CHAPTER - IV
MATERIALS AND METHODS

In order to achieve the objectives outlined in chapter III, different experiments were carried out following the methodologies mentioned below.

4.1. Study Area

Soil sample was collected from waste disposal site of Barrackpore Municipality in Barrackpore (latitude 22°44’ N and longitude 88°30’E) in North 24 Parganas District of West Bengal State in Eastern India and from Dhapa situated at 88°20’E-88°35’E and 20°25’N-20°35’N at Kolkata District in West Bengal.
4.2. Chemicals.

Casein for protease assay was from Sigma (St. Louis, MO, USA). All other analytical reagents and media components used were of highest purity grade available commercially in India.

4.3. Collection of samples

A total of 10 waste samples were collected 5 from waste disposal site of Barrackpore Municipality and rest 5 from Dhapa, landfill site of city of Kolkata. Sample (soil mixed with waste) was collected in sterile zip-lock plastic maintaining aseptical condition, stored at 4°C and marked accordingly to their source and location. The collected samples were brought to the laboratory for isolation of soil bacteria and the moisture content and pH of sample were documented.

4.4. Determination of moisture content (%) and pH of waste samples

Freshly collected samples were kept in filter paper and the initial weight was recorded. After that these samples were kept inside a hot air incubator at 110°C. The samples were weighed several times until a constant weight was achieved. Samples moisture content was calculated employing the following formula provided by AWPA (AWPA, 1986).
\[ \frac{W-w}{w} \times 100 \]

Where, MC is moisture content, \( W \) is the original weight and \( w \) is the constant weight after oven drying.

\( \text{pH} \) was determined in Electrometric method with the help of a pH meter using combination glass electrode.

4.5. Chemical analysis of waste

Chemical characteristic of sample were analyzed for the following properties, Organic matter (%), total N (%), P(%) and K (%). Organic carbon was determined following a rapid titration method Walkey and Black (Walkey and Black 1934). Determination of total Nitrogen content was performed by digestion of the waste with a mixture of acids (\( \text{HClO}_4, \text{HNO}_3, \text{H}_2\text{SO}_4 \)) and then using Kjeldahl procedure according to the method described by Bremner (Bremner 1960). Total Phosphorous was estimated by Colorimetric method using ammonium molybdate and stannous Chloride (APHA, 1992). Total Potassium was estimated by Flame photometric method ([Jackson, 1973]).

4.6. Isolation of bacteria from waste Samples

Serial dilution techniques were used for the isolation of bacteria. In this technique sample suspension was prepared by adding soil mixed with waste (1g) was added to 10 ml of sterile water (the stock) and shaken vigorously for atleast 1 minute. The dilute was then sedimented for a short period. Sterile dilution blanks were marked sequentially starting from stock and \( 10^{-1} \) to \( 10^{-4} \). One ml from the stock was transferred to the \( 10^{-1} \) dilution blank using a fresh sterile pipette. One ml from the \( 10^{-1} \) dilution was transferred to the \( 10^{-2} \) tube for each succeeding step then from the \( 10^{-2} \) to the \( 10^{-3} \), then from the \( 10^{-3} \) to the \( 10^{-4} \). From each dilution tube 0.1 ml of dilution fluid was transferred into Nutrient Agar culture media (composition in [g/l] 10.0 g Bacteriological peptone; 1.0 g Glucose; 5.0 g NaCl; 10.0 g Beef Extract, pH 7-7.5) and incubated at 37\(^\circ\)C for 24 hours. Nutrient Agar (NA) culture
media contained 0.5% Peptone, 0.3% Yeast extract, 0.5% NaCl, 0.25% Glucose, 1.5% Agar, distilled water and pH was adjusted to 7 at room temperature. After successful growth of microorganisms the pure cultures of bacteria were sub-cultured in NA slants; incubated at 37°C to achieve vigorous growth and then preserved in 20% glycerol vials at -80°C (Williams and Cross 1971).

4.7. Microbiological and biochemical characteristic of isolated bacteria

After successful growth of microorganisms, each colony morphology e.g., size, shape, margin elevation, consistency, colour, transparency was determined. Gram stain was performed to observe the cellular morphology and gram nature of the bacteria and biochemical characterization of the strains were also carried out. The biochemical tests of sugar utilization; amino acid decarboxylation; nitrate reduction; hydrogen sulfide production; starch, casein, and urea hydrolysis; and IMVIC tests were performed (Pacarynuk et al., 2004; Collins et al. 1989).

