MATERIALS AND METHODS

3.1. Collection of soil samples

In order to isolate rhizosphere microorganisms, soil samples were collected from the different jute growing districts of North Bengal. The soil samples have been collected mainly from North and South Dinajpur, Malda and plains area of Darjeeling districts. From the field soil samples were collected in polythene bags and brought to the laboratory for the purpose of isolation.

3.2. Isolation of bacteria from rhizosphere

Rhizosphere soils of healthy jute plants were chosen for the isolation of rhizospheric bacteria. 10 gm of soil particles loosely adhering to the roots were collected. The soil suspension was made by dissolving the soil sample in 100 ml of sterile distilled water using magnetic stirrer for 1 h. The suspension was allowed to settle down till the two distinct layers were clearly visible. Then the upper light brown colored layer was drawn by pipette and serial dilutions were prepared. 0.1 ml of each of 10^{-3} and 10^{-8} dilutions was actually used for isolation by dilution plate technique (Kobayashi et al. 2000) using Nutrient Agar (NA- peptone 5 g, NaCl 5 g, beef extract 1.5 g, yeast extract 1.5 g, agar 12 g and distilled water 1 L; pH 7.2 ± 0.2) as the growth media. The petriplates were then incubated for 1-5 days at 30 ± 2^0C for the observation of appearance of bacterial colonies.

3.3. Characterization and identification of selected isolates

3.3.1. Gram staining

Gram staining technique was followed for microscopic characterization of rhizobacterial cultures. Bacterial isolates were smeared separately on a clean glass slide and heat-fixed after air drying. One drop of crystal violet solution was put onto and the smear was allowed to stand for about minute. Excess stain was then washed off with sterile distilled water. After that one drop of Gram's iodine solution was put and allowed to stand for 45 sec, followed by washing with distilled water and dipping in absolute alcohol for 1 minute. Eventually, one drop of safranine (counter stain)

was applied, and allowed to stand for 1 min, washed gently with sterile distilled water, air dried and examined under oil immersion.

3.3.2. Endospore staining

For the purpose of endospore staining, bacterial smear was prepared on the slide and passed over the flame for fixing. The fixed slide was flooded with the solution of malachite green and placed over hot plate for 5 min. After rinsing, the smear was counter stained with safranine. It was washed gently with sterile water, air dried, mounted and examined under oil immersion.

3.3.3. SEM studies of bacterial isolates

For scanning electron microscopy of the bacterial cells, isolates were grown in Luria Bertani broth for 48 hours and collected by centrifugation at 6000 r.p.m. for 15 minutes. The pellet was collected and washed with 0.1 M phosphate buffer saline. Then the samples were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 6.8 followed by dehydrolysis of the samples with different gradation of ethanol starting from 30%, 50%, 70%, 80%, 90% and 100% for 10 minutes in each. After serial dehydration samples were subjected to critical drying in CO_2 then mounted on sample stab, coated with gold palladium alloy in a mini sputter coater and examined under a JEOL JSM-6610LV Scanning Electron Microscope.

3.3.4. Biochemical tests

3.3.4.1. Catalase activity

A small amount of 48 h old culture of the bacterial isolate was aseptically placed on a clean glass slide. Then a drop of H_2O_2 (3%) was added. Production of effervescence of oxygen around the bacterial isolates was observed and recorded (Graham and Parker, 1964).

3.3.4.2. Oxidase activity

Few drops of p-amino dimethyl aniline oxylate (1%) was dropped on 48 h old culture of bacterial isolate and examined for the development of colour (Kovacs, 1956).

3.3.4.3. Voges-Proskauer reaction

Culture tubes containing 10 ml sterile VP broth were inoculated with the bacterial isolates and incubated at 37°C for 3 days. To the cultures 0.6 ml of 5% w/v ethanolic α-napthol and 0.2 ml of 40% w/v aqueous potassium hydroxide were added and kept at room temperature for 1 h for production of pink color indicating positive reaction.

3.3.4.4. Urea digestion

Streaks were made on the slants containing urea medium and incubated at 37°C for 3-7 days. The change in color of the medium indicates the presence of urease.

3.3.4.5. Casein hydrolysis

The milk agar was streaked with the bacteria and was incubated at 37°C and observed for the clear zone around the streaks.

3.3.4.6. Starch hydrolysis

The bacteria were streaked on starch agar plates (NA + 0.1% soluble starch) and incubated for 5 days at 37°C. The plates were flooded with Lugol's iodine solution. The clear zone underneath and around the growth indicates the starch hydrolysis.

3.3.4.7. Indole test

10 ml of Davis Mingoli's broth supplemented with 0.1% tryptophan were inoculated with the isolates and incubated at 37°C for 7 days. The cultures were layered carefully with 2 ml of Ehrlich-Bohme (p-dimethylaminobenzaldehyde 10 g, concentrated HCl 100 ml) reagent on the surface, allowed to stand for a few minutes and observed for the formation of a ring at the medium reagent interface indicating the production of indole.

3.3.4.8. Nitrate reduction

Sterile nitrate broth was inoculated with test isolates and incubated at 37^{O}C till the medium became turbid. Three drops of nitrate reduction test reagent [Sol A: H_2SO_4 0.8 g, 100 ml 5 N Acetic acid; Sol B: 0.5 g α-napthylamine and 100 ml acetic acid. The solutions A and B were mixed in equal volume just before use] was mixed

in 1 ml of the cultures and observed for the development of red or yellow color indicating the presence of nitrate.

3.3.4.9. Gelatin hydrolysis

Culture tubes containing gelatin agar (0.4% (w/v) gelatin) was prepared and inoculated by exponentially grown culture of bacterial isolates followed by incubation at 37 ± 1^{o} C for 7 days. The tubes were then observed for liquefaction of gelatin (Sadowsky et al. 1983).

