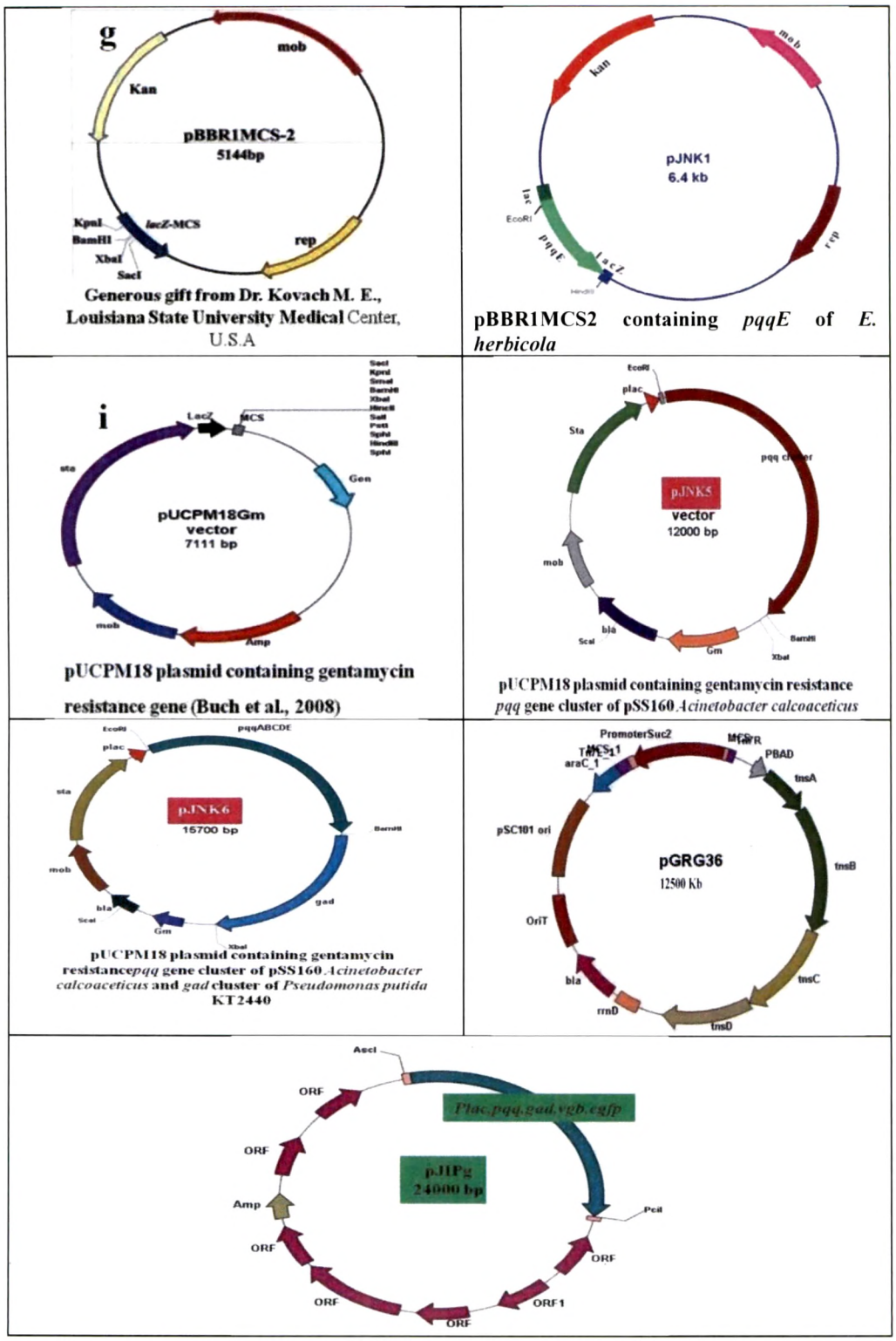

Fig. 2.1: Restriction maps of the plasmids used in this study. All plasmids are broad host- range vectors used for expression of heterologous overepression of genes under *lac* promoter in *Rhizobium* strains (*Bradyrhizobium japonicum* USDA 110; *Mesorhizobium loti* MAFF303099 and *Sinorhizobium freddi* (NGR234)

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 37°C while all *Rhizobium* strains (*Bradyrhizobium japonicum* USDA 110; *Mesorhizobium loti* MAFF303099 and *Sinorhizobium freddi* (NGR234) cultures were grown at 30°C. 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 *Rhizobium* strains (*Bradyrhizobium japonicum* USDA 110; *Mesorhizobium loti* MAFF303099 and *Sinorhizobium freddi* (NGR234) 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 -20°C.