4.8. Optimization of growth conditions of the isolates

Three semi-solid media as NA (Nutrient Agar), BCDA (Czapeck Dox Agar medium [Basic]) and ACDA (Czapeck Dox Agar medium [Acidic]) were used to optimize the cultural media of isolated bacteria. The pH were adjusted to 5.2, 6.5, 7.2, 8.9 and 10.2 in NA medium; 2.2, 3.2, 4.0, 5.5 and 6.9 in BCDA medium; and 7.1, 7.6, 9.1, 10.06 and 12.10 in ACDA medium. For optimization of incubation period and temperature the culture plates were incubated at 25, 29, 33, 37 and 41°C for 6 – 72 hours (Zaved et al., 2008).

4.9. Antagonism of bacterial strains

Each strain was grown at room temperature and subsequently tested by the cross-streaking method at room temperature and at 37°C. The cross-streaking method was performed as described by (Gillies et al., 1966) and (Govan et al., 1969) with two modifications. The strain to be tested was inoculated as a 1.5-cm-wide streak (instead of 1 cm) diametrically across duplicate nutrient agar plates. The plates were incubated overnight at either room temperature or 37°C. A wider streak of the original inoculum was used because the inhibitory zones produced were larger and clearer. After overnight incubation, the inoculum was removed with a glass slide,
and remaining viable growth was killed by exposure to UV light for 30 min. The indicator strains were streaked singly at right angles to the original inoculum by using a wire loop (8 strains per plate). The plates were incubated at room temperature or 37°C overnight, and inhibition was recorded where the indicator strains crossed the original inoculum. This procedure was followed until all of the strains had been tested against each other.

### 4.10. Metal tolerance assay

Study of MIC (Minimum Inhibitory Concentration) of heavy metals viz. Arsenic (As), Zinc (Zn), Lead (Pb), Cadmium (Cd), Mercury (Hg) was carried out for the bacterial strains through Cup Assay method (Smania et al., 1999). The sterilized agar medium (Beef extract-10 gm, Peptone-10 gm, Sodium chloride-5 gm, Glucose-1 gm, Agar-20 gm per litre of distilled water, pH-7.5) was prepared for bacterial growth. The plates were inoculated with bacterial suspension through spread plate method. On each plate cups were made by sterilized cup borer. Various concentrations of each metal compounds poured on cups of pre-inoculated plates. For each metal concentration separate Petri plate with pre-inoculated bacteria were used. All the plates were incubated at 37±2°C for 48 hours. The diameters (milimetre) of inhibition zone around each cup were measured against each concentration and also against each bacterium. Plates without any metal concentration were treated as reference plates.

### 4.11. Antibiotic sensitivity assay

The cup assay method (Cooper 1955) was used for antibiotic sensitivity testing. The sterilized agar medium (Beef extract-10 gm, Peptone-10 gm, Sodium chloride-5 gm, Glucose-1 gm, Agar-20 gm per litre of distilled water, pH-7.5) was prepared for bacterial growth. The plates were inoculated with bacterial suspension through spread plate method. On each plate cups were made by sterilized cup borer. A constant concentration of 100 ppm of each antibiotic (Gentamycin, Oxytetracyclin, Penicillin, Streptomycin) was poured on cups of pre-inoculated plates. For each antibiotic concentration separate petri plate with pre-inoculated bacteria were used. All the plates were incubated at 37±2°C for 48 hours. The inhibition zones around each cup were observed against each antibiotic and also
against each bacterium. Plates without any antibiotic concentration were treated as reference plates.

4.12. Antimicrobial activity

The search for new antimicrobial agents is a field of utmost importance. The prevalence of antimicrobial resistance among key microbial pathogens is increasing at an alarming rate worldwide. All the 9 isolates were screened for antibacterial and antifungal activity by cross streak method (Nakano et al., 1990). In the cross streak method, the soil isolates were streaked on modified nutrient agar as a straight line in the left side corner of the Petri plate and the plates were incubated overnight at either room temperature or 37°C. After incubation, the test human bacterial pathogens (Klebsiella pneumoniae, Staphylococcus aureus, Salmonella sp.) and fungal pathogens (Fusarium sp., Alternaria sp., Helminthosporium sp.) were streaked at right angle to the original streak of the bacterial isolates. The zone of inhibition against human bacterial pathogens and fungal pathogens were observed after 48hrs of incubation. Plates with the same medium without inoculation of bacterial isolates but with simultaneous streaking of test organisms were maintained for controls.

4.13. Enzymological characterization of the strains

The bacterial isolates were screened for enzyme production and optimization, purification and characterization of produced enzyme was carried out applying following procedures.

4.13.1. Qualitative assay of extracellular enzyme production

All the isolated bacterial strains were screened qualitatively for the production of eight important enzymes such as protease, lecinthinase, DNase, lipase, cellulase, amylase, catalase, and oxidase. Each bacterial strain was streaked on the four corners of the respective substrates such as milk, egg yolk, toluidine, tributyrin, casein, tween 80 amended agar plates separately and for catalase and oxidase discs were used. The Petri plates were incubated overnight at either room temperature or 37°C. Then the plates were flooded with indicator solution and the development of
clear zone around the growth of organism was considered positive for enzyme activity (Peterson et al., 1994; Pointing, 1999).