3.3.4.10. Utilization of carbon sources

The carbon source utilization profile of selected isolates was prepared by using Himedia Carbohydrate^{TM} Kit (Himedia Laboratories, Mumbai, India). It contained the following carbon sources:

Strip 'A'- Lactose, xylose, maltose, fructose, dextrose, galactose, rhaffinose, trehalose, melibiose, sucrose, L-arabinose, mannose.

Strip 'B'- Inulin, sodium gluconate, glycerol, salicin, glucosamine, dulcitol, inositol, sorbitol, mannitol, adonitol, α-methyl-D-glucoside, and ribose.

Strip'C'-Rhamnose, cellobiose, melizotose, α-methyle-D-mannoside, xylitol, ONPG, esculin, D-arabinose, citrate, malonate, sorbose.

For experimentation, nutrient broth medium was prepared and inoculated with selected isolates and kept at 37ºC for 4-6 h until inoculum density attained 0.5 OD at 620 nm. Then the kits were opened aseptically inside the laminar air flow cabinet and 50 µl inocula of each strain of all bacteria were inoculated in each well as per the instructions of manufacturer.

The test is based on the principle of pH change after substrate utilization. After 24 hrs of incubation strips were observed for any change in colour of each well.

3.3.5. Protein pattern analysis

Whole cell protein patterns of selected strains were also analysed with log phase grown culture. Soluble protein was extracted by sonication of cell suspension at continuous exposure of a fixed frequency with alternate pulse (3.0 sec) on and pulse (2.0 sec) off up to 5 mins in a sonicator. Sonicated cell suspension was used as protein source.

3.3.5.1. SDS-PAGE analysis of protein

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed for the detailed analysis of bacterial protein profile following the method of Laemmli (1970). The gel was prepared by using the following stock solution.

(A) Acrylamide and N' N'- methylene bis-acrylamide

A stock solution containing 29% acrylamide and 1% bisacrylamide was prepared in distilled water. The pH of the solution was kept below 7.0 and the stock solution was filtered through Whatman No. 1 filter paper, kept in brown bottle and stored at 4ºC and used within one month.

(B) Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was freshly prepared just before use in luke warm water and stored at room temperature.

(C) Tris Buffer

(i) 1.5 M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with conc HCl and stored at 4ºC for further use.

(ii) 1.0 M Tris buffer was prepared for stacking gel. The pH of this buffer was adjusted to 6.8 with conc HCl and stored at 4ºC for use.

(D) Ammonium Persulphate (APS)

Fresh 10 % APS solution was prepared with distilled water before use.

(E) Tris –Glycine electrophoresis buffer

Tris running buffer consists of 25 mM Tris base, 250 mM Glycine (pH 8.3) and 0.1% SDS. A 1X solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10% SDS in 1 L of distilled water.

(F) SDS gel loading buffer

This buffer contains 50 mM Tris-HCl (pH 6.8), 10 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1X solution was prepared by dissolving 0.5 ml of 1 M Tris buffer (pH 6.8), 0.5 ml of 14.4 M β-mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1 ml glycerol in 6.8 ml of distilled water.

(G) Preparation of gel

Mini slab gel (plate size 8 cm x10 cm) was prepared for the analysis of protein patterns by SDS-PAGE. For gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed with high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by pipette leaving sufficient space for comb in the stacking gel (comb + 1cm).

Name of the compound	10% Resolving gel (ml)	5% Stacking gel (ml)
Distilled water	2.85	2.10
30% acrylamide	2.55	0.50
Tris*	1.95	0.38
10% SDS	0.075	0.030
10% APS	0.075	0.030
TEMED**	0.003	0.003

*For 1.5 M Tris pH 8.8 in resolving gel and for 1 M Tris pH 6.8 in stacking gel

** N, N, N', N'- Tetramethyl ethylene diamine

After pouring the resolving gel solution inside the gel plates, it was immediately over layered with isobutanol and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 minutes. After polymerization of the stacking gel the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris-Glycine buffer was added sufficiently in both upper and lower

reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

(H) Sample preparation

Protein **s**ample (50 µl) was prepared by mixing the sample protein (35 µl) with 1X SDS gel loading buffer (15 µl) in cyclomixer. All the samples were floated in boiling water bath for 4 min in order to denature the protein sample. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a microliter syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b-97,4000; Bovine Serum Albumin-68,000; Ovalbumin-43,000; Carbolic Anhydrase-29,000; Soyabean Trypsin inhibitor-20,000; Lysozyme-14,300) was treated as the other sample and loaded in a separate well.

(I) Electrophoresis

Electrophoresis was performed at a constant 15 mA current for a period of three hours until the dye front reached the bottom of the gel.

(J) Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was removed from the resolving gel with the help of scalpel and finally fixed in glacial acetic acid : methanol : water (10 : 20 : 70) for overnight. The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper. The gel was removed from the fixer and stained in this stain solution for 4 hours at 37°C with constant shaking at low speed. After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5: 4.5: 1) at 40°C with constant shaking until the background became clear.

3.3.6. Molecular identification

3.3.6.1. Extraction of DNA

Genomic DNA of the isolates was performed following the method of Sambrook and Russel (2001). Single colonies of the isolates were inoculated into the

nutrient broth and incubated over night at 28ºC. Then the broth cultures of bacterial isolates were centrifuged at 10,000 r.p.m. at 28ºC for 5 mins and the pellets were collected by discarding the supernatant. The pellets were washed repeatedly with distilled water and resuspended in 0.5 ml of lysis solution (100mM TrisHcl, pH-7.5, 20 mM EDTA, 250 mM NaCl, 2% SDS, 1 mg/ml lysozyme). To it 5 µl of RNase (50 mg/ml) was added and incubated at 37ºC for 3 hr. Then 10µl proteinase K solution (20 mg/ml) was added and incubated at 65ºC for 3 min. The lysate was extracted with equal volume of water saturated phenol: chloroform: isoamylalcohol (25:24:1) and then centrifuged at 10,000 r.p.m. for 5 min. The aqueous phase was collected in clean tube and 2 volume of chilled absolute ethanol was added to this aqueous phase. The mixture was centrifuged at 10,000 r.p.m. for 5 min at 4ºC, the pellet was air dried and finally dissolved in 40 µl TE buffer and stored at 4ºC.

