

# Contents

<b>Chapter 1 INTRODUCTION .....</b>	<b>1-10</b>
<b>Chapter 2 REVIEW OF LITERATURE.....</b>	<b>11-32</b>
<b>Chapter 3 MATERIALS &amp; METHODS .....</b>	<b>33-80</b>
3.1. Maintenance of Bacterial strains.....	53
3.1a. Maintenance of <i>E. coli</i> cells: .....	53
3.1b. Maintenance of <i>B. subtilis</i> : .....	53
3.1c. Permanent Stock Preparation of bacterial culture:.....	53
3.1d. Growth conditions for <i>E. coli</i> and <i>B. subtilis</i> cells: .....	53
3.1e. Sterilization: .....	54
3.2. Evaluation of colonizing capacity of <i>B. subtilis</i> strains in the intercellular regions of <i>Hevea</i> .....	54
3.2a. Preparation of poly bag plants for plant application studies of <i>B. subtilis</i> .....	54
3.2b. Culture preparation of <i>B. subtilis</i> for plant application.....	54
3.2c. Inoculation of leaves with <i>B. subtilis</i> .....	54
3.2d. Surface disinfection of leaves.....	55
3.3. Standardization of an efficient transformation protocol for endosymbiotic <i>B. subtilis</i> .....	55
3.3a. Transformation of <i>B. subtilis</i> : .....	55
3.3a.1. Protocol 1 .....	55
3.3a.2. Protocol 2 .....	56
3.3b. Transformation of <i>E. coli</i> cells: .....	56
3.3c. Slot Lysis: .....	57
3.3c.1. For <i>B. subtilis</i> .....	57
3.3c.2. For <i>E. coli</i> .....	57
3.4. Sample collection, mRNA isolation and cDNA synthesis from <i>Corynespora cassiicola</i> infected leaves of <i>Hevea</i> and PCR amplification of chitinase gene.....	57
3.4a. Sample collection .....	57
3.4b. mRNA isolation:.....	58
3.4c. Formaldehyde Denaturing Agarose gel Electrophoresis for RNA (Formaldehyde Gel): .....	58
3.4d. cDNA synthesis from mRNA of <i>Hevea</i> leaves .....	59
3.4e.1. Amplification of chitinase gene .....	59

3.4e.1a. Primer designing for chitinase gene.....	59
3.4e.1b. Gradient PCR.....	59
3.4e.2. PCR Amplification .....	59
3.4f. Agarose Gel Electrophoresis of PCR product:.....	60
3.4g. Purification of PCR amplified products .....	60
3.5. Cloning chitinase gene into expression vector and its characterization by sequencing.....	61
3.5a. Ligation of purified amplicon of chitinase gene into cloning vector (pGEM-T Easy Vector): .....	61
3.5b. Ligation of PCR product into TOPO cloning vector:.....	61
3.5c. Preparation of competent <i>E. coli</i> cells:.....	61
3.5c.1. Using CaCl <sub>2</sub> method .....	61
3.5c.2. Using MgCl <sub>2</sub> and CaCl <sub>2</sub> method.....	62
3.5c.3. Using Transform Aid Bacterial transformation Kit of Fermentas.....	62
3.5d. Transformation Protocols: .....	63
3.5d.1. For freshly prepared competent <i>E. coli</i> cells.....	63
3.5d.2. For Genhunter cells, DH5 alpha cells, JM109 cells and TOP 10F cells .....	63
3.5e. Selection of transformed colonies.....	64
3.5e.1. Colony PCR.....	64
3.5e.2. Agarose Gel Electrophoresis of PCR product .....	64
3.5e.3. Isolation of Plasmids from <i>E. coli</i> cells .....	64
a) Alkaline lysis method .....	64
b) Using Gen Elute Plasmid Miniprep Kit (Sigma) .....	65
c) Mega Preparation .....	66
3.5e.4. Restriction Digestion.....	67
3.5e.5. Agarose Gel Electrophoresis of isolated plasmid and restricted plasmid .....	67
3.5e.6. Sequencing .....	67
3.6. Removal of <i>lacI</i> repressor coding region from pHT 43 expression secretion vector .....	67
3.6a. Alkaline lysis method for plasmid isolation from <i>E. coli</i> .....	67
3.6a.1. Mega Preparation .....	67
3.6a.2. Mini preparation .....	67
3.6b. Restriction Digestion .....	67
3.6c. Agarose Gel Electrophoresis of isolated plasmid and restricted plasmid.....	68
3.6d. Preparatory agarose gel electrophoresis for elution of restricted DNA.....	68
3.6e. Gel Elution using Gen Elute™ Gel Extraction Kit-Sigma .....	68
3.6f. Filling of cohesive ends of DNA.....	68

