Chapter 3

Cloning *Escherichia coli* DH10B Gamma glutamyl Cysteine synthetase (YbdK) in rhizobia to enhance glutathione synthesis in rhizobia

3.1 Introduction

3.1.1 Biosynthesis of Glutathione

Glutathione (GSH), y-L-glutamyl-L-cysteinyl-glycine, is the most abundant nonprotein thiol found in all organisms.[1] It has carboxyls, one amine, and one thiol group, which makes it highly soluble in aqueous solutions and in polar solvents. The thiol group of GSH is responsible for its biological activity, whereas the gamma linkage between glutamic acid and cysteine prevents its degradation by any kind of proteases, except y-glutamyl transpeptidase (GGT), which hydrolyzes GSH by breaking the gamma linkage between glutamate and cysteine and transferring the glutamyl residue to another amino acid.

Glutathione exists in a reduced form (GSH) as well as in a disulfide-bonded dimeric form, aka the oxidised state (GSSG), in any cell. The cell precisely controls the ratio of GSH to GSSG, where the GSH is always higher than GSSG. The ratio of GSH/GSSG in the cytoplasm of *E. coli* growing in LB medium under normal condition is 200. In almost all types of cells, in stress condition this ratio can go down to 10:1 or sometimes as low as 1:1, but that totally depends on the condition and stress level in which the cell is present at that particular time. [2]

In *E. coli* bacteria the synthesis of glutathione happens in two main steps, and the reaction is catalysed by the product of two genes *gshA* and *gshB* sequentially. The gshA gene encodes an ATP dependent enzyme y-glutamylcysteine synthetase (GCS) [EC:6.3.2.2], which catalyses the fusion of glutamic acid and cysteine to form y-glutamylcysteine. The GSC of E. coli is a 58.3 kDa monomer unlike the GCS characterized from the rat kidneys which is a heterodimer. Both bacterial and eukaryotic GCS can be operated by a feed-back loop mechanism, where the glutathione binds the active site of glutamic acid and prevents further binding of glutamic acid. The thiol group of glutathione GSH interacts inside the active site of GCS enzyme. The second step in glutathione synthesis is the fusion of y-glutamylcysteine and glycine which make a tripeptide glutathione molecule. The second step is catalysed by glutathione synthase (GS) [EC:6.3.2.3], which is also an ATP dependent enzyme. This enzyme is a tetra dimer with four identical subunits of 35.6 kDa.[3]

3.1.2 Roles of Glutathione in bacteria

Glutathione is one of the most abundant thiols present in cyanobacteria and proteobacteria and in all mitochondria and chloroplast-bearing eukaryotic cells. In bacterial cells, its main role is to maintain the proper oxidation state of protein thiols and protect the cells from oxidative

stress, osmotic stress, chlorine compounds, and low pH. One of its most important functions is the post-translational regulation of protein function under oxidative stress conditions via the glutathionylation process.[2]In bacteria the physiological concentration of glutathione ranges from 0.1mM to 10mM.[4]

3.1.3 Roles of glutathione in plant-rhizobia interaction

The growth and protection of plants not only depends on the defence mechanism of plants but, it also depends on the plant growth promoting bacteria (PGPB) / Plant growth promoting rhizobacteria (PGPR) which lives in harmony within the root-rhizosphere plane. Some of them can even infect plant roots through cracks or through root hairs (e.g., Rhizobium) and establish a commensal relationship with plants. Rhizosphere is colonized by different soil bacteria species.[5] PGPR/PGPB bring about growth promotion in plants, shows antimicrobial effects and protects plants from stresses like heavy metals/hydrocarbons/salinity/drought.[6] PGPR can combat heavy metals in varieties of ways as they possess a mechanism such as efflux pump[7], Siderophore production for heavy metal chelation [8] and exopolysaccharides production[5,9]. PGPR also produces glutathione which is one of most important antioxidants in all the living systems. It helps in chelating the heavy metals and also directly neutralizes the ROS produced by the oxidative actions of these heavy metals. It also helps in maintaining Ascorbate-Glutathione cycle, Redoxin cycle, helps in reducing the oxidized protein thiols and is a building block of Phytochelatins which chelates heavy metal ions.[10] Rhizobium interacts with legumes and forms root nodules in legume corps where it fixes the atmospheric nitrogen and makes it available to the plants.

Besides this, glutathione is also very important for the normal physiology of bacteria and formation of nitrogen fixing nodule. A study indicated that *S. meliloti* which had a mutation in *gshA* and *gshB* genes were unable to grow in minimal medium under laboratory conditions.[11] It is also known that the glutathione produced by rhizobium is extremely important for the formation and proper function of root nodules, and its need cannot be complemented by the glutathione produced by the plant. It was observed that when a gshA mutant rhizobium was inoculated with *M. sativa*, plant was unable to form the root nodules, while the gshB mutant showed a delayed nodulation with 75% decrease in Nitrogen fixation efficiency.[11] After infecting the root hairs, bacteria penetrate inside the plant cells and forms a nodule. In a study it was observed that when a legume was infected with a *gshB* mutant rhizobium, it showed a decrease in the nodule occupancy and the observation was reversed on transforming that mutant

with a plasmid containing *gshB* gene.[12] All this evidence proves a point that the glutathione of rhizobium is extremely important for formation and maintenance of healthy root nodules which can perform efficient nitrogen fixation. The main reason behind this is a weakly permeable peribacterial membrane which prevents an uptake of plant glutathione. [13]

3.1.4 *YbdK* is a Carboxylate-amine ligase found in *E. coli*

An open reading frame YbdK, of E. coli encodes a member of large bacterial protein family. Initially its function was unknown. In a study, the ligase activity of YbdK was tested between glutamic acid and each of the 20 amino acid residues on HPLC by following the hydrolysis of ATP to ADP, and the catalysis was only observed with Cysteine. [14]Thus, it become established that *YbdK* has the y-glutamylcysteine synthetase activity, which is the first catalytic and rate limiting step in glutathione synthesis. But the catalytic activity of YbdK was 500 folds slower compared to the authentic y-glutamyl cysteine synthetase enzyme [14]. Comparison between YbdK and y-GCS structures by DALI showed a high similarity between their sequences. It was also observed that the authentic y-GCS possesses the active site lid, which is absent in YbdK due to somewhat disordered loops in its structure. This arrangement makes an active site of YbdK more open compared to the active site of y-GCS. [15] This might be the reason behind its low catalytic activity in comparison to y-GCS.

3.1.5 Rationale behind using *YbdK* in this study

y-GCS is the rate limiting enzyme in glutathione biosynthesis pathway. Excess glutathione binds to the glutamate binding site in the active site of y-GCS, which will halt the glutathione biosynthesis pathway. Since the active site of YbdK is more open, there is a possibility that the feedback inhibition by glutathione towards YbdK enzyme might be slower. If a steady state expression of YbdK through a recombinant plasmid in a rhizobium is able to produce higher levels of glutathione compared to the wild type, then it would be beneficial to plants as well as bacteria in heavy metal stress. In this study, we transformed two rhizobium bacteria (*Sinorhizobium fredii* NGR 234 and *Sinorhizobium meliloti* (NIAMCC B-00836)) with a recombinant pPAT plasmid (Constructed by cloning *ybdK* gene form *E. coli* in a low copy number plasmid pBBR1MCS2). pBBR1MCS2 plasmid (empty backbone) is a broad host range plasmid, with a derepressed lac promoter which do not require any induction. It is one of the best choices for getting a constitutive expression of the gene of our interest. Hence this study aims to clone *E. coli* DH10B *ybdK* gene in rhizobium bacteria for an enhanced production of glutathione and using it as a PGPR for fenugreek seedlings growing in heavy metal (Arsenic

and Cadmium) contaminated soil, as well as using it for *invitro* biosynthesis of cadmium sulphide nanoparticles. Both of these have not been attempted yet, thus our work is novel.