3.3.6.2. Quantification

The genomic DNA of selected isolates was checked for their purities by A260/A280 ratio. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. The reading at 280 nm gives the amount of protein in the sample. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 to 2.0, respectively.

3.3.6.3. Agarose gel electrophoresis

The quality of the genomic DNAs were also checked on 0.8% agarose gel electrophoresis. For this purpose, 1X TAE buffer was prepared from 50X TAE buffer by dilution. 0.8% agarose was prepared in 100 ml of 1X TAE buffer by melting in a microwave oven. 2 µl ethidium bromide was then added after cooling the agarose solution. After cooling, the solution was poured in to a casting tray with a comb and allowed to solidify at room temperature. DNA sample was prepared by adding 1 µl of tracking dye to a volume of 5 µl sample.

The gel was horizontally placed on the tray and the electrophoresis chamber was filled with fresh running buffer (TAE) to cover the gel. The wells were loaded with DNA samples with the help of the pipette and 60V current was applied for the purpose of electrophoresis.

3.3.6.4. Amplification of 16S rDNA by PCR

For 16S rDNA amplification in a total volume of 100 µl reaction mixture, 78 µl deionized water, 10 µl 10 X Taq polymerase buffer, 1 µl of 1U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM forward PA:5'-AGAGTTTGATCCTGGCTCAG-3'; PH:5'-AGGAGGTGATCCAGCCGCA-3' primers and 3.5 µl of 50 ng template DNA. PCR was programmed with an initial denaturation phase of 5 min at 94^0C followed by 30 cycles of denaturation at 94^0C for 60 s, annealing at 59^0C for 60 s and extension at 70^0C for 2 min and the final extension at 72^0C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40% w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination with horizontal electrophoresis. The PCR products were then sent for sequencing to Credora Life Sciences, Bangalore, India.

3.3.6.5. 16S rDNA sequencing and phylogenetic analyses

For the confirmation of identification, 16S rDNA sequences obtained from PCR products were subjected to BLAST analyses and aligned with extype isolate sequences from NCBI GenBank. The DNA sequences were deposited to NCBI GenBank through BankIt procedure and approved as the sequence after complete annotation and given accession numbers.

The evolutionary history was inferred using the UPGMA / NJ method (Sneath and Sokal, 1973). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and these are represented in the units of number of base substitutions per site. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analysis was done in MEGA 4.1 software (Tamura et al. 2007).

3.4. Fungal culture

For *in vitro* evaluation of antagonistic activity of rhizobacterial isolates, following root fungal pathogens viz. *Macrophomina phaseolina, Fusarium oxysporum, Fusarium semitectum,* and *Alternaria alternata* were used.

3.4.1. Source and maintenance

The culture of *M. phaseolina* obtained from the Central Research Institute for Jute and Allied Fibers, Barrackpore, West Bengal, India and culture of *Fusarium oxysporum* was obtained from Immuno-Phytopathology laboratory, Department of Botany, North Bengal University, and *Fusarium semitectum, Alternaria alternata* were obtained from Microbiology and Microbial Biotechnology laboratory of University of Gour Banga, Malda. All the cultures were maintained in PDA with frequent sub-culturing.

3.4.2. Assessment of mycelial growth

Mycelial growth of fungus was assessed in both on solid media and liquid media to know their culture characteristic.

3.4.2.1. Solid media

To assess the mycelial growth of *Macrophomina phaseolina* in solid media, the fungus was first grown on petridishes, each containing 20 ml of PDA and incubated for 5-8 days at 30 ± 2^0C. Agar block (6 mm diameter) containing the mycelia was cut with sterile cork borer from the actively growing zone of mycelial mat and transferred to each petridish containing 20 ml of sterilized solid media. Finally diameter of mycelia was measured at regular interval of time. The media were as follows: Potato Dextrose Agar, PDA (Peeled potato-40.00 g, Dextrose-2.00 g, Agar- 2.00 g, Distilled water-100 ml); Richard's Agar, RA (KNO_3-1.00 g, KH_2PO_4- 0.50 g, $MgSO_4$, $7H_20$ 0.25 g Sucrose- 3.00 g, Agar-1.50 g, Distilled Water-100 ml); Carrot Juice Agar, CJA (Grated carrot-20.00 g, Agar-2.00 g, Water-100 ml), Potato Sucrose Agar, PSA (Peeled potato-40.00 g, Sucrose 2.00 g, Agar-2.00 g, Water-100 ml); Czapek-Dox Agar, CDA ($NaNO_3$ -0.20 g, K_2HPO_4 -0.10 g, KCl-0.05 g , $FeSO_4$, $7H_20$-0.05 g, Sucrose-3.00 g, Agar-2.00 g, Water-100 ml); Yeast Dextrose Agar ,YDA (Yeast extract-0.75 g, Dextrose-2.00 g, Agar-1.50 g, Agar-1.50 g)

3.4.2.2. Liquid media

Further, to assess the mycelial growth of *M. phaseolina* in liquid media, the fungus was first grown on petridishes, each containing 20 ml of PDA and incubated for 3 days at 30 ± 2^0C. From the advancing zone, mycelial block (6 mm) was cut with sterilized cork borer and transferred to 250 ml Erlenmeyer flask containing 50 ml of

sterilized liquid PDB and incubated for 6-8 days with constant stirring at room temperature. After incubation the mycelia were strained through muslin cloth, collected in aluminum foil cup of known weight and dried at 60^0C for 96 hr. cooled in desiccators and weighed.

