

## List of Contents

<b>Chapter</b>	<b>Review of Literature and Introduction</b>	<b>Page No.</b>
<b>1</b>		
<b>1.1</b>	Rhizobia: The master microbe	1
<b>1.1.1</b>	General Taxonomy	1
<b>1.1.2</b>	Host Specificity and Nodulation	1
<b>1.1.3</b>	<i>Rhizobium</i> as nitrogen-fixer	8
<b>1.1.4</b>	Plant Growth Promoting Rhizobacteria (PGPR )	10
<b>1.1.4.1</b>	<i>Rhizobium</i> as PGPR for non-legumes.	14
<b>1.1.5</b>	Effect of P on nodulation	15
<b>1.1.6</b>	Phosphorus deficiency and Nitrogen fixation	16
<b>1.1.7</b>	<i>Rhizobium</i> - PSM co-inoculation	17
<b>1.1.8</b>	Phosphate solubilization by <i>Rhizobium</i> spp.	22
<b>1.1.9</b>	Phosphorus in agriculture	23
<b>1.2</b>	Central Carbon Metabolism	30
<b>1.2.1</b>	<i>Escherichia coli</i> –as the Model Organism	32
<b>1.2.2</b>	Glucose catabolic pathways in pseudomonads	33
<b>1.2.3</b>	Glucose Metabolism in <i>Bacillus subtilis</i>	34
<b>1.2.4</b>	Glucose Metabolism in <i>Rhizobium</i>	35
<b>1.2.5</b>	Importance of PEP-Pyruvate-OAA branch point in the cellular metabolism	42
<b>1.2.5.1</b>	PEP-Pyruvate-OAA node in <i>E. coli</i>	45
<b>1.2.5.2</b>	PEP-Pyruvate-OAA node in <i>B. subtilis</i> and <i>Corynebacterium glutamicum</i>	46
<b>1.2.6</b>	Comparison of Central Carbon metabolism	49
<b>1.3</b>	Metabolic engineering	51
<b>1.3.1</b>	Metabolic engineering of organic acids for P solubilization.	55
<b>1.3.2</b>	Genetic manipulations at the anaplerotic node in <i>E. coli</i> , <i>B. subtilis</i> and <i>C. glutamicum</i>	56
<b>1.3.3</b>	Metabolic engineering of organic acids for P solubilization.	58

<b>1.3.3.1</b>	Role of citric acid in P solubilization	58
<b>1.3.3.2</b>	Metabolic engineering of rhizobacteria for citric acid secretion.	59
<b>1.4:</b>	Rationale	61
<b>1.5</b>	Objectives of the present study	63
<b>Chapter 2</b>	<b>Materials and Methods</b>	<b>Page No.</b>
<b>2.1</b>	Bacterial strains / Plasmids	65
<b>2.2.1</b>	M9 minimal medium	70
<b>2.2.2</b>	Tris buffered medium	71
<b>2.2.3</b>	Pikovskaya's (PVK) Agar	71
<b>2.2.4</b>	Yeast Extract Mannitol Broth (YEMB)	71
<b>2.2.5</b>	Tryptone Yeast Extract Medium (TYE)	72
<b>2.3</b>	Morphological characterization and antibiotic sensitivity profile	72
<b>2.4</b>	Molecular biology tools and techniques	72
<b>2.4.1</b>	Isolation of plasmid and genomic DNA	72
<b>2.4.1.1</b>	Plasmid DNA isolation from <i>E. coli</i> and <i>B. japonicum USDA110</i> and <i>M. loti MAFF0300669 MAFF030669</i> transformants	72
<b>2.4.1.2</b>	Genomic DNA isolation from <i>Rhizobium</i>	73
<b>2.4.2</b>	Transformation of plasmid DNA	73
<b>2.4.2.2</b>	Transformation of plasmid DNA by Electroporation	73
<b>2.4.3</b>	Transfer of plasmid DNA by conjugation	74
<b>2.4.4</b>	Agarose gel electrophoresis	74
<b>2.4.5</b>	Restriction enzyme digestion analysis	75
<b>2.4.6</b>	Gel elution and purification	
<b>2.4.7</b>	Ligation	75
<b>2.4.8</b>	Polymerase Chain Reaction (PCR)	76
<b>2.4.9</b>	Genome integration.	78