3.2 Materials and Methods

3.2.1 Microorganisms, plasmids and primers used in the study

Bacteria used in the study are mentioned in **Table 3.1.** Plasmids and Primers used in the study are described briefly in **Table 3.2**

Table 3.1 Bacteria used in this study

Bacterial strain (Wild type /GMOs) and their antibiotic selection	**Growth media used**	**Optimum growth temperature**	**Source and description**
Escherichia coli DH10B Streptomycin (50ug/ml)	Luria Bertani broth/agar (LB)	37 °C	The department of Biochemistry, MSU Baroda
Sinorhizobium fredii NGR 234 Rifampicin (18ug/ml)	Yeast extract Mannitol broth /agar (YEM)	28 °C	Generous gift by Dr. José Mª Vinardell González, Facultad de Biología, Universidad de Sevilla, Spain
Sinorhizobium meliloti (NIAMCC B-00836)	Yeast extract Mannitol broth /agar (YEM)	28 °C	Purchased from NIAMCC
Escherichia coli DH10B (pPAT) Kanamycin (25ug/ml)	Luria Bertani broth/agar (LB)	37 °C	*Escherichia coli* DH10B transformed with the recombinant plasmid (pPAT)

Sinorhizobium fredii NGR 234 (pPAT) Rifampicin(18ug/ml)/Kanamycin(25ug/ml)	Yeast extract Mannitol broth /agar (YEM)	28 °C	*Sinorhizobium fredii* NGR 234 transformed with the recombinant plasmid (pPAT)
Sinorhizobium meliloti (NAIMCC B-00836) (pPAT) Rifampicin(18ug/ml)/Kanamycin(25ug/ml)	Yeast extract Mannitol broth /agar (YEM)	28 °C	*Sinorhizobium meliloti* (NAIMCC B-00836) transformed with the recombinant plasmid (pPAT)
Pseudomonas fluorescence (NAIMCC B-00342)	King's B broth/agar	28°C	Purchased from NIAMCC

Table 3.2 Plasmids and primers used in the study

Plasmid/Primers	**Size in base pairs (bp)**	**Description**	**Antibiotic selection used**	**Source**
pBBR1MCS2	5148 bp	Low copy number cloning and expression vector having derepressed lac promoter.	Kanamycin 25ug/ml	S.P. University, Anand.
pPAT-*ybdK*	6264 bp	1119 bp *ybdK* ligated in pBBR1MCS2 plasmid	Kanamycin 25ug/ml	*ybdK* gene was amplified by PCR using suitable primers from *Escherichia coli* DH10B genome and then ligated in pBBR1MCS2 plasmid after a restriction digestion of vector

				and PCR amplified gene
Forward primer	34 bp	CGCTCGAGAGGAGGATAGTCATGCCATTACCCGA 34 base pairs primer flanking Xho1(CTCGAG) restriction enzyme site at 5' end	NA	Thermoscientific
Reverse primer	28 bp	TTAAGCTTTTAGTCACCGGCCCAGATCT 28 base pairs primer flanking Hind3 (AAGCTT) restriction enzyme site at 5'end	NA	Thermoscientific

3.2.2 Bacterial growth media composition

Luria -Bertani (LB) media was used to grow and maintain the *E. coli* DH10B bacteria used in this study. LB is a nutrient media and it is composed of the components such as Tryptone (10g/L); Yeast extract (5g/L) and Sodium chloride (10g/L). LB broth as well as LB agar, also known as Luria agar were used in this study. The LB agar media also contains Agar- agar (15g/L) for the solidification of media to prepare plates.[16] Yeast extract mannitol (YEM) media was used to grow and maintain the rhizobium bacteria (mentioned in Table 3.1) used in this study. YEM is a nutrient media generally used for the isolation and selection of rhizobium bacteria. YEM broth as well as YEM agar were used in this study. YEM contains the media components such as yeast extract (1g/L); mannitol (10g/L); dipotassium phosphate (0.5g/L); magnesium sulphate (0.2g/L) and sodium chloride (0.1g/L). The YEM agar also contains Agar-

agar (15g/L) for the solidification of media to prepare plates. [17] Some experiments were conducted in the M9 minimal media. The working concentration of the media components are components such as 1X M9 salts, 1X trace elements, 0.4 % Glucose, 1mM MgSO4, 0.03 mM CaCl2 and 1ug/ml Vitamin (B1/B7).[18] King's B is a nutrient media and it is used for detection of the fluorescing bacteria, particularly *Pseudomonas fluorescence*.[19] King's B broth and King's B were used to grow and maintain the *P. fluorescence* (NAIMCC B-00342) bacteria used in this study. This growth media is composed of the components such as Peptone (20g/L); Dipotassium phosphate (1.5g/L) and Magnesium sulphate (1.5g/L). The King's B agar also contains Agar-agar (15g/L) for the solidification of media to prepare plates. All the media were sterilized by autoclaving it at 121 ℃ for 15 minutes before inoculation.

3.2.3 Genomic DNA extraction and PCR amplification

Single colony of *E. coli DH10B* form the plate was picked up and inoculated in a test tube containing 5 ml LB broth. It was allowed to grow overnight at 37 °C in an incubator shaker at 150 rpm. Cells were pelleted down at 5000 g for 5 minutes. Genomic DNA from the pellet was extracted by a standard CTAB method.[20] The genomic DNA of *E. coli* DH10B was used as a template and the gene of interest was amplified by a PCR method[21] using a thermocycler machine (Applied Biosystems, Veriti^{TM}). Gradient PCR protocol was set up to confirm the proper annealing temperature of the primers. A range of annealing temperature was used for the primers (Table 3.3). The genomic DNA and the amplified gene of interest were quantified using a nanodrop spectrophotometer. Details regarding the primers used in a PCR reaction is mentioned in Table 2. For a PCR reaction, 20ul system was used which comprised of 2ul PCR buffer (15mM MgCl2); 1.6ul DNTP's; 1ul forward primer; 1ul reverse primer; 0.2ul Taq. Polymerase enzyme; 14.2 ul Water. PCR reaction mixture the reaction mixture was subjected to electrophoresis on the agarose gel (1% w/v), supplemented with ethidium bromide (0.5ug/ml) at 100 V for 1.5 hours. The FASTA sequence of the gene of our interest, which is 1119 base pairs long *ybdK* gene is shown in Figure 3.1.

Table 3.3 PCR protocol for amplification of *E. coli* DH10B *ybdK* gene

PCR amplification protocol for 25 cycles		
1.	Initial denaturation	94 °C for 5 minutes
2.	Denaturation	94 °C for 30 seconds
	Annealing **(Gradient)**	57-61 °C for 30 seconds
	Extension	72 °C for 90 seconds
3.	Final extension	72 °C for 10 minutes

5’

ATGCCATTACCCGATTTTCATGTTTCTGAACCTTTTACCCTCGGTATTGAACTGG
AAATGCAGGTGGTTAATCCGCCGGGCTATGACTTAAGCCAGGACTCTTCAATG
CTGATTGACGCGGTTAAAAATAAGATCACGGCCGGAGAGGTAAAGCACGATAT
CACCGAAAGTATGCTGGAGCTGGCGACGGATGTTTGCCGTGATATCAACCAGG
CTGCCGGGCAGTTTTCAGCGATGCAGAAAGTCGTATTGCAGGCAGCCACAGAC
CATCATCTGGAAATTTGCGGCGGTGGCACGCACCCGTTTCAGAAATGGCAGCG
TCAGGAGGTATGCGATAACGAACGCTATCAACGCACGCTGGAAAACTTTGGTT
ATCTCATTCAGCAGGCGACCGTTTTTGGTCAGCATGTCCATGTTGGCTGCGCCA
GTGGCGATGACGCCATTTATTTGCTGCACGGCTTGTCACGATTTGTGCCGCACT
TTATCGCCCTTTCCGCCGCGTCGCCATATATGCAGGGAACGGATACGCGTTTTG
CCTCCTCACGACCGAATATTTTTTCCGCCTTTCCTGATAATGGCCCGATGCCGT
GGGTCAGTAACTGGCAACAATTTGAAGCCCTGTTTCGCTGTCTGAGTTACACCA
CGATGATCGACAGCATTAAAGATCTGCACTGGGATATTCGCCCCAGTCCTCATT
TTGGCACGGTGGAGGTTCGGGTGATGGATACCCCGTTAACCCTTAGCCACGCA
GTAAATATGGCGGGATTAATTCAGGCTACCGCCCACTGGTTACTGACGGAACG
CCCGTTTAAACATCAGGAAAAAGATTACCTGCTGTATAAATTCAACCGTTTCCA
GGCCTGTCGCTATGGGCTTGAAGGCGTCATCACCGATCCGCACACTGGAGATC
GTCGACCGCTAACGGAAGATACCTTGCGATTGCTGGAAAAAATCGCCCCTTCC
GCACATAAAATTGGTGCATCGAGCGCGATTGAGGCCCTGCATCGCCAGGTCGT
CAGCGGTCTGAATGAAGCGCAGCTAATGCGCGATTTCGTCGCCGATGGCGGCT
CGCTGATTGGGCTGGTGAAAAAGCATTGTGAGATCTGGGCCGGTGACTAA