3.5. *In vitro* characterization of PGPR activity of selected bacterial isolates

3.5.1. IAA production

The production of indole acetic acid (IAA) was estimated by inoculation of bacterial suspension into Luria-Bertani (LB) broth containing 0.01 mM tryptophan for 3-5 days. It was then centrifuge at 10000 r.p.m. 20 mins. Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaere et al. (1999). For the reaction, 1 ml of reagent, consisting of 12 g $FeCl_3$ per liter in 7.9 M H_2SO_4 was added to 1 ml of sample supernatant, mixed well and kept in the dark for 30 min at room temperature. Absorbance was measured at 530 nm. The IAA production of bacteria was calculated from the regression equation of standard curve and the result was expressed as µg/ml over control.

3.5.2. Phosphate solubilisation

The phosphate solubilization activities were determined in both Pikovskaya and NBRIP medium. Primary phosphate solubilizing activities of both the isolates were carried out by allowing the bacteria to grow in Pikovskaya's agar (Himedia-M520; ingredients- yeast extract-0.50 g/l, dextrose- 10.00 g/l, calcium phosphate- 5.00 g/l, ammonium sulphate- 0.50 g/l, potassium chloride- 0.20 g/l, magnesium sulphate- 0.10 g/l, manganese sulphate- 0.0001 g/l, ferrous sulphate- 0.0001 g/l and agar- 15.00 g/l) for 7 to 10 days at 37°C (Pikovskaya, 1948).The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

The efficacy of the isolates as phosphate solubilizer was assessed by inoculating in National Botanical Research Institute's Phosphate (NBRIP) (Nautiyal, 1999) growth medium containing insoluble tricalcium phosphate $Ca_3(PO_4)_2$. Inoculated NBRIP media was incubated at 30° C in a shaker incubator at 150 r.p.m. for 5 days. Autoclaved, un-inoculated NBRIP medium served as control. For assay, the broth was centrifuged at 13,000 rpm for 10 min to obtain a clear supernatant. The presence of

soluble phosphate was determined using the Fiske and Subbarow method (Fiske and Subbarow, 1925).

3.5.3. Siderophore production

Production of siderophore was detected following standard method (Schwyn and Neiland, 1987) using blue indicator dye chrome azurol S (CAS). For production CAS agar, 1 L, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCL_3$. $6H_20$ in 10 mM HCl).With constant stirring this solution was added to 72.9 mg hexa-decyltrimethyl ammonium bromide (HDTMA), dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. The dye solution was mixed into the medium along the glass wall with enough agitation to achieve mixing without the generation of foam, and poured into sterile petriplates. The bacteria were spot inoculated at the center of the plates and incubated for 12-15 days. The change in the colour of the medium around the bacterial spot is an indication of siderophore production.

3.5.4. HCN production

Method as described by Wei et al. (1991) was followed with slight modification for determination of HCN production by selected bacterial isolates. Bacteria were grown on NA medium supplemented with glycine (4.4 g L^{-1}) in a Petri plate. Sterilized filter paper (Whatman no.1) saturated with a solution containing picric acid 0.5% and sodium carbonate 2% placed on upper part of petriplate. The plates were sealed with parafilm and incubated for 5 days at 28° C. HCN production was indicated by the changes in color of the filter paper from yellow to brown to red.

3.5.5. Volatile production

To study the volatile production, the bacterial antagonist and pathogen were inoculated in different Petri plates on PDA. Petri plate containing pathogen was inverted over the Petri plate-containing antagonist and sealed with adhesive tape (parafilm) keeping antagonist in lower and pathogen in upper Petri plate (Dennis and Webster, 1971). The plate without antagonist served as control. Each experiment considering a single bacterial isolate was performed in triplicate.

3.5.6. Ammonia production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly growing cultures were inoculated in 10ml sterilized peptone water in each tube separately and incubated for 48-72 hrs. at 28 ± 2^0 C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was positive test for ammonia production (Cappuccino et al. 1992).

3.6. Extracellular hydrolytic enzyme production

3.6.1. Chitinase

3.6.1.1. Qualitative assay

Production of chitinase by the selected bacterial isolates was determined following conventional plate method using chitinase detection agar (CDA). Plates were prepared by mixing 10 g colloidal chitin with 20 g of agar in M9 medium (Na_2HPO_4 0.65 g KH_2PO_4 1.50 g NaCl 0.25 g, NH_4Cl 0.50 g $MgSO_4$ 0.12 g, $CaCl_2$ 0.005 g and distilled water 1 L; pH 6.5).The CDA plate was spot inoculated with organism followed by incubation at 30 ± 2^0C for 7-8 days. Formation of clear zone around the bacteria was considered positive reaction (West and Colwell, 1984).

The colloidal chitin was prepared by adding 40 g chitin in 500ml of concentrated HCl in a 1000 ml beaker and continuously stirred at 4^0 C for 1hour. The hydrolyzed chitin in the beaker was washed several times with distilled water to attain a pH near to 7. Thereafter, it was filtered using Whatman No. 1 filter paper. The filtrate was stored in 4^0C (Berger and Reynolds, 1958).

3.6.1.2. Quantitative assay

The chitinase production by the isolate was also quantified in liquid medium following the method of Kuddus and Ahmad (2013). The isolate was inoculated in 100 ml of mineral salt broth supplemented with colloidal chitin (1% w/v) and incubated at 30 ± 2°C, 120 rpm for 3 days. The cell free supernatant, obtained by centrifugation at 10,000 rpm for 20 min at 4°C was used as crude enzyme. For assay, the crude enzyme was mixed with 1 ml of 1% (w/v) colloidal chitin in citrate phosphate buffer (0.1 M, pH 5.5) and incubated at 50°C for 30 min. To stop the reaction, the reaction mixture was put into boiling water bath for 3 min. The solution

was centrifuged at 5000 rpm for 10 min. The amount of reducing sugar in the supernatants was determined by dinitrosalicylic acid method (Miller, 1959). One enzyme unit was calculated as the amount of enzyme that liberated 1 μmol of N-acetyl-D-glucosamine per minute under the standard assay conditions.