4.13.2. Quantitative assay of enzyme produced

The isolates were screened for quantitative production of enzymes to select the hyperactive enzyme producers.

4.13.2.1. Assay of protease

Protease activity was determined by caseinolytic method (Mohawed et al., 1986). One unit (U) of enzyme is defined as the amount of protease that liberates peptide fragments equivalent to one mg of bovine serum albumin (BSA) per unit time (min-1) under the assay conditions (Patil et al., 1981).

4.13.2.2. Assay of lignin peroxidase

LiP activity was determined spectrophotometrically by veratryl alcohol oxidation according to Tien and Kirk (Tien et al., 1984). One unit (U) of enzyme was defined as 1 micromol of veratryl alcohol oxidized in 1 min, determined at 310 nm.

4.13.3. Selection of the potent producer and study of its growth characteristics

All of the bacterial isolates were screened for selecting the bacteria with the highest protease activity and also selecting the isolate having lignin peroxidase activity. The growth characteristics of the potent strain were studied by measuring the optical density (of the medium where bacteria is grown) per hour depicting the bacterial growth.

4.13.4. Optimization of protease and lignin peroxidase enzyme production in sub-merged fermentation

To enhance the production of both the enzymes, the process parameters (viz, media, pH, inoculums volume, incubation period and temperature) was optimized (Vidyalakshmi et al., 2009).
Effect of Media
Enzyme activity was estimated in the four fermentation medium (Czapeck Dox broth, Nutrient broth, Nutrient broth + Yeast Extract [0.5%] and Nutrient broth + Peptone [0.5%]) to select the best media (Vidyalakshmi et al., 2009).

Effect of pH
The fermentation medium was prepared by varying the pH values (6, 7, 8 and 9) for the production of enzyme (Vidyalakshmi et al., 2009).

Effect of Inoculum Volume
The effect of inoculums volume on enzyme production was studied by carrying out the fermentation process applying different inoculums volumes (1%, 2%, 3%, 4% and 5%) (Vidyalakshmi et al., 2009).

Effect of Incubation Period
The incubation period was varied (8 hours, 10 hours, 12 hours, 14 hours, 16 hours, 18 hours, 20 hours, 22 hours and 24 hours) for studying the effect of incubation period on enzyme production (Vidyalakshmi et al., 2009).

Effect of Temperature
To study the effect of temperature on enzyme production, the submerged fermentation was carried out at different temperatures (30°C, 32°C, 34°C, 37°C and 40°C) (Vidyalakshmi et al., 2009).

4.13.5. Optimization of enzyme production in solid state fermentation
Solid state fermentation (SSF) holds tremendous potential for the production of enzyme especially where the raw fermented product may be used directly as the enzyme source (Tengerdy, 1998). This system offers numerous advantages over submerged fermentation (SmF) system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipments, etc (Pandey, 1994).
4.13.5.1. Selection of substrate for enzyme production

Saw dust, cabbage waste, orange peel waste, jack fruit seed waste, banana waste, potato waste, soyawhey and rice straw were purchased from the grain market of Kharagpur in West Bengal and the substrates were dried and grounded to powder form (40 mm mesh size). The substrate was then stored in plastic jar for subsequent use in a fermentation medium (Mehboob et al., 2011).

4.13.5.2. Fermentative microorganism and inoculum preparation

The pure culture of the hyperactive bacterial strain *Paenibacillus mucilaginosus* S4 isolated from the waste dumping site of Barrackpore municipality (north 24 pgs) was screened for high yield of extracellular alkaline protease and lignin peroxidise production. The bacteria was cultured on minimal media (Gradisar et al., 2005) and the culture obtained was preserved at 4°C in the refrigerator.

The 12-h-old culture prepared in minimal media (pH 8) (with the composition of: sucrose-2%, lactose-1%, peptone-1%, sodium nitrate-0.25%, potassium chloride-0.05%, manganese sulphate-0.05%, sodium chloride-0.05%, ferric chloride-0.05%) at 37°C under shaking (200 rpm) was used to inoculate production flasks.

4.13.5.3. Enzyme production in solid state fermentation

For the production of protease and lignin peroxidise in solid state fermentation flasks (250 ml Erlenmeyer flasks) were prepared in triplicate each containing 5g of the substrate moistened with the minimal medium (pH-8) in the ratio of 1:4 and autoclaved at 121°C for 15 minutes. On gradual cooling to room temperature, the inoculum was aseptically added to each flask in laminar air flow. Then the inoculated flasks were incubated for 13 h at 37°C, under shaking [200 rpm] (Mehboob et al., 2011).