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: M9 minimal medium

Composition of M9 minimal broth was according to Sambrook and Russell (2001) including Na_2HPO_4 $7H_2O$, 34g/L; KH_2PO_4, 15g/L; NH_4Cl, 5g/L; NaCl, 2.5g/L; 2mM $MgSO_4$; 0.1mM $CaCl_2$ and micronutrient cocktail. The micronutrient cocktail was constituted of $FeSO_4.7H_2O$, 3.5 mg/L; $ZnSO_4.7H_2O$, 0.16 mg/L; $CuSO_4.5H_2O$, 0.08 mg/L; H_3BO_3, 0.5 mg/L; $CaCl_2.2H_2O$, 0.03 mg/L and $MnSO_4.4H_2O$, 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.

2.2.2: TrisCl buffered medium

The media composition included TrisCl (pH=8.0), 100 mM; NH_4Cl, 10 mM; KCl,10 mM; $MgSO_4$, 2 mM; $CaCl_2$, 0.1 mM; micronutrient cocktail; Glucose, 100mM and phosphate (P) sources (Sharma et al., 2005). 1mg/ml Senegal Rock phosphate (RP) or KH_2PO_4 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.

2.2.3: 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 *Rhizobium* spp. by heterologous expression of *pqq* gene cluster, *gad* operon and *vgb* gene 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.

2.2.4: Murashige-Skoog's Medium

Murashige-Skoog's (MS) medium was composed of macro elements and micro elements. The macro elements included $CaCl_2.2H_20$, 0.440g/L; KH_2PO_4, 0.17g/L; KNO_3, 1.9g/L; $MgSO_4.7H_2O$, 0.37g/L; NH_4NO_3, 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.

2.2.5: YEMA medium for *Rhizobium* strains

Yeast Mannitol Agar with congo red was used to maintain *Rhizobium* species on plates, YEMA medium contains ingredients gms/ L. Yeast extract 1.000, Mannitol 10.000, Dipotassium phosphate 0.500, Magnesium sulphate 0.200, Sodium chloride 0.100, Congo red 0.025, Agar 20.000, Final pH (at 25°C) 6.8±0.2 (Vincent, J.M. 1970).

2.2.6 Tryptone Yeast Extract Medium (TYE)

TY medium contained **0.5** % (w/v) Difco Bacto-Tryptone, 0.3 % (w/v) Difco Bacto-Yeast Extract and 7 mM-CaCl, Liquid PA medium contained 0.4 % (w/v) Difco Bacto-Peptone and **2** mM-$MgSO_4$, (Hirsch et al., 1980)

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 *Rhizobium* strains.

The plasmid DNA from *E. coli* and *Rhizobium* was isolated by the boiling alkali lysis method, (Sambrook and Russell, 2001). Engineering the glucose metabolism of *Rhizobium* strains by heterologous expression of and *pqq* gene clusters and *gad* operon.

2.3.1.2: Genomic DNA from *Rhizobium* strains.

The plasmid DNA from *E. coli* and *Rhizobium* strains was isolated by the method, (Sambrook and Russell, 2001).

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 $MgCl_2$-$CaCl_2$ 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 *Rhizobium* strains.

Plasmid transformation in *Rhizobium* strains was done by electroporation as described by (Unge et al., 1998) *Rhizobium* strains cells grown till early stationary phase, cells were harvested (at 4°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 Electroporator 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°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.

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

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)

2.3.8: Genomic Integration.

Integration of genes in genome was done by using miniTn7 based transposon integration vector, which is temperature sensitive. After electroporation of plasmid in Rhizobium strains culture was allowed to grown on YEMA plate and kept at 42°C for 24 h for integration of genes at *att* sites of genome. (Nancy et al., 2006) confirmation of integrants was done by PCR amplification from genomic DNA.

2.4: P-solubilization phenotype

Fresh culture of *Rhizobium* strains transformants obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30°C 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) TrisCl buffered RP-Methyl red as pH indicator dye and 1.5% agar (TRP) agar plates with addition of yeast extract 0.1% (Gyaneshwar et al., 1998). Culture was aseptically spotted on the TRP 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 TRP Methyl red agar plates.

2.5: Physiological experiments

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

2.5.1: Inoculum preparation

The inoculum for M9 and HEPES minimal media containing rock phosphate were prepared by growing the *Rhizobium* strains overnight at 30°C in 3ml LB broth. Inoculum for the buffered RP broth (HRP) was prepared by growing the *Rhizobium* strains 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.

2.5.2: Growth characteristics and pH profile

Growth parameters and pH profile of the native as well as *Rhizobium* strains transformants were determined using TRP 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 Bert and Priefer (2004). Flasks were sealed with rubber septum and incubated at 30°C by shaking at 200 r.p.m. and the syringe was used to take out samples.

2.6: Estimation of Plant growth promoting factors

2.6.1: Culture conditions for EPS production and quantification

Fresh overnight grown culture of *Rhizobium* strains transformants as well as native obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30°C 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 30°C 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.