3’

Figure 3.1 FASTA sequence of *E. coli* DH10B *ybdK* gene

3.2.4 Restriction cloning

The plasmid pBBR1MCS2 (vector/backbone) and the amplified PCR product (gene of interest) were subjected to restriction digestion[22] by Xho1 (10U/uL) and Hind III (10U/uL) procured from ThermoFisher scientific. Sequential digestion was carried out in a 20ul system. The protocol for restriction digestion used in the study is described in brief in **Table 3.4.** Digestion was confirmed by loading the digested mixture on a 1% agarose gel for electrophoresis and observing it under UV illumination on a transilluminator. Digested products were eluted form the gel by a gel elution method and their concentration was measured by a nanodrop spectrophotometer. Elution was done in 10ul deionized water. Eluted vector and the gene of interest were ligated using a T4 DNA ligase (ThermoFisher Scientific). Ligation reaction was carried out in a 20 ul volume system which had the 1 ul digested pBBR1MCS2; 5 ul digested *ybdK*; 2 ul T4 buffer; 0.8 ul T4 DNA ligase and 11.2 ul Water. Ligation mixture was incubated at 25 °C for 15 minutes.

Table 3.4 Protocol for Sequential digestion of a Vector and the gene of interest

Step 1		
Materials	**pBBR1MCS2 plasmid (600ng/ul)**	***ybdK* gene amplicon (200 ng/ul)**
DNA	3.3 ul	10 ul
XhoI	1ul	1ul
Water	13.7 ul	7 ul
1X R buffer	2ul	2ul
Total volume	**20 ul**	**20ul**
Incubate it at 37°C for 14 hours and then deactivate it by heating the mixture at 80°C for 10 minutes		
After digestion with XhoI enzyme, add 1ul HindIII enzyme as shown in step 2		
Step 2		
HindIII	1ul	1ul
1X R buffer	1ul	1ul
Incubate it at 37°C for 14 hours and then deactivate it by heating the mixture at 80°C for 10 minutes		

3.2.5 Bacterial transformation and plasmid purification

Standard calcium chloride transformation method[23] was used to transform *E. coli* DH10B. The ligation mixture (mixture obtained after ligation of vector and the gene of interest) was used to transform the *E. coli* DH10B. After the transformation procedure, cells were spreaded on the LB agar plates supplemented with kanamycin (25µg/ml) for selection and were allowed to grow overnight at 37 °C. Single colony was selected from the plate and inoculated in a test tube containing 3 ml LB broth. It was incubated in an incubator shaker at 37 °C and allowed to grow at 120 rpm till its OD reach 0.8. Cells were pelleted down and the recombinant plasmid was isolated from them by using a modified alkaline lysis method involving silicone dioxide powder. [24]

3.2.6 Plasmid validation by restriction digestion

To verify the construction of a recombinant plasmid, it was subjected to restriction digestion.[25] Recombinant plasmid was sequentially digested with the enzymes Xho1 and Hind3, as shown in Table 4. The digested products were validated by electrophoresis on agarose gel (1% w/v) at 100 V for 1 hour. Agarose gel was stained with ethidium bromide (0.5ug/ml) and visualized under the UV transilluminator. Empty vector/backbone was also sequentially digested with Xho1 and Hind3, which was used as a control in electrophoresis. To further verify the presence of *ybdK* gene in the recombinant plasmid, sangers sequencing was done.[26] Sequencing was done using BigDye terminator cycle sequencing kit on 3730xl DNA analyser (Applied Biosystems, CA, USA). For sequencing gene specific primers were used.

3.2.7 Transformation of rhizobium by electroporation

Electroporation technique was used to transform *S. fredii* NGR 234 and *S. meliloti* (NIAMCC B-00836) with the recombinant plasmid.[27,28] Bio-Rad, Genepulsar X-cell total system was used for this purpose. Pulse of 12kv/cm for the fixed time of 5 milliseconds was used in order to get a maximum transformation efficiency.

3.2.8 Bacterial growth curve

A series of Erlenmeyer flask (100 ml) containing 30 ml YEM medium and M9 minimal medium, supplemented with respective antibiotics were inoculated with 1% (v/v) freshly grown bacterial culture. They were incubated at 28 °C in an incubator shaker at 120rpm. The overnight culture used for inoculation were prepared in the corresponding media. Growth was

monitored by measuring the OD_{600} of growing culture at a definite time interval. The time interval for different bacteria depends on their multiplying time.

3.2.9 Glutathione estimation from bacteria

Bacteria were grown in 30 ml YEM and M9 minimal media broth, supplemented with required antibiotics at 28 °C in a shaking condition at 120 rpm. Samples of cell suspension (1 ml) were collected by pelleting them down during logarithmic growth phases (0.5 OD and 0.8 OD) at 8000 g for 10 minutes. Cell pellet was washed twice with phosphate buffer and resuspended in 1ml cell lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 1% v/v Tween 20, adjust pH to 8.0 using NaOH and filter before use and store at 4°C. This is supplemented with 1mg/ml Lysozyme and 400 units/ml of DNAse Type I before using) followed by sonication at 0 °C, using a 30s pulse for 6 cycles. The amplitude of the pulse was set to 40% for efficient lysis of the bacterial cells. The lysate was treated with 20% w/v TCA solution (final concentration of TCA is 5% in the mixture) to precipitate all the proteins. Precipitates were removed by centrifugation at 13000 g for 15 minutes and supernatant was collected (whole process was done in dark condition at 0 °C) and stored at -80 °C for glutathione analysis. Intracellular total glutathione was determined by a colorimetric method which measures GSH before and after the reduction of GSSG to GSH by $NaBH_4$. [29]

3.3 Results and Discussion

3.3.1 Construction of a recombinant plasmid pPAT

The concentration of *E. coli* DH10B genomic DNA extracted by a CTAB method was recorded about 400ng/ul of DNA and its 260/280 ratio was 2.015. Since the quality of DNA was good, it was used as a template DNA in a PCR reaction. The primers (Table 3.2) were used to amplify the 1119 bp *ybdK* gene from the template DNA. A gradient PCR rection was set up and a range of annealing temperature (57 °C - 61 °C) was used for the primers as shown in Table 3.3. Proper amplification of the 1.1 kb gene was seen in every well of 1% agarose gel as shown in Figure 3.2. Schematic representation of the amplified product is shown in Figure 3.3. Since the annealing temperature points showed the positive result, anyone of them can be used as the annealing temperature. The PCR reaction was repeated by setting up the annealing temperature to 61 °C. The amplicon yielded by this reaction was stored at -20°C for further use.

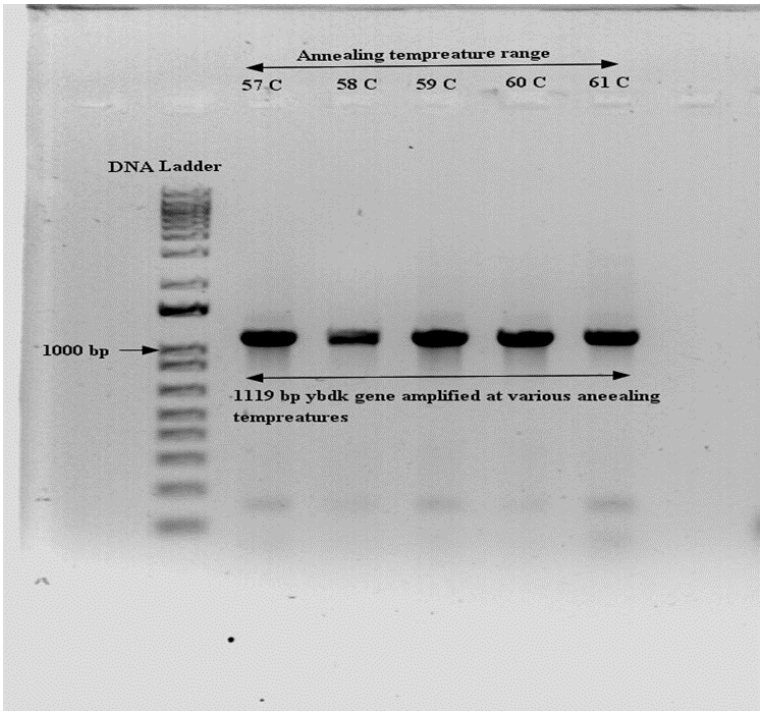


Figure 3.2 Agarose gel electrophoresis profile of *ybdK* gene amplicons generated by gradient PCR.