The effect of different parameters such as incubation period, temperature, pH and the nitrogen source on chitinase production was also determined.

The effect of incubation period on the chitinase production was determined at different time intervals. The culture broth was pulled at 24, 48, 72, 96 and 120 hours of incubation and enzyme activity was determined.

The effect of initial pH of medium on the enzyme production was determined by maintaining the pH of culture media at different level, from pH 4.0 to pH 10.0.

The effect of nitrogen sources on the chitinase production by the isolate was also determined. Effect of nitrogen sources such as peptone, tryptone, yeast extract, beef extract, urea and sodium nitrate (0.1% w/v) was examined by replacing the nitrogen source in basal medium.

3.6.2. Protease

3.6.2.1. Qualitative assay

The qualitative assay for protease production was performed on sterile skim milk agar plates (Panc. Digest of caseine 5.0 g, Yeast extracts 2.5 g, Glucose 1.0 g, Agar 15.0 g, Distilled water 1000 ml, Skim milk 7% was added as inducer). Isolates were spot inoculated and followed by incubation at 30^0C and zone of clearance around the colony indicating the enzymatic degradation of protease (Chaiharn et al. 2008).

3.6.2.2. Quantitative assay

The protease activity was assayed by adding 5 ml of 0.65% casein as substrate (prepared in 50 mM potassium phosphate buffer pH 7.5), which was incubated at 37ºC. Then 1ml crude enzyme was added and mixed thoroughly and was incubated for 10 minutes at 37ºC. After incubation, 5ml of 110 mM trichloroacetic acid (TCA) was added and mixed thoroughly to terminate the enzyme reaction and incubated for 30 minutes at 37ºC. Then the solution was centrifuged at 5000 r.p.m. for 10 minutes.

Next the solution was filtered, then 2 ml of filtrate solution was taken, after that 5 ml 500 mM Na_2CO_3 solution and 15% Folin ciocalteau reagent was added mixed thoroughly and incubated at 37ºC for 30 minutes. After incubation the OD was taken at 660 nm. Simultaneous controls containing enzyme, heat-killed enzyme and substrate were maintained. One unit of protease activity was calculated as the amount of enzyme required to liberate 1 μmol of tyrosine min^{-1} ml^{-1}. The protein content of the culture filtrate was estimated by the dye-binding method of Bradford.

All enzyme assay experiments were carried out in triplicate, and the mean values were recorded. The enzyme activity was calculated by the following formula (Pant et al. 2015).

Units/ml= (μmol tyrosine equivalent x total volume of assay) / volume of enzyme used in assay length of assay x volume used in colorimetric determination

Among the extracellular enzymes, chitinase and protease is considered most important for antagonistic activity. So, optimization studies were carried out for these two enzymes.

3.6.2.3. Optimization of protease production

Optimization of protease production was done to study the effect of different parameters on protease production. There are about 6 parameters, which were tested for optimizing the protease enzyme production.

3.6.2.3.1. Effect of incubation period

To study the effect of incubation period on protease production, the bacterium was grown in skim milk broth at 37ºC temperature at 150 rpm. Enzyme assay was carried out at 12 hours intervals ranging from 12-96 hours.

3.6.2.3.2. Effect of incubation temperature

Effect of temperature on protease production was studied by growing the isolate in the skim milk broth for protease production at different temperature ranging from 30ºC-60ºC at 5ºC intervals.

3.6.2.3.3. Effect of pH on protease production

The effect of pH on protease production in relation to initial medium pH was studied by inoculating the isolate in skim milk broth, by adjusting the pH ranging from 5.0 to 10.

3.6.2.3.4. Effect of carbon source on production of protease

To determine the influence of different carbon sources on protease production, the production medium was substituted with other carbon sources, including 1% (w/v) glucose, fructose, mannitol, sucrose.

3.6.2.3.5. Effect of nitrogen sources on production of protease

The effect of nitrogen sources on production of protease of the organisms were determined by using different organic and inorganic nitrogen sources (0.5%). Nitrogen compounds are very important for growth and enzyme production. The nitrogen source of the skim milk broth was substituted with –beef extract, yeast extract, peptone and urea.

3.6.2.3.6. Effect of metal ions on production of protease

To determine the effect of metal ions on protease production, media was substituted with different metal ions-calcium chloride, magnesium chloride, manganese chloride, zinc chloride.

3.6.3. Amylase

The bacterial isolates were spot inoculated on starch agar (Beef extract 3.0 g, Peptone 5.0 g, soluble starch 2.0 g, Agar 15 g, Distilled water 1 lit) medium plates and incubated at 30°C for 48 h. At the end of the incubation period, the plates were flooded with iodine solution, kept for a minute and then poured off. Iodine reacts with starch to form a blue colour compound. The blue colour fades rapidly. Hence the colour less zone surrounding colonies indicates the production of amylase**.**

3.6.4. β-1-4-glucanse

For the detection of β-1-4-glucanse activity the bacterial isolates were spot inoculated on CMC (Carboxy methyl cellulose) agar (KH_2PO_4 1.0 g, $MgSO_4.7H_2O$ 0.5 g, NaCl 0.5 g, $FeSO_4.7H_2O$ 0.01 g, $MnSO_4.H_2O$ 0.01 g, NH_4NO_3 0.3 g, CMC 10 g, Agar 15 g, distilled water 1000 ml pH 7). Streaked CMC agar plates were incubated at 30^0 C for 5 days. At the end of the incubation, agar media was flooded with an aqueous solution of Congo red (1% w/v). Formation of clear zone indicated cellulose production.