<b>2.5</b>	P-solubilization phenotype	78
<b>2.6</b>	Physiological experiments	79
<b>2.6.1</b>	Inoculum preparation	79
<b>2.6.2</b>	Growth characteristics and pH profile	79
<b>2.7</b>	Estimation of Plant growth promoting factors	80
<b>2.7.1</b>	Culture conditions for EPS production and quantification	80
<b>2.7.2</b>	Biofilm assay <i>Biofilm</i>	81
<b>2.7.3</b>	Indole acetic acid (IAA) production and estimation	81
<b>2.8</b>	Analytical techniques	82
<b>2.9</b>	Enzyme assays	84
<b>2.9.1</b>	Preparation of cells and cell free extracts	84
<b>2.9.2</b>	Enzyme Assay Protocols	85
<b>2.9.2.1</b>	PPC assay	85
<b>2.9.2.2</b>	PYC assay	85
<b>2.9.2.3</b>	G-6-PDH assay	86
<b>2.9.2.4</b>	GDH assay	86
<b>2.9.2.5</b>	ICL assay	86
<b>2.9.2.6</b>	ICDH assay	86
<b>2.9.2.7</b>	CS assay	87
<b>2.10</b>	Inoculation of mung beans ( <i>Vigna radiata</i> )	88
<b>2.11</b>	Pot experiments- Interaction with mung beans	88
<b>2.11.1</b>	Preparation of inoculum	87
<b>2.11.2</b>	Growth Analysis	88
<b>2.11.2.1</b>	Growth Analysis: Above ground parts	88
<b>2.11.2.2</b>	Growth Analysis: Below ground parts	88
<b>2.11.3</b>	Antioxidant Enzymes / ROS scavenging enzyme activity:	88
<b>2.11.3.1</b>	Superoxide Dismutase (SOD)	90
<b>2.11.3.2</b>	Catalase	90
<b>2.11.3.3</b>	Guaiacol Peroxidase (POX)	91
<b>2.11.3.4</b>	Ascorbate peroxidase (APX)	91

*B. japonicum*  
USDA110

*M. loti* MAFF  
303099

<b>2.11.4</b>	Acetylene Reduction Assay (ARA):	92
<b>2.11.5</b>	Chlorophyll Content	92
<b>2.11.6</b>	Estimation of Water Soluble Protein Content	93
<b>Chapter 3</b>	<b>Effect of constitutive overexpression of <i>ppc</i> gene of <i>Synechococcus elongatus</i> PCC 6301 on production of organic acid in <i>B. japonicum</i> USDA110 and <i>M. loti</i> MAFF030669.</b>	<b>Page No.</b>
<b>3.1</b>	INTRODUCTION	94
<b>3.1.1</b>	Effects of <i>ppc</i> gene overexpression in <i>E. coli</i> and other organisms.	94
<b>3.1.2</b>	Why heterologous <i>ppc</i> gene?	95
<b>3.2</b>	EXPERIMENTAL DESIGN	100
<b>3.2.1</b>	Bacterial strains used in this study	101
<b>3.2.2</b>	Development of <i>B. japonicum</i> USDA110 and <i>M. loti</i> MAFF030669 strains harboring <i>ppc</i> gene of <i>S. elongatus</i> PCC6301	103
<b>3.2.3</b>	Growth and MPS phenotype of transformant strains of <i>Rhizobium</i>	103
<b>3.2.4</b>	Effect of heterologous <i>ppc</i> gene expression on the physiology and glucose metabolism.	103
<b>3.3</b>	RESULTS	103
<b>3.3.1</b>	Heterologous overexpression of <i>S. elongatus</i> PCC 6301 <i>ppc</i> gene in <i>Rhizobium</i> strains.	103
<b>3.3.2</b>	Effect of <i>S. elongatus</i> PCC 6301 <i>ppc</i> gene overexpression on growth pattern and pH profile in TRP medium.	106
<b>3.3.5:</b>	Physiological effects of <i>ppc</i> overexpression in TRP medium	108
<b>3.3.6</b>	Biofilm, exopolysaccharide and indole acetic acid production by <i>Bj</i> (pAB3) and <i>Ml</i> (pAB3) transformants in TRP medium.	109
<b>3.3.7</b>	P solubilization and organic acid secretion in TRP medium.	109
<b>3.3.8</b>	Alterations in enzyme activities in <i>Bj</i> (pAB3) and <i>Ml</i> (pAB3) transformants.	111