Figure 3.3 Schematic representation of the *ybdK* gene amplicon

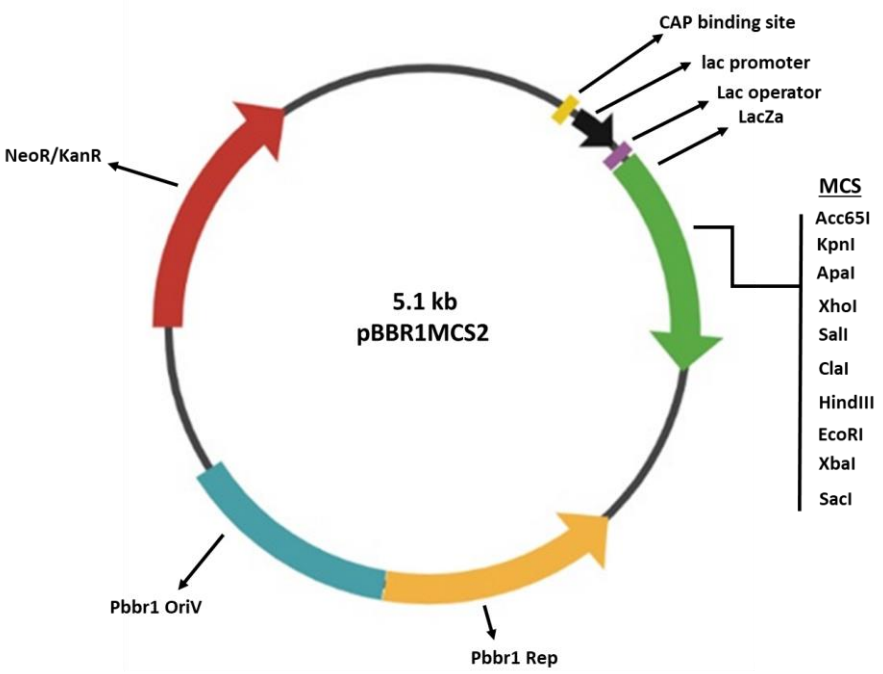


Figure 3.4 Schematic representation of an empty vector pBBR1MCS2 (Made in Biorender website)

PCR amplified product, *ybdK* gene (Figure 3.3) and a plasmid vector pBBR1MCS2 were sequentially digested by XhoI and Hind III enzymes. Schematic representation of an empty vector pBBR1MCS2 is shown in Figure 3.4. Amplicon and vector backbone were sequentially digested and validated by electrophoresis (Figure 3.5). Sequential digestion protocol was performed as shown in Table 3.4. Vector pBBR1MCS2 plasmid was linearized (5.1kb) after restriction digestion as seen in the lane 4. Undigested vector was loaded in lane 3 and 7 as a control. Digested amplicon *ybdK* gene (1.1 kb) was loaded in lane 6, while undigested in lane 8 (Figure 3.5). The size of the digested and the undigested DNA were checked by comparing them with an Invitrogen E gel 1kb plus DNA ladder, loaded in lane 5 (Figure 3.5). Digested vector and amplicon were purified from agarose gel by a gel elution method. After elution about 1.26 ug vector and 1.31 ug of amplicon was recovered, which is 37% loss for vector and 34.5% loss for amplicon.

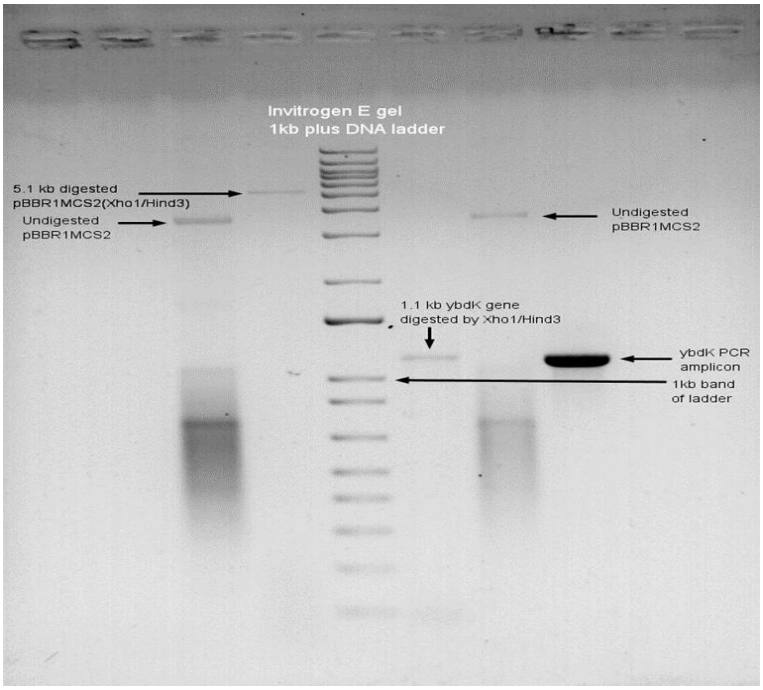


Figure 3.5 Agarose gel electrophoresis profile of the digested vector (pBBR1MCS2) and amplicon (*ybdK* gene).

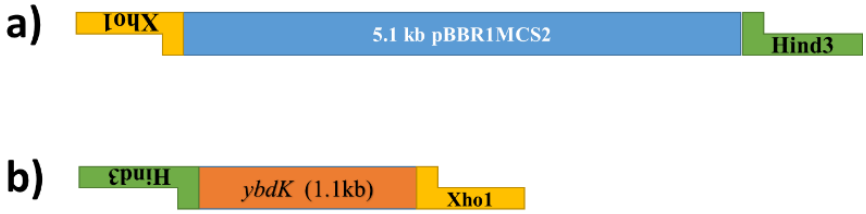


Figure 3.6 Schematic representation of digested vector pBBR1MCS2 (a) and the *ybdK* gene amplicon (b). Both vector and the gene of interest were linearized after digestion

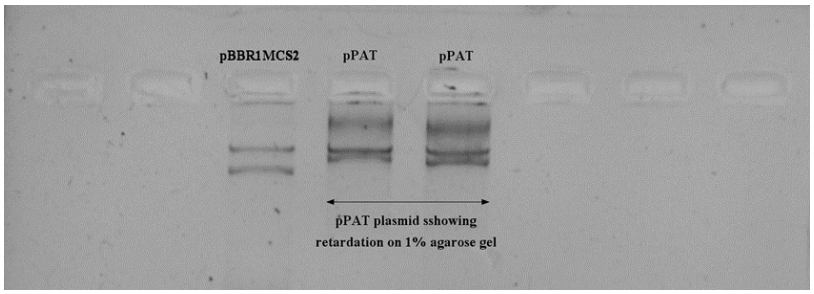


Figure 3.7 Agarose gel electrophoresis profile of a recombinant plasmid pPAT. Recombinant plasmid loaded in well 2 and 3 showing the shift on gel as compared to the empty vector (pBBR1MCS 2)

Schematic representation of linearized eluted vector and amplicon is shown in Figure 3.6.a and Figure 3.6.b respectively. Linearized vector and amplicon were ligated again by T4 DNA ligase in 1:5 ratio for an efficient ligation. 5ul of ligation mixture was used to transform wild type *E. coli* DH10B. The colonies harbouring the recombinant plasmid were isolated on LB-agar plates supplemented with 25µg/mL Kanamycin. Plasmid DNA was extracted from individual colonies by a modified alkaline lysis method and subjected to 1% agarose gel electrophoresis. The recombinant clones harbouring the insert were assessed by gel shift assay where the putative positive clone showed retarded movement compared to the control plasmid pBBR1MCS2 (Figure 3.7).