3.7. *In vitro* testing of antagonism

3.7.1. Solid medium

For antagonistic tests the bacterial isolates were streak at a distance of 3.5 cm from rim of individual Petri plate containing PDA medium. A 6 mm mycelial disc from a 7 day old PDA culture of fungal pathogen was then placed on the other side of the Petri dish and the plates were incubated ay 28^0 C for 7 days (Rabindran et. al, 1996). Antifungal activity was estimated from the inhibition of mycelial growth of fungus in the direction of actively growing bacteria. The level of inhibition was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist from the fungal radius. The percent inhibition was calculated using the formula:

PI = (R-r) × 100 / R

Where,

PI = Percent inhibition

r = Redial growth of the fungal colony opposite the bacterial colony and

R = The radial growth of the pathogen in control plate.

3.7.2. Liquid medium

The antagonistic activity in liquid medium was determined by culturing the pathogen in PDB.

One agar block (6 mm dia.) containing 7 day old mycelia of the test fungus and 0.5 ml of bacterial suspension ($1x10^6$ cfu/ml) were used as inocula for each 250 ml flask containing 50 ml PDB. The mycelium grown without the bacterial strain in the similar medium was taken as the control sample. The cultures were inoculated at 28 ± 2^0C and after 96 hrs. of incubation mycelia were washed thoroughly with sterile distilled water to remove bacteria as far as possible and harvested by staining through muslin cloth and mycelial dry weights were determined.

3.7.3. SEM analyses

For scanning electron microscopic observation, the mycelia were aseptically removed from the site of interaction for the study of infected hyphae and from

growing fungal plate for the study of control. The mycelia were then placed on small square slides and they were fixed and air dried. To fix the fungal mycelia first it was fixed with 2.5% gluteraldehyde for 2 hrs. Then it was dehydrated with 50% alcohol for 10 minutes, therefore with 70% and 90% alcohol respectively, each for 1 hour. After that it was dehydrated with absolute alcohol for 1 hour. Lastly it was air dried.

For scanning electron microscopic (SEM) studies, samples were mounted on the specimen stubs using adhesive. Then they were coated with gold to a thickness of 100 angstrom. Coating was done by IB2 iron coater. Coated samples were analyzed in a Hitachi Scanning Electron Microscopes model S-530 operated at various magnifications and photographed.

3.8. Extraction of antifungal compounds from bacteria

3.8.1. Preparation of cell free culture filtrate

The antagonistic isolate was grown in NB medium for 96 h at 37°C in shaking condition. Centrifugation was done at 15000 r.p.m. for 20 min and supernatant was collected and passed through the micro filter (0.22 μm pore size). Portion of this was further used for further bioassays.

The cell free culture filtrate (200 ml) was extracted separately with equal volumes of acetone, benzene, chloroform, ethyl acetate and diethyl ether. The culture filtrate was mixed with equal volumes of organic solvent and taken in separating funnel. The mixture was shaken vigorously and allowed to stand for few minutes and observed for the separation of two liquid phases. The organic as well as aqueous fraction was collected in beakers. The extraction procedure was repeated thrice with each solvent. The organic fraction and corresponding aqueous fractions were evaporated to complete dryness in a rotary evaporator at room temperature and residue in each case was dissolved in 2 ml of respective solvent used for the assessment of antifungal activity. A control was maintained with the solvent only at the time of assessment of antifungal activity.

3.9. Partial characterization of active principle

3.9.1. UV-spectrophotometry

The antifungal compounds were analyzed in UV spectrophotometer at a range of 200 to 900 nm and maximum absorption was determined.

3.9.2. GC-MS analysis of crude cell free extract

Identification of the antimicrobial metabolites was done by Gas Chromatography-Mass Spectrometry (GC-MS) analysis with JEOL GC MATE II GC System (Agilent Technologies 6890N Network GC system for gas chromatography). 1µL of sample solution was injected into the GC system provided with HP 5 Ms column at 220^{o}C and high pure helium was used as a carrier gas at a flow rate of 1 ml/min. For GC–MS the GC oven was held at 50°C and then ramped from 50°C to 250°C at 10°C/min. temperature of ion chamber was held at 250°C. The chromatogram and mass spectra were recorded and analyzed by quadruple double focusing mass analyzer with a photon multiplier tube detector. The m/z peaks representing mass to charge ratio characteristic of the antimicrobial fractions were compared with those in the mass spectrum of NIST (National Institute for Standards and Technology) library of the corresponding organic compound.

3.10. Plant materials

3.10.1. Source of seeds

The seeds of the jute varieties were collected from the Central Research Institute for Jute and Allied Fibers (CRIJAF), Barrackpore, West Bengal, India. Seeds of two varieties of Tossa jute (*Corchorus olitorius*) i.e. JRO-524, JRO-8432 and two varieties of White jute (*C. capsularis*) i.e. JRC-212 & JRC-321 were procured from CRIJAF.

3.10.2. Growth of plants

The pot experiments were conducted in 8” earthen pot containing 2 kg of soil collected from agricultural field. For each treatment, 10 pots were taken as replicate. Plants were maintained under natural conditions of light and temperature (31 ± 2°C). Plants were irrigated at regular intervals.

3.11. Soil analysis

The soil used for experimental study was collected from the nearest agricultural field. The physical properties of the soil were determined by gravitational sedimentation analysis method (Gee and Or, 2002). 25 gm of soil sample used in pot experiment was macerated and taken in a 1000 ml measuring cylinder. Then distilled water was added and shaken carefully until the volume reached up to 100 ml. Then, the total volume of soil sample solution was retained for gradual intervals of 2

minutes, 60 minutes and 72 hours respectively. After each time interval the length of deposited soil was recorded using a graduated scale. From this the physical properties such percentage of sand, silt and clay in respect of successive time intervals of the soil were determined.