3.6g. Ligation of the end filled DNA .....	69
3.6i. Agarose Gel Electrophoresis of ligated DNA .....	69
3.6j. Transformation of <i>E. coli</i> cells with the modified plasmid DNA .....	69
3.6k. Plasmid Isolation .....	69
3.6l. Restriction Digestion analysis of the isolated modified plasmid pHT43... ..	69
3.6m. Agarose Gel Electrophoresis of restricted plasmid .....	69
3.7. Construction of a recombinant vector for constitutive expression of heterologous protein ( <i>Hevea</i> chitinase) in endosymbiotic <i>B. subtilis</i> and its sequencing .....	70
3.7a. Isolation of Plasmid from transformed <i>E. coli</i> cells.....	70
3.7b. Restriction Digestion .....	70
3.7c. Agarose Gel Electrophoresis of isolated plasmid and restricted plasmid .....	70
3.7d. Preparatory agarose gel electrophoresis for elution of restricted DNA .....	71
3.7e. Gel Elution of the double digested plasmids .....	71
3.7f. Ligation of the double digested modified pHT43 vector and chitinase .....	71
3.7g. Transformation of <i>E. coli</i> with the recombinant vector pHT43 – <i>lacI</i>   chitinase gene .....	71
3.7h. Selection of transformed colonies.....	71
3.7h.1. Primer designing .....	71
3.7h.1a. For pHT 43 Vector.....	71
3.7h.1b. Gradient PCR.....	72
3.7h.2. Colony PCR.....	72
3.7h.3. Agarose Gel Electrophoresis of PCR product .....	72
3.7h.4. Plasmid Isolation .....	72
3.7h.5. Restriction Digestion .....	72
3.7h.6. Agarose Gel Electrophoresis of isolated plasmid and restricted plasmid .....	72
3.7i. Sequencing.....	73
3.8. Transformation of WB800N strain of <i>B. subtilis</i> with the recombinant vector pHT43– <i>lacI</i> chitinase gene and confirmation of efficiency of the recombinant vector.....	73
3.8a. Transformation of WB800N strain of <i>B. subtilis</i> .....	73
3.8b. Colony PCR .....	73
3.8c. Agarose Gel Electrophoresis of PCR product.....	73
3.8d. Slot Lysis: .....	73
3.8e. Isolation of Plasmids from transformed <i>B. subtilis</i> :.....	73
3.8f. Isolation of Plasmids from transformed <i>E. coli</i> cells: .....	74
3.8g. Agarose Gel Electrophoresis of the isolated plasmid .....	74

3.8h. Study of Growth performance of the transformed <i>B. subtilis</i> cells .....	75
3.8i. Confirmation of efficiency of the constructed recombinant vector by expression studies .....	75
3.8i.1a. Total RNA isolation from transformed <i>B. subtilis</i> .....	75
3.8i.1b. Formaldehyde Denaturing Agarose gel Electrophoresis for RNA (Formaldehyde Gel) .....	76
3.8i.1c. PCR analysis of RNA from the transformed <i>B. subtilis</i> / recombinant vector .....	76
3.8i.2. Assay of chitinase activity .....	76
3.8i.3. SDS PAGE analysis .....	77
3.8i.3.a. TCA Precipitation of protein .....	77
3.8i.3.b. SDS-PAGE .....	78
3.8i.3.c. Coomassie Brilliant blue Staining of SDS-PAGE Gel .....	78
3.8i.3.d. Silver staining Method for SDS-PAGE Gel .....	78
3.8j. Antagonism .....	79
3.9. Transformation of endosymbiotic strain (8LK) of <i>B. subtilis</i> with the recombinant vector pHT43 – <i>lacI</i> chitinase construct .....	79
3.9a. Transformation of endosymbiotic <i>B. subtilis</i> and ATCC strain 6633 .....	79
3.9b. Colony PCR .....	79
3.9c. Agarose Gel Electrophoresis of PCR product .....	79
3.9d. Slot Lysis .....	79
3.9e. Isolation of Plasmids from transformed <i>B. subtilis</i> .....	79
3.9f. Isolation of Plasmids from transformed <i>E. coli</i> cells .....	80
3.9g. Agarose Gel Electrophoresis of the isolated plasmid .....	80
<b>Chapter 4 RESULTS .....</b>	<b>81-116</b>
<b>Chapter 5 DISCUSSION .....</b>	<b>117-122</b>
<b>Chapter 6 SUMMARY AND CONCLUSION .....</b>	<b>123-125</b>
<b>BIBLIOGRAPHY .....</b>	<b>126-145</b>