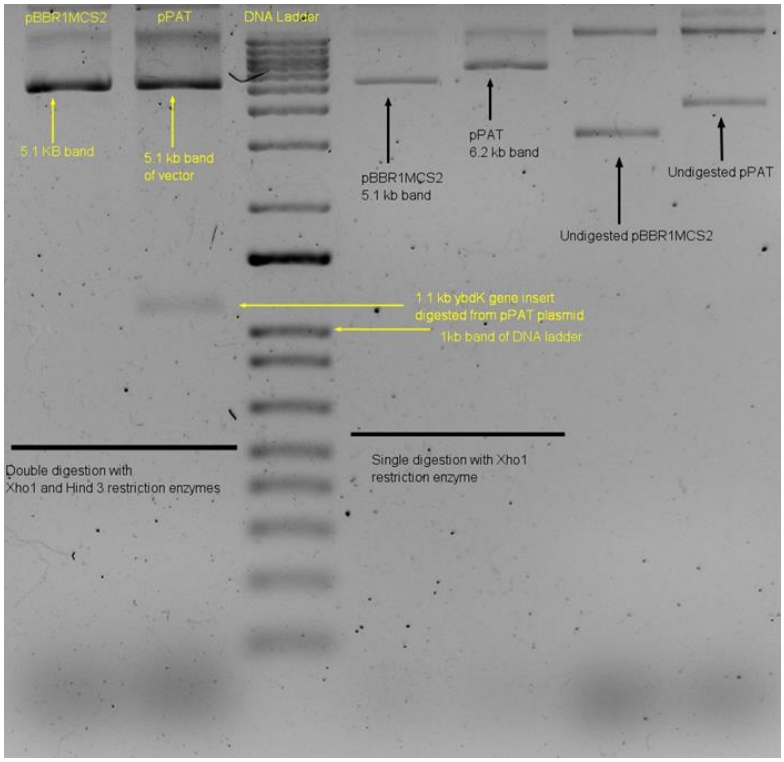


Figure 3.8 Agarose gel electrophoresis profile of the validation of recombinant plasmid by digestion. Digestion of pPAT gave 2 fragments of 5.1 kb and 1.1kb, while pBBR1MCS2 gave only one band of 5.1 kb

Integration of the insert was validated by restriction digestion of the DNA isolated from the clones. It was observed that pPAT on sequential digestion yielded 2 bands, 5.1kb of pBBR1MCS2 vector and 1.1 kb of *ybdK* gene (lane). While, pBBR1MCS2 sequential digestion gave only one band near 5.1 kb. This proves that the gene has been successfully integrated in pBBR1MCS2 to for a recombinant plasmid pPAT. Lane 3 is a DNA marker while lane 4 and

5 are the pBBR1MCS2 and pPAT digested by only XhoI restriction enzyme. Lane 4 shows a band of linearized pBBR1MCS2 vector at 5.1 kb and lane 5 shows a band of pPAT plasmid at 6.2 kb. Lane 7 and 8 shows the undigested pBBR1MCS2 and pPAT (recombinant plasmid respectively (Figure 3.8). This proves that the gene of interest *ybdK* has been successfully integrated in pBBR1MCS2 and a recombinant plasmid pPAT is formed. The schematic representation of pPAT is shown in Figure 3.9.

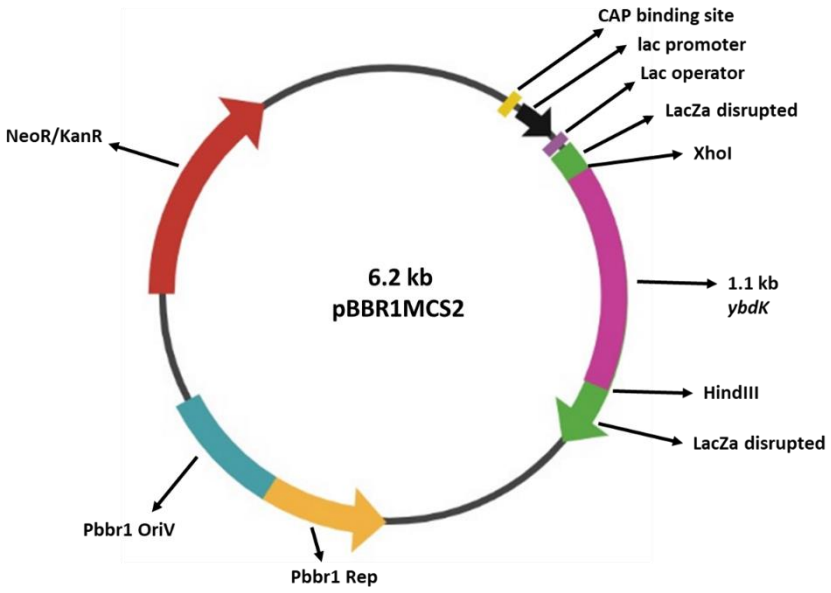


Figure 3.9 Schematic representation of pPAT plasmid

Gene specific forward primer ATGCCATTACCCGATTTTCATGTT and reverse primer TTAGTCACCGGCCCAGATCT were used for sanger sequencing of *ybdK* gene in the recombinant plasmid pPAT. FASTA format of forward and reverse sequences were obtained after carefully examining the electropherogram in Bioedit sequence alignment editor software. These sequences were blasted against *E. coli* DH10B genome (CP000948.1) database to check sequence homology, which is mentioned in the Table 3.5. Both the query sequences showed 100 % homology with the subject sequence. As only the gene specific primers were used, an entire ORF was not sequenced (Due to the lack of funding). Sequencing was only done with a purpose to confirm the presence of *ybdK* gene in the recombinant plasmid pPAT. The part which was sequenced by forward and reverse primers were absolutely identical with the subject sequence as shown in Table 3.5. Thus, by the results obtained from sequential digestion (Figure 3.8) and sequencing (Table 3.5), it was proved that *ybdK* gene is successfully integrated in pPAT plasmid.

Table 3.5 BLASTN of *ybdK* gene insert from pPAT plasmid with *E. coli* genome

Sr.no.	Query	Subject	Subject accession number	Query coverage	Percent identity	E value
1.	Forward sequence	*E. coli* genome	CP000948.1	100 %	100 %	0.0
2.	Reverse sequence	*E. coli* genome	CP000948.1	100 %	100 %	0.0

3.3.2 Transformation of rhizobium bacteria by the recombinant plasmid pPAT

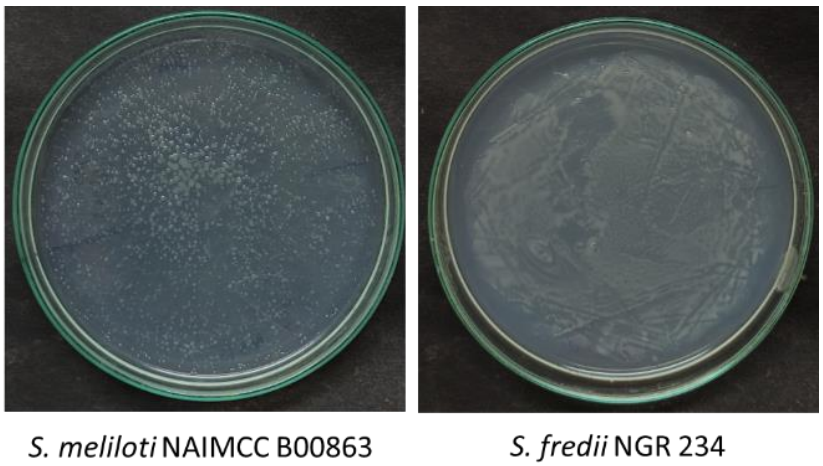


Figure 3.10 Transformation by electroporation. Rhizobial strains were electroporated with pPAT plasmid.