3.12. Application of bacteria

The bacterial isolates were cultured in Nutrient Broth medium and were allowed to grow with shaking at 30°C, 120 r.p.m. for 48 hr. At the end of the log phase, bacterial culture was centrifuged at 10,000 r.p.m. for 15 min and the supernatant was discarded, selecting the bacterial pellet. Pellet was scraped into sterile distilled water. The aqueous suspensions were diluted as necessary to maintain the bacterial concentration at 1 x 10^8 cfu/ml. The aqueous suspension was then applied as a soil drench @ 200 ml pot^{-1} to the rhizosphere of the seedlings in potted conditions twice after 7 and 15 days of sowing. The bacterial suspension was applied to the rhizosphere of potted plants either singly or in combination.

3.13. Inoculation of pathogen

3.13.1. Preparation of pathogen inoculum

To prepare the inoculum of *M. phaseolina* the method of Chakraborty and Purkayastha (1984) was followed. The mature jute plants were collected from jute fields and washed thoroughly after defoliation. Then the stems were cut into small pieces of 2-3 cm and sterilized by autoclaving in conical flasks. After sterilization, a block of *M. phaseolina* from the actively growing edge was inoculated into the flasks and incubated at 26°C for seven days.

3.13.2. Inoculation to healthy jute plants

The jute stem pieces covered with *M. phaseolina* were used as source of inoculum. The jute stem pieces were inserted in the soil around the healthy jute plants. In each pot 4-5 stem pieces were used to inoculate the pathogen.

3.14. Disease assessment

Disease assessment was done by computing a disease index based on the symptoms.

The disease index was computed by development of disease symptoms was observed and recorded. Percent disease index (PDI) was determined by using the formula:

$$\% \text{ disease index} = \frac{\text{No of plants showing disease symptoms}}{\text{Total number of plants}} \text{X100}$$

Observations were made at growth period of 15 days.

3.15. Determination of plant growth promoting activity

Determination of plant growth promoting activity by the bacteria was assessed under greenhouse condition. Growth promotion was studied in terms of increase in shoot length, root length, area of leaves, shoot and root dry mass in potted plants. Plants were grown under natural conditions of light and temperature (31±2°C). Observations were recorded after 15, 30 and 45 days of bacterial application. 10 replicates were taken for each treatment and the average of the 10 replicate plants were analyzed.

3.16. Biochemical analyses

3.16.1. Extraction and estimation of biochemical components

3.16.1.1. Extraction and estimation of chlorophyll

For the extraction of chlorophyll from leaves of plants the method of Harborne (1973) with modifications was used. 1 g leaf tissue was crushed in a mortar and pestles using 80% acetone in the dark to prevent the photo oxidation of chlorophyll. The crushed samples were filtered thorough Whitman No.1 filter paper. Final volume was made up 25 ml with adding sufficient amount of acetone. A tube containing 80% acetone was used as blank.

Estimation of chlorophyll was done by measuring the absorbance at 645 nm and 663 nm respectively in a UV-VIS spectrophotometer against a blank of 80% acetone and calculated using the formula as given by Arnon (1949).

$$\textbf{Total chlorophyll} = (20.2\ A645 + 8.02\ A_{663})\ \text{mg g}^{-1}\textbf{fr. wt.}$$

3.16.1.2. Extraction of carbohydrate

Total and reducing sugar was extracted following the method of Harborne (1973). 1 g of leaf and root tissue was extracted in 10 ml of 95% ethanol and the alcoholic fraction was evaporated on a boiling water bath. The residue was again extracted with ethanol and the same process was repeated 3 times. Then the residue was dissolved in dH_2O and the final volume was made up to 5 ml which was centrifuged at 5000 rpm for 10 min. The supernatant was collected and used for estimation.

3.16.1.2.1. Estimation of total sugar

Estimation of total sugar was done by Anthrone reagent following the method of Plummer (1978). To 1 ml of test solution, 4 ml of Anthrone reagent (0.2% Anthrone in conc. H_2SO_4) was added. The reaction mixture was mixed thoroughly and was incubated in boiling water bath for 10 mins. Then the reaction mixture was cooled under running tap water and absorbance was measured in a colorimeter at a wavelength of 620 nm and sugar content was quantified using a standard curve of D-glucose.

3.16.1.2.2. Estimation of reducing sugar

Reducing sugar was estimated by Nelson-Somogyi method as described by Plummer (1978). 1 ml of the test solution was mixed with 1 ml of alkaline copper tartarate solution (2 g $CuSO_4$, 12 g Na_2CO_3 anhydrous, 8 g Na-K tartarate, 90 g Na_2SO_4 anhydrous in 500 ml of dH_2O) and heated over a boiling water bath for 20 mins. The reaction mixture was then cooled under running tap water and 1 ml Nelson's Arsenomolybdate reagent was added along with 2 ml of dH_2O and mixed vigorously. A blue colour was developed, the absorbance of which was the measured in a colorimeter at 515 nm and reducing sugar content was estimated using a standard curve of D-glucose.

3.16.1.3. Extraction and estimation of protein

Soluble protein from the leaves and roots were extracted by following the method of Chakraborty et al. (1995). 1 g fresh plant tissue was taken and homogenized in a pre-chilled mortar with liquid nitrogen using 5 ml of 50 mM sodium phosphate buffer (pH 7.2) and PVP (polyvinylpyrrolidone) under ice

cold condition and centrifuged at 10,000 r.p.m. at 4ºC for 15 mins. The supernatant obtained was used as the crude extract for estimation.