<b>3.4</b>	DISCUSSION	113
<b>Chapter 4</b>	<b>Effect of overexpression of <i>E. coli</i> cs gene on production of organic acid in <i>B. japonicum</i> USDA110 and <i>M. loti</i> MAFF030669</b>	<b>Page No.</b>
<b>4.1</b>	INTRODUCTION	119
<b>4.1.1</b>	Biochemical basis of citric acid accumulation in bacteria	121
<b>4.1.2</b>	Genetic manipulations for citric acid overproduction	121
<b>4.1.4</b>	Rationale for cs gene overexpression in Rhizobium spp.	124
<b>4.2</b>	EXPERIMENTAL DESIGN	125
<b>4.2.1</b>	Bacterial strains and plasmids used in this study	125
<b>4.2.2</b>	Development of <i>B. japonicum</i> USDA110 and <i>M. loti</i> MAFF0300669 harboring <i>E. coli</i> cs gene	127
<b>4.2.3</b>	Effect of <i>E. coli</i> cs gene expression on the physiology and glucose metabolism of <i>B. japonicum</i> USDA110 and <i>M. loti</i> MAFF0300669	127
<b>4.3</b>	RESULTS	128
<b>4.3.1</b>	Heterologous overexpression of <i>E. coli</i> cs gene in <i>B. japonicum</i> USDA110 and <i>M. loti</i> MAFF030669.	128
<b>4.3.2</b>	Effect of <i>E. coli</i> cs gene overexpression on growth pattern and pH profile in TRP medium.	131
<b>4.3.3</b>	Physiological effects of <i>E. coli</i> cs overexpression in TRP medium .	133
<b>4.3.4</b>	Biofilm, exopolysaccharide and indole acetic acid production by in <i>Bj</i> (pAB7) and <i>Ml</i> (pAB7) transformants in TRP medium.	134
<b>4.3.5</b>	P Solubilization and Organic acid secretion by <i>Bj</i> (pAB7) and <i>Ml</i> (pAB7) transformants in TRP medium.	134
<b>4.3.6</b>	Alterations in enzyme activities in <i>Bj</i> (pAB7) and <i>Ml</i> (pAB7) transformants.	137
<b>4.4</b>	DISCUSSION	139

<b>Chapter 5</b>	<b>Effect of overexpression of <i>E. coli</i> NADH insensitive Y145F cs gene on production of organic acid in <i>B. japonicum</i> USDA110 and <i>M. loti</i> MAFF030669.</b>	<b>Page No.</b>
<b>5.1</b>	INTRODUCTION	144
<b>5.1.1</b>	CS and NADH sensitivity	144
<b>5.1.2</b>	NADH insensitive CS	145
<b>5.1.3</b>	Increase in production of Citric acid	148
<b>5.1.4</b>	Rational of the present study	154
<b>5.2</b>	Experimental design	155
<b>5.2.1</b>	List of bacterial strains used	155
<b>5.2.2</b>	Details of Plasmid used	156
<b>5.3</b>	RESULTS	157
<b>5.3.1</b>	Heterologous overexpression of <i>E. coli</i> NADH insensitive cs* gene in <i>Rhizobium</i> spp.	159
<b>5.3.2</b>	Effect of <i>E. coli</i> NADH insensitive cs gene overexpression on growth pattern and pH profile in TRP medium	159
<b>5.3.3</b>	Physiological effects of <i>E. coli</i> NADH insensitive cs gene overexpression in TRP medium .	161
<b>5.3.4</b>	Biofilm, exopolysaccharide and indole acetic acid production by <i>Bj</i> (pJNK3) and <i>Ml</i> (pJNK3) transformants in TRP medium.	162
<b>5.3.5</b>	P Solubilization and Organic acid secretion by <i>Bj</i> (pJNK3) and <i>Ml</i> (pJNK3) transformants in TRP medium	162
<b>5.4</b>	DISCUSSION	166
<b>Chapter 6</b>	<b>Effect of overexpression of <i>E. coli</i> NADH insensitive Y145F cs along with Na<sup>+</sup> dependant citrate transporter citC gene in <i>B. japonicum</i> USDA110 and <i>M. loti</i> MAFF030669.</b>	<b>Page No.</b>
<b>6.1</b>	INTRODUCTION	170
<b>6.1.1</b>	Citrate transporters in fungi and bacteria	170