S. meliloti (NAIMCC B 00863) and *S. fredii* NGR 234 were transformed with the recombinant plasmid pPAT by an electroporation method. 12 kv/cm at a fixed time of 5 ms gave maximum transformation efficiency (Figure 3.10). To validate a proper transformation of rhizobia, the recombinant plasmid pPAT was extracted from the transformed colonies by a modified alkaline lysis method and checked of 1% agarose gel, with pBBR1MCS2 plasmid as a control. The recombinant plasmid was assessed by gel shift assay where the recombinant plasmid showed a retarded movement compared to the control pBBR1MCS2 plasmid (Figure 3.11) which confirmed a proper transformation of rhizobial strains. Recombinant plasmid extracted from rhizobia was also validated by PCR method (Table 3.3) by amplifying the *ybdK* gene by using the same set of primers used above. 1.1 *ybdK* gene was amplified form the pPAT plasmid extracted from *S. meliloti* (NAIMCC B 00863) (Figure 3.12.a) as well as *S. fredii* NGR 234

(Figure 3.12.b). After a successful transformation, *S. meliloti* (NAIMCC B 00863) and *S. fredii* NGR 234 were designated as *S. meliloti* (NAIMCC B 00863) (pPAT) and *S. fredii* NGR 234 (pPAT). Our study involving the use of *S. fredii* NGR 234 (pPAT), capable of secreting higher levels of glutathione in media for synthesis of cadmium sulphide nanoparticles is already published.[30]

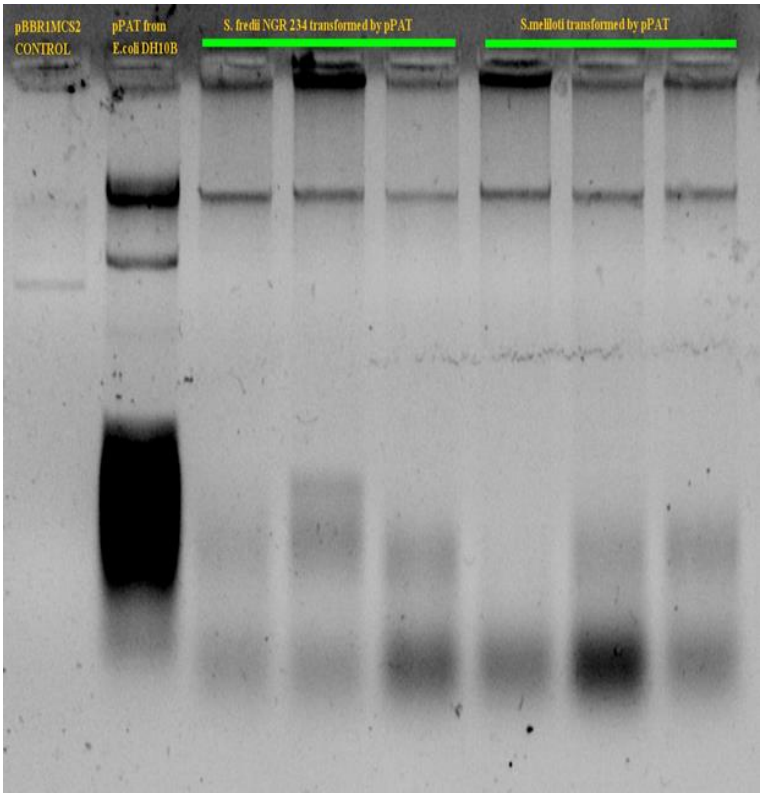


Figure 3.11 pPAT isolated from Rhizobial transformants. Agarose gel electrophoresis profile of a recombinant plasmid pPAT isolated from *S. fredii* NGR 234 and *S. meliloti* (NAIMCC B 00863). pBBR1MCS2 (negative control) is loaded in well no 1 and pPAT isolated form *E. coli* (positive control) is loaded in well no 2. pPAT isolated from *S. fredii* NGR234 is loaded in well no 3,4 and 5. pPAT isolated from *S. meliloti* (NAIMCC B 00863) is loaded in well no 6,7 and 8.

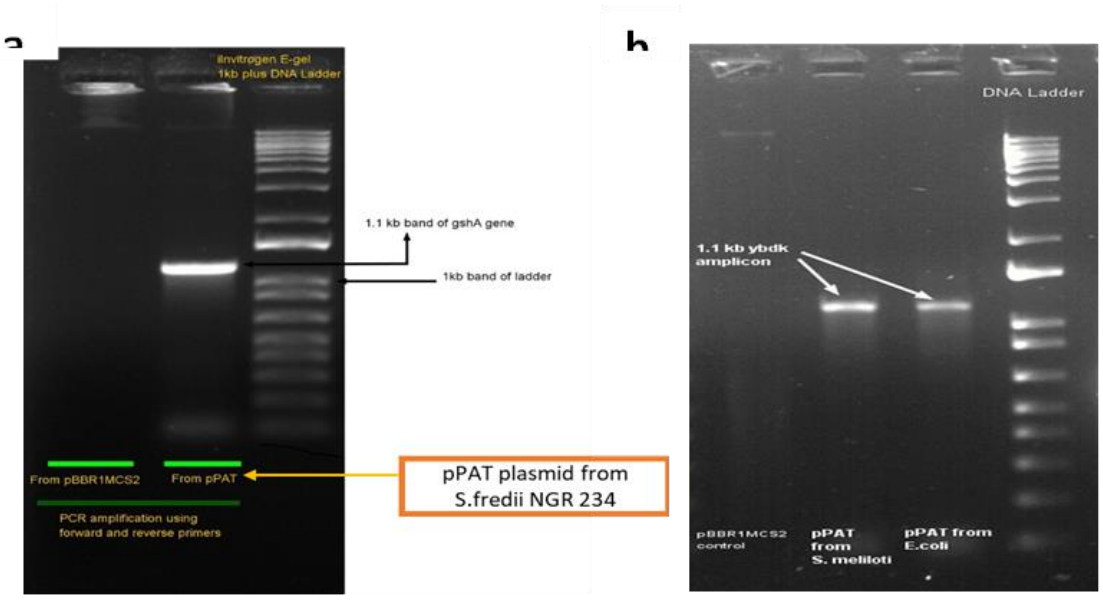
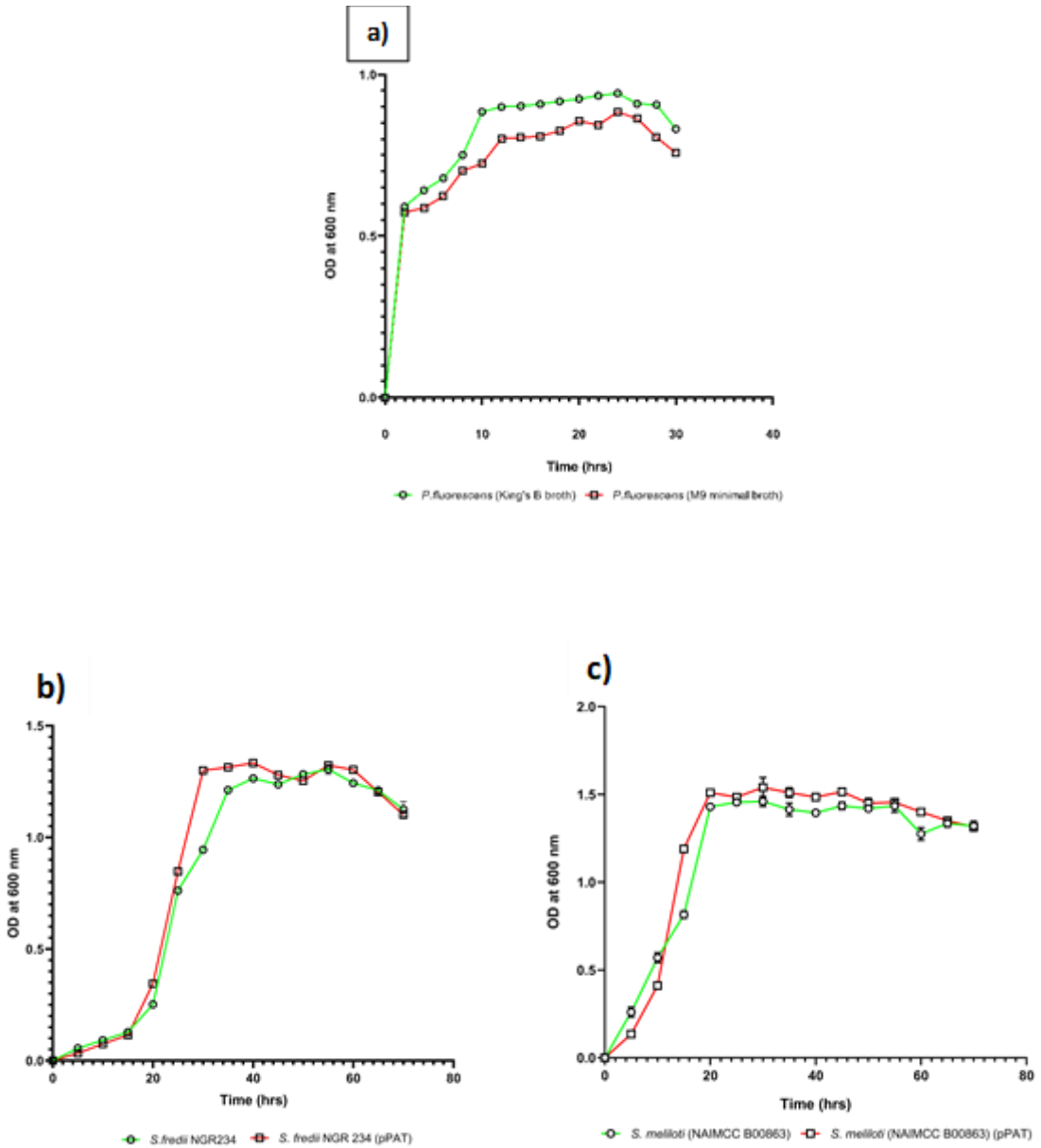