Estimation was done by the method of Lowry et al. (1951). To 1 ml of protein sample 5 ml of alkaline reagent (1 ml of 1% $CuSO_4$ and 1 ml of 2% sodium-potassium tartarate, added to 100 ml of 2% Na_2CO_3 in 0.1 N NaOH) was added. This was incubated for 15 minutes at room temperature and then 0.5 ml of 1N Folin-Ciocalteau reagent was added and again incubated for further 15 minutes following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.16.1.4. Extraction of phenol

The phenols were extracted by the method given by Mahadevan and Sridhar (1982). 1 g of leaf tissue was dipped in 5 ml of boiling absolute alcohol in dark for 10 mins. After cooling the sample was crushed with 80% alcohol and then filtered in a dark chamber. The residue was reextracted with 80% alcohol and then final volume was made up to 10 ml with 80% alcohol.

3.16.1.4.1. Estimation of total phenol

Total phenol content was estimated by Folin Ciocalteau's reagent, following the method of Mahadevan and Sridhar (1982). To 1 ml of the alcoholic extract, 1 ml of 1N Folin Ciocalteau's reagent followed by 2 ml of 20% sodium carbonate solution was added in a test tube. The test tube was shaken and heated on a boiling water bath for 1 minute. After cooling, the volume of the reaction mixture was raised to 25 ml. Absorbance of the blue colored solution was measured at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.16.1.4.2. Estimation of ortho-phenol

Orthodihydroxy phenol was also estimated following the method of Mahadevan and Sridhar (1982). 1 ml of alcoholic extract was mixed with 2 ml of 0.05 N HCl, 1 ml of Arnow's reagent ($NaNO_2$-10 g, Na_2MoO_4-10 g, distilled water -100 ml) and 2 ml of 1 N NaOH and mixed thoroughly at room temperature following which the volume of the reaction mixture was raised to 10 ml. Absorbance of the colored solution was recorded at 515 nm. Quantity of the o-dihydroxy phenol was estimated using caffeic acid as standard.

3.16.2. Extraction and assay of enzyme activities

3.16.2.1. Chitinase

Extraction of chitinase was done by following the method described by Boller and Mauch (1988) with modifications. 1g of leaf sample from the jute plants were crushed and extracted using 5 ml of chilled 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged at 12000 r.p.m. for 10 min at 4°C, and precipitate was discarded. The supernatant was used as enzyme source.

Chitinase activity was measured according to the method described by Boller and Mauch (1988). Assay mixture consisted of 10 µl of 1 M Na-acetate buffer (pH 4) 0.4 ml enzyme solution and 0.1 ml colloidal chitin. Colloidal chitin was prepared as per the method of Roberts and Selitrennikoff (1988). Incubation was done for 2 hrs at 37°C and centrifuged at 10,000 r.p.m. for 3 min 0.3 ml supernatant, 30 µl of 1M K-PO_4 buffer (pH 7.1) and 20 µl Helicase (3%) were mixed and allowed to incubate for 1h at 37°C. 70 µl of 1M Na-borate buffer (pH 9.8) was added to the reaction mixture. The mixture was again incubated in a boiling water bath for 3 min and rapidly cooled in ice water bath. 2 ml DMAB (2% di methyl amino benzaldehyde in 20% HCl) was finally added and incubated for 20 min at 37°C. The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg GlcNAc released $min^{-1}g^{-1}$fresh wt. tissue.

3.16.2.2. Phenylalanine ammonia lyase

Enzyme was extracted by the method described by Chakraborty et al. (1993). Leaf samples were crushed and extracted by using 5 ml of sodium borate buffer (pH 8.8) containing 2 mM β-mercaptoethanol in ice followed by centrifugation at 15000 r.p.m. for 20 min at 4°C. The supernatant was collected and after recording its volume, used immediately for assay or stored -20°C.

Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300 µM sodium borate (pH 8.8), 0.3 ml of 30 µM L-phenylalanine and 0.5 ml of supernatant in a total volume of 3 ml. Following incubation for 1hr at 40°C the absorbance at 290 nm was

read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid produced min^{-1} g^{-1} fresh wt. of tissue.

3.16.2.3. β-1, 3-Glucanase

β-1, 3-glucanase was extracted from leaf samples following the method of Pan et al. (1991). Leaf sample (1g) was crushed and extracted using 5 ml of chilled 0.05 M sodium acetate buffer (pH 5.0). Finally the extract was centrifuged at 10000 rpm for 15 min at 4°C and the supernatant was used as crude enzyme extract.

Estimation of β-1,3-glucanase activity was done by following the laminarin dinitrosalicylate method described by Pan et al. (1991). The crude enzyme extract of 62.5 µl was added to 62.5 µl of laminarin (4%) and then incubated at 40°C for 10 min. The reaction was then stopped by adding 375 µl of dinitrosalicylic reagent and heating on a boiling water bath for 5 min. The resulting colored solution was diluted and vortexed with 4.5 ml of water, and absorbance was recorded at 500 nm. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh wt. tissue.

3.16.2.4. Peroxidase

For the extraction of peroxidase the plant tissues were macerated and extracted in 0.1 M odium borate buffer (pH 8.8) containing 2 mM β-mercaptoethanol under ice cold conditions. The homogenate was centrifuged immediately at 15000 r.p.m. for 20 minutes at 4ºC. After centrifugation the supernatant was collected and after recording its volume was immediately used for assay or stored at -20ºC (Chakraborty et al. 1993).

The reaction mixture contained 1 ml of 0.2M Na-phosphate buffer (pH 5.4), 1.7 ml dH_2O, 100 µl crude enzyme, 100 µl O-dianisidine (5 mg/ml methanol) and 0.1 ml of 4 mM H_2O_2. O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakraborty et al. 1993). Specific activity expressed as the increase in ΔA 465 $min^{-1}g^{-1}$fresh wt. tissue.