2.6.2: Biofilm 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., (**2006**).

2.6.3: Indole acetic acid (IAA) production and estimation

Overnight grown all cultures were pulled in sterile 1.5ml eppendorff 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.

2.7: PQQ determination

PQQ production was estimated using the method of Rajpurohit et al., (2008). *Rhizobium* strains transformants and 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.

2.8: Analytical techniques

Modified Lowry's method (Peterson 1979) was used for total protein estimation. *Rhizobium* strains native as well as transformants increased in cell growth density were 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 **1966**) 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):

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

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

ΔGlucose (t1-t2) is the amount of glucose consumed over the time interval t1-t2; Δdcw (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)

2.9: Enzyme assays

2.9.1: 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. 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.

2.9.2: Enzyme Assay Protocols

2.9.2.1: GDH (1.1.99.17) 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 600 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.

2.9.2.2: Gluconate dehydrogenase assay (GADH) (1.1.99.3)

Rhizobium strains 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.

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

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

where,

ΔA_y nm is the difference in the absorbance at any given wavelengths (y nm) and ϵ 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.

2.10: Pot experiments - Interaction with (*Mung bean*).

2.10.1: Plant Inoculation Experiments:

Pure bacterial cultures were grown in nutrient broth at 30°C, centrifuged, and diluted to a final concentration of 10^8 CFU/ml in sterile distilled water. Mung bean 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°C.and then immersed in a bacterial suspension for 5 h. Germinated seeds were sown in pot with 10Kg of unsterilized locally collected soil. Before sowing seeds in pots initial physical and chemical properties in experimental of soil is presented in **(Table. 2.4)**.The plants were grown at 30°C in Green house and were irrigated with 50 mL of sterile distilled water every day. The experiment was conducted on six replicates (three pots per replicate, two plants per pot) for each treatment and was completely randomized. The plants were harvested after 45 days and growth parameters (plant height and plant biomass) were recorded. Total N, was measured by micro Kjeldahl's method, by Vepodest- 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.

Leaf area

Leaf area of 3rd leaf was calculated according to method described by Khalilzadeh et al., 2012.

2.10.2. Acetylene Reduction Assay (ARA): Measuring Nitrogenase Activity

ARA assay was performed on GC at SICART method described by Castle 2010. Nitrogenase assays were conducted by acetylene reduction, using 0.1-mL samples injected into a PerkinElmer Clarus 680 Gas Chromatograph (GC) with dual flame ionization detectors (FID) were used in these experiments. Elite-U PLOT column. Acetylene was generated from calcium carbide and using 10% (w/v)

2.10.3. Chlorophyll Content:

Total cholophyll content of fresh leaves were estimated by using method described by (Khaleghi et al., 2012)

Table 2.4: Physical and chemical properties of experimental soil used for *Rhizobium* strains integrants

pH	P Kg/hac^{-1}	N Kg/hac^{-1}	K Kg/hac^{-1}
8.0	12.9	158.7	338.7

2.10.4.1: 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 Giannopolitis and Ries (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 H_2O_2 (ε = 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.

Guaiacol Peroxidase (POX)

Two hundred mg of leaves were homogenized in a pre-chilled mortar and pestle under ice cold condition in 2.0 ml of extraction buffer, containing 0.1M sodium phosphate buffer (pH 7.2) with the addition of 1 mM β-mercaptoethanol and 1% (w/v) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay.

POX (EC 1.11.1.7) activity was determined in the supernatants of centrifuged homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (ϵ=26.6mM^{-1}cm^{-1}) in a reaction mixture containing 50 mM sodium phosphate buffer pH 7.0, 0.1 mM EDTA, 0.05 ml enzyme extract, and 10 mM H_2O_2 (Costa et al., 2002).

(4) Ascorbate peroxidase (APX)

Mung bean leaf samples were crushed with chilled acetone in pre-chilled mortar-pestle. To obtain fine acetone powder the homogenates were filtered and stored immediately at - 20o C. Hundred mg of acetone powder was then homogenized in a pre-chilled mortar and pestle under ice cold condition using 2.0 ml of extraction buffer, containing 0.1 M sodium phosphate buffer (pH 7.2), and 1% (w/v) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay (Mahatma et al., 2011).

APX (EC 1.11.1.11) activity was measured immediately in fresh crude extracts and assayed by procedure described by Nakano and Asada (1981). Three ml of the reaction mixture contained 50 mM sodium phosphate buffer pH 7.0, 0.1 mM H_2O_2, 0.5 mM ascorbic acid, 0.1 mM EDTA and 0.1 ml enzyme extract. The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in the

absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). The enzyme unit of APX was defined similar to that of CAT activity as described.

2.10.4.2: Estimation of Water Soluble Protein Content

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

(a) Reagents for Lowry'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 Lowry'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.