<b>6.1.2</b>	Structural Model of 2-HCT transporters	177
<b>6.1.3</b>	Rationale of the present work	184
<b>6.2</b>	EXPERIMENTAL DESIGN	185
<b>6.2.1</b>	Bacterial strains used in this study	185
<b>6.3</b>	RESULTS	186
<b>6.3.1</b>	Heterologous overexpression of <i>E. coli</i> NADH insensitive <i>cs*</i> gene along with citrate transporter <i>citC</i> gene of <i>S. typhimurium</i> in <i>Rhizobium</i> spp.	186
<b>6.3.2</b>	Effect of <i>E. coli</i> NADH insensitive <i>cs*</i> gene and citrate transporter <i>citC</i> gene of <i>S. typhimurium</i> gene overexpression on growth pattern and pH profile in TRP medium.	189
<b>6.3.3</b>	Physiological effects of <i>E. coli</i> NADH insensitive <i>cs*</i> gene along with citrate transporter <i>citC</i> gene of <i>S. typhimurium</i> gene overexpression in TRP medium.	191
<b>6.3.4</b>	Biofilm, exopolysaccharide and indole acetic acid production by <i>Bj</i> (pJNK4) and <i>Ml</i> (pJNK4) transformants in TRP medium.	192
<b>6.3.5</b>	P Solubilization and organic acid secretion by <i>Bj</i> (pJNK4) and <i>Ml</i> (pJNK4) transformants in TRP medium.	192
<b>6.4</b>	DISCUSSION	197
<b>Chapter 7</b>	Genomic integration of <i>E. coli</i> NADH insensitive <i>cs</i> and <i>Salmonella typhimurium</i> Na <sup>+</sup> dependent citrate transporter with <i>vgb</i> , <i>egfp</i> in <i>B. japonicum</i> USDA110 <i>M. loti</i> MAFF030669 and <i>S. fredii</i> NGR 234	<b>Page No.</b>
<b>7.1</b>	Introduction	200
<b>7.2</b>	Experimental design	204
<b>7.2.1</b>	Bacterial strains used in this study	204
<b>7.2.2</b>	Cloning of artificial citrate operon in integration vector.	205
<b>7.3</b>	RESULTS	206

<b>7.3.1</b>	Construction of Genome integrants of <i>B. japonicum</i> USDA110, <i>M. loti</i> MAFF0300669 . and <i>S. fredii</i> NGR234	206
<b>7.3.2</b>	CS activity and MPS ability of <i>B. japonicum</i> USDA110, <i>M. loti</i> MAFF0300669 . and <i>S. fredii</i> NGR234 integrants on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.	208
<b>7.3.3</b>	Growth pattern and pH profile of <i>B. japonicum</i> USDA110, <i>M. loti</i> MAFF0300669 . and <i>S. fredii</i> NGR234integrants on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.	210
<b>7.3.4</b>	Physiological effects of genomic integration on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.	212
<b>7.3.5</b>	P solubilization and organic acid by <i>B. japonicum</i> USDA110, <i>M. loti</i> MAFF0300669 and <i>S. fredii</i> NGR234 integrants in 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.	213
<b>7.3.6</b>	Alterations in enzyme activities in <i>B. japonicum</i> USDA110, <i>M. loti</i> MAFF0300669 and <i>S. fredii</i> NGR234 integrants	216
<b>7.4</b>	DISCUSSION	219
<b>Chapter 8</b>	<b>Effect of <i>Sinorhizobium fredii</i> NGR 234 genomic integrant containing <i>E. coli</i> NADH insensitive cs and <i>S. typhimurium</i> citC, vgb and egfp gene cluster on growth promotion of Mung bean (<i>Vigna radiata</i>) plants</b>	<b>Page No.</b>
<b>8.1</b>	Introduction	221
<b>8.1.2</b>	Rationale of the Study	228
<b>8.2</b>	Experimental design	228
<b>8.2.1</b>	Bacterial strains used in this study	228
<b>8.2.2</b>	Plant inoculation experiments	228

<b>8.2.3</b>	Greenhouse experiment	229
<b>8.2.4</b>	Growth parameter assessment	229
<b>8.2.5</b>	Biochemical characterization	229
<b>8.2.6</b>	PQQ determination	230
<b>8.2.7</b>	Isolation of bacteria from rhizospheric soil and nodules.	230
<b>8.2.8</b>	Statistical analysis	230
<b>8.3</b>	RESULTS	230
<b>8.3.1</b>	Effect of genomic integrant on bacteria of Rhizospheric soil and bacteroids of nodules.	230
<b>8.3.2</b>	Effect <i>S. fredii</i> NGR 234 genomic integrant on nitrogenase activity and available soil P and N, K Content	232
<b>8.3.3</b>	Effect of genomic integrant on N P K content in plant and pods	233
<b>8.3.4</b>	Growth parameters	234
<b>8.3.4.1.</b>	Effect of <i>S. fredii</i> NGR 234 genomic integrant on growth parameters of mung bean leaves	234
<b>8.3.4.2</b>	Effect <i>S. fredii</i> NGR 234 genomic integrant antioxidant enzyme activities.	240
<b>8.4</b>	Discussion	243
	<b>Summary</b>	246
	<b>Summary Table</b>	250
<b>Chapter 9</b>	<b>Bibliography</b>	253