Figure 3.12 Validation of pPAT by PCR method. a) Agarose gel electrophoresis profile for the PCR validation of the recombinant plasmid pPAT isolated form S. fredii NGR 234. ybdK gene amplification from S. fredii NGR 234 (pPAT) b) Agarose gel electrophoresis profile for the PCR validation of the recombinant plasmid pPAT isolated form S. fredii NGR 234. ybdK gene amplification from S. fredii NGR 234 (pPAT)

3.3.3 Growth curve of wild type and transformed rhizobium

Figure 3.13 (a-e) shows the growth curve of different organisms in enriched and minimal media broth. This experiment was conducted to compare the growth pattern between wild type and transformants in order to check the effect of cloning *ybdK* gene. Growth pattern of *P. fluorescens* NAIMCC B00342 was also monitored which is used as a positive control in upcoming experiments. Figure 3.13.a. shows the growth curve of *P. fluorescens* NAIMCC B00342 in King's B broth and M9 minimal broth. It was observed that from 2nd hour onwards till late stationary phase, *P. fluorescens* NAIMCC B00342 maintained accelerated growth in King's B broth. Figure 3.13.b and c shows the growth pattern of *S. meliloti* NAIMCC B 00863, *S. fredii* NGR 234, *S. meliloti* NAIMCC B 00863 (pPAT) and *S. fredii* NGR 234 (pPAT) in YEM broth. Initially both transformants showed retarded growth compared to their respective wild type organisms, but later in the log phase the growth of transformants accelerated significantly compared to the wild type organisms. Similar growth trend was observed when the bacteria were grown in the M9 minimal broth, which is shown in Figure 3.13.d and e. Similar interpretations were reported when the growth pattern of *gshA* mutant and *gshA* complemented rhizobium were compared. Rhizobium having a *gshA* gene showed a better

growth in the log and late stationary phase compared to the rhizobium having a mutation in *gshA* gene. [31] Glutathione is very essential to maintain the normal growth in all the living beings including bacteria. [32]Since overexpression of YbdK protein leads to enhanced glutathione accumulation in the transformants compared to wild type (Figure 14), it might have protected the transformants efficiently from the generated ROS in broth during the course of its growth. Also, our results correlate to a fact that an engineered *E. coli* DH10B, capable of enhanced glutathione synthesis also showed enhanced growth in LB broth during the log phase in comparison to its wild type counterpart.[33]



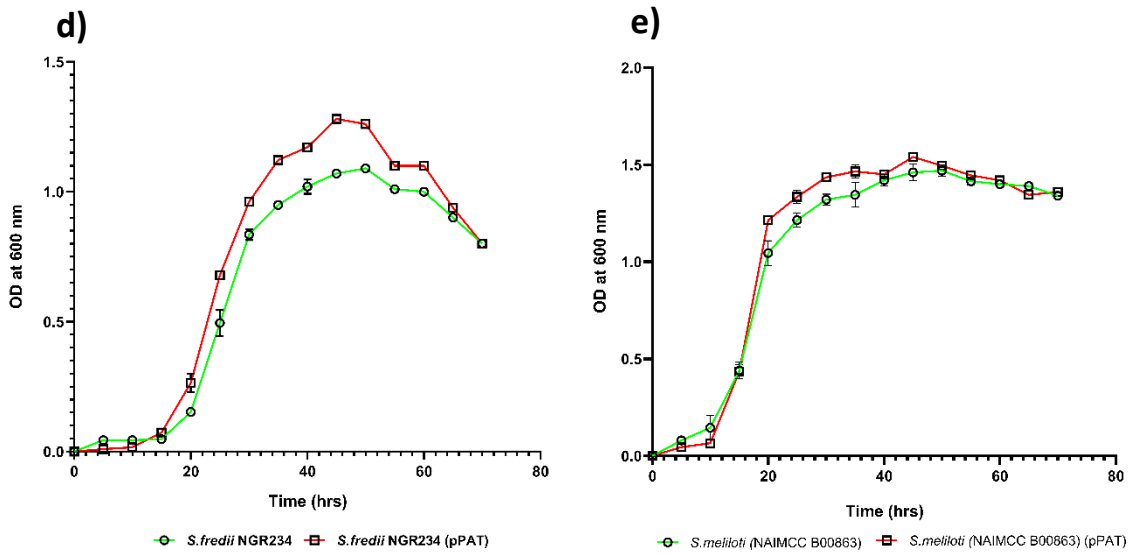


Figure 3.13 Growth curve of PGPR in YEM and M9 minimal medium. a) P. fluorescens growth curve in King's B (green) and minimal media (red) b) S. fredii NGR 234 (green) and S. fredii NGR 234 (pPAT) (red) growth curve in YEM medium c) S. meliloti (green) and S. meliloti (pPAT) growth curve in YEM media d) S. fredii NGR 234 (green) and S. fredii NGR 234 (pPAT) (red) growth curve in M9 minimal media e) S. meliloti (green) and S. meliloti (pPAT) growth curve in M9 minimal media.

3.3.4 Estimation of glutathione from the wild type and transformed rhizobium bacteria

Intracellular and extracellular glutathione was estimated from the wild type and GMO bacteria. Rhizobium bacteria were grown in YEM and M9 minimal media, while *Pseudomonas fluorescence* (NAIMCC B-00342) was grown in King's B and M9 minimal media. Bacteria were grown till OD_{600} value reached 0.6 and the cells were pelleted down by centrifugation at 5000 rpm for 5 minutes followed by lysis. 20ul lysate was aliquoted for the protein estimation by Bradford method and the remaining lysate was treated with TCA (to precipitate the proteins) and glutathione was estimated. Bacteria M1-M5 are mentioned on the x-axis of graphs, 3.14.a and 3.14.b. They are, M1; Pseudomonas fluorescence (NAIMCC B-00342), M2; Sinorhizobium fredii NGR 234, M3; Sinorhizobium fredii NGR 234 (pPAT), M4; Sinorhizobium meliloti (NIAMCC B-00836), M5; Sinorhizobium meliloti (NIAMCC B-00836) (pPAT). It was reported that M3 and M5 produced significantly higher levels of glutathione compared to M2 and M4 respectively, in YEM and M9 media. In YEM media M3 accumulated 1.41 folds more glutathione compared to M2, while M5 accumulated 1.13 folds more glutathione compared to M4. Similarly, in M9 minimal media M3 acumulated1.42 folds higher glutathione compared to M2, while M5 accumulated 1.20 folds higher glutathione

compared to M4. This result indicates that the transformants were able to accumulate higher amount of total glutathione inside the cells compared to their wild type counterparts (Figure 3.14.a).

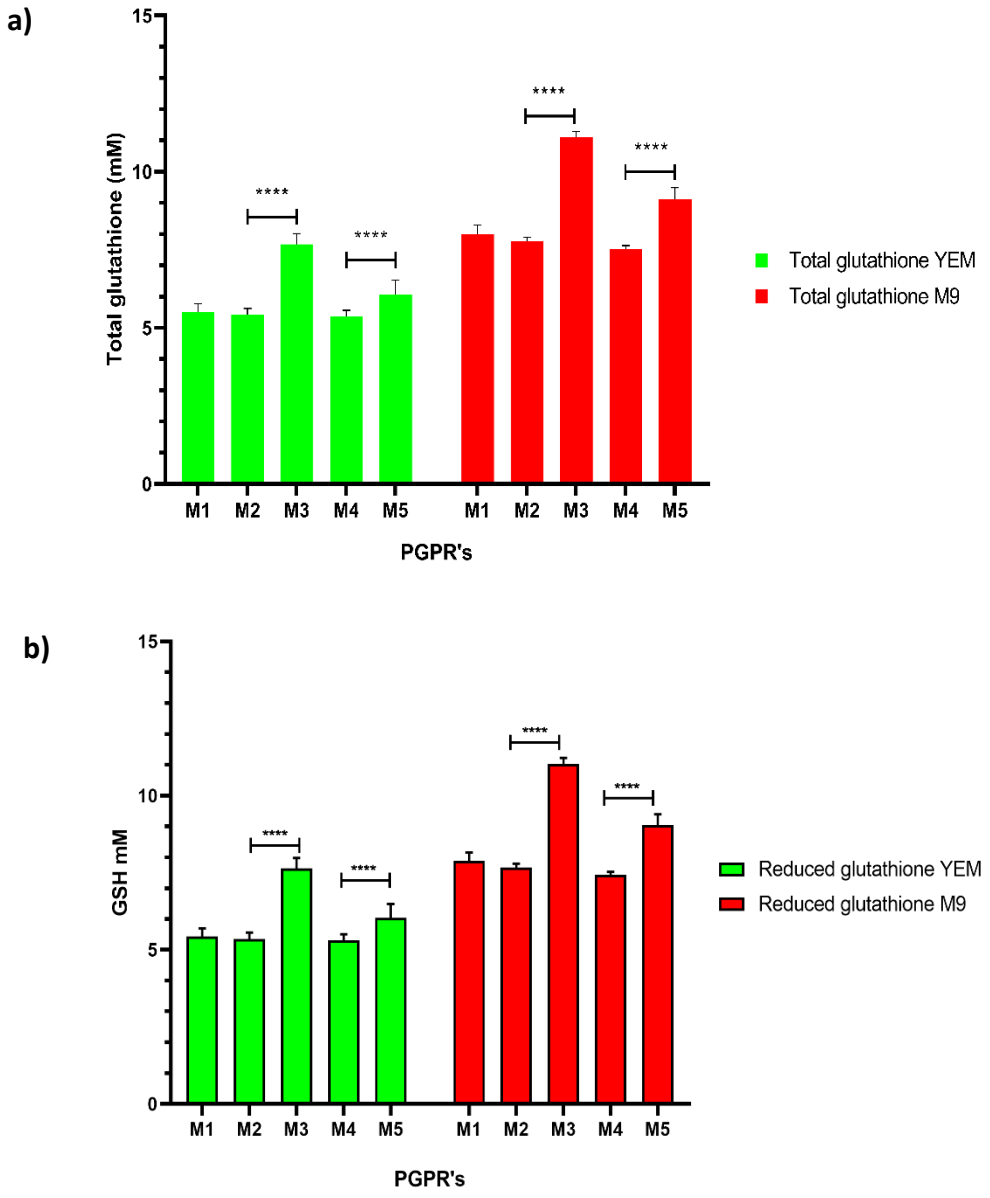


Figure 3.14 a) Estimation of Total glutathione (intracellular) from different PGPR b) Estimation of Reduced glutathione (intracellular) estimation from different PGPR.

Reduced glutathione (GSH) is an important marker for cellular health and oxidative stress level. Transformants growing in nutrient media as well as M9 minimal media were able to accumulate significantly higher amount of reduced glutathione compared to their wildtype

counterparts. In YEM media M3 accumulated about 1.43 folds more GSH compared to M2, while M5 accumulated around 1.13 folds more GSH compared to M4. Similarly, in M9 minimal media M3 accumulated 1.43 folds more GSH compared to M2, while M5 accumulated about 1.21 folds more GSH compared to M4 (Figure 3.1.4.b). This proves that the oxidative stress levels in transformants have significantly decreased. Besides this, all organisms successfully accumulated > 98% reduced glutathione of total glutathione. These results are similar to the results obtained for the gram-negative aerobic *E. coli* grown in laboratory conditions.[34] These results proves that rhizobial transformants containing *ybdK* gene were able to produce higher levels of intracellular glutathione compared to the wild type. Data (n=3) was analysed by GraphPad prism 8.0 software using Two-way ANOVA test and $p<0.05$ was considered to be statistically significant.

Table 3.6.a Estimation of extracellular glutathione from nutrient media

Organism	Total glutathione	Reduced glutathione	Oxidized glutathione	%Total extracellular glutathione of total internal glutathione
M1	0.402 ± 0.05	0.352 ± 0.03	0.050 ± 0.01	7.3
M2	1.083 ± 0.10	0.954 ± 0.08	0.129 ± 0.02	20.0
M3	1.654 ± 0.17	1.543 ± 0.16	0.110 ± 0.04	21.6
M4	1.007 ± 0.07	0.882 ± 0.04	0.125 ± 0.02	18.8
M5	1.206 ± 0.11	1.133 ± 0.09	0.073 ± 0.03	19.9

Table 3.6.b Estimation of extracellular glutathione from M9 media

Organism	Total glutathione	Reduced glutathione	Oxidized glutathione	%Total external glutathione of total internal glutathione
M1	0.109 ± 0.018	0.084 ± 0.016	0.025 ± 0.002	1.4
M2	0.166 ± 0.012	0.144 ± 0.013	0.022 ± 0.001	2.1
M3	0.374 ± 0.023	0.347 ± 0.027	0.027 ± 0.007	3.4
M4	0.156 ± 0.010	0.120 ± 0.011	0.035 ± 0.001	2.1
M5	0.224 ± 0.006	0.196 ± 0.004	0.028 ± 0.004	2.5

Table 3.6.a and 3.6.b shows the levels of glutathione secreted by different bacteria in the nutrient media and M9 minimal media respectively during log phase (OD_{600} = 0.6). Total extracellular/excreted glutathione was estimated from the growth media directly. In nutrient media, bacteria M1, M2, M3, M4 and M5 secreted 7.3 %, 20 %, 21.6 %, 18.8 % and 19.9 % total glutathione of its total intracellular glutathione respectively (Table 3.5.a). While in M9 minimal media, bacteria M1, M2, M3, M4 and M5 secreted 1.4 %, 2.1 %, 3.4 %, 2.1 % and 2.5 % total glutathione of its total intracellular glutathione respectively (Table 3.5.b). The data of the table represents mean ± SD (n=3), and $p<0.05$ were considered to be statistically significant. The concentration of excreted glutathione in the media was in accordance with various findings where aerobic gram-negative bacteria excreted around 1 % - 40 % of the total glutathione in the growth media.[35,36] In spite of significantly higher levels of intracellular glutathione in bacteria growing in M9 media, they secreted less glutathione in the media compared to the bacteria growing in YEM media. This could be due to the presence of glutathione in the yeast extract which was used to make YEM media. It contains a notable amount of glutathione.[37] There is a possibility that this additional amount of glutathione which is already present in the YEM media could be the reason for elevated levels of extracellular glutathione in YEM media compared to the M9 media. Also, the transformants growing in both media were able to secrete more glutathione compared to the wild type and the secretion by *S. fredii* NGR 234 (wild type and transformants) was highest, which could be due to its extensively developed secretion system. [38]

3.4 Conclusion

We conclude that after a successful and proper integration of the *ybdK* gene in pBBR1MCS2 plasmid, a 6.2 kb recombinant plasmid pPAT was created. Confirmation of YbdK enzyme expression was monitored by the estimation of glutathione levels from transformants (rhizobial strains bearing pPAT plasmid) and wildtype rhizobia. We observed that the transformation of the rhizobial strains (mentioned in Table 3.1) by pPAT plasmid significantly increased the glutathione production compared to their wild type counterparts. Thus, it is established that cloning *ybdK* gene in rhizobia (mentioned in Table 3.1) increases their capacity to produce glutathione in minimal as well as nutrient medium. Their enhanced capacity to excrete significantly higher levels of glutathione is also discussed in the results of Chapter 5.

3.5 References

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