4.13.5.4. Extraction of enzyme and sample harvesting

After incubation, triplicate flasks were harvested after every 13 h. The fermented biomass was soaked in extraction media comprising of deionized water (pH 7) for two hours and extracted in the tincture press. The extracted enzyme was centrifuged 10,000 rpm for 10 min. to remove the particulate matters and bacterial
cells. The supernatants were collected and stored at 4°C for further use and analysis (Mehboob et al., 2011).

4.13.5.5. Optimization of process parameters

The various parameters that may influence the production of protease and lignin peroxidase during SSF process were optimized. The classical method was adopted for optimization of fermentation parameters by varying one parameter in an experiment and to incorporate it at a standardized level before optimizing the next parameter. Different process parameters that were standardized include the effect of incubation period, inoculum volume, temperature, pH, and substrate to media ratio (Mehboob et al., 2011).

pH

Triplicate flasks containing 5 g of rice straw were moistened with 20 ml of minimal medium of varying pH (6.0, 7.0, 8.0, 9.0 and 10.0). Prior to inoculation, all flasks were autoclaved and then were incubated in an incubator at 37°C. After 12 hours of incubation, samples were harvested; the contents of the flasks were diluted five times with distilled water and were analyzed to determine the protease and lignin peroxidase activity (Mohawed et al., 1986; Kirk et al., 1988).

Incubation period

For the optimization of incubation period, the culture was grown in a set of ten flasks. All flasks were autoclaved before inoculation with the bacterial suspension. All flasks were incubated at 37°C. Triplicate flasks were harvested after every 12 hours, and culture supernatants were subjected to protease and LiP assay (Mohawed et al., 1986; Kirk et al., 1988).

Incubation temperature

In order to determine the most suitable incubation temperature for the efficient production of protease and lignin peroxidase, the media was adjusted to pH 8.0. After autoclaving, all flasks were inoculated and incubated at varying temperatures (30, 32, 34, 37, 38 and 40°C) for 13 hours. The samples were removed after 12
hours of incubation and were diluted five times to measure the enzyme activity (Mohawed et al., 1986; Kirk et al., 1988).

**Inoculum volume**

To investigate the effect of inoculum volume on the production of protease and lignin peroxidase, four different levels of inoculum (1, 2, 3, 4, 5, 7 and 10%) were tested in triplicate and compared with a control without inoculum. The culture was harvested on the 13th hour of incubation and subjected to enzyme assay. Each 1 ml-1 of bacterial suspension contained (7' 10^3 cell/ml-1).

**Substrate to media ratio**

For the optimization of substrate to water ratio, 5 g substrate was moistened with varying volumes (5, 10, 15, 20, 25 and 30) of minimal medium. The flasks were autoclaved and then incubated at 35°C. After four days of incubation (optimum incubation period) samples were removed and assayed for lignin peroxidase (Mohawed et al., 1986; Kirk et al., 1988).

### 4.13.5.6. Response surface methodology in production optimization

Response surface methodology (RSM) is an empirical modeling technique used to obtain an optimal response of a sequence of designed experiments. It explores the relationship between explanatory variables and response variables. This optimization process consists of three major steps: (i) performing statistically designed experiments, (ii) estimating the coefficients in a mathematical model and (iii) predicting the response and checking the adequacy of the model (Box et al., 1960). For determining the optimal levels of five crucial variables, viz., pH (A), incubation time (B), temperature (C), inoculum volume (D) and solid to liquid ratio (E) on protease and lignin peroixidase production along with enhancements of its total production, the RSM was adopted. The statistical software package used a set of experimental design (FCCCD:Face Centered Central Composite Design) using multiple factors each studied at two different levels (-1,+1). The five parameters of , pH (A), incubation time (B), temperature (C), inoculums volume (D) and solid to liquid ratio (E) were chosen to be crucial based on the results from one factor at a time approach in optimizing the enzyme production. The boundary
conditions for each parameter are as follows: pH (6 and 10), incubation time (8 and 24), temperature (30°C and 40°C), inoculum volume (1% and 10%), and solid to liquid ratio (1 and 6).

4.13.6. Purification of protease enzyme

a. Enzyme purification

The protease purification steps were described as previously mentioned by El-Safey, (1994). This included the following steps:

Step 1. Enzyme production and preparation of cell free filtrate

*Paenibacillus mucilaginosus* S4 was grown under optimized conditions. The filtrate broth (crude protease) was collected and centrifuged at 10,000 rpm for 15 min at 4°C in order to obtain a cell free filtrate (cff). After performing a test for sterility, 200 ml of the cell free filtrate (CFF) containing protease were collected and their proteolytic activities and protein content were determined.

Step 2. Ammonium sulfate fractionation

200 ml-1 of the crude protease enzyme were first brought to 20% (w/v) saturation with solid ammonium sulfate (enzyme grade) according to the chart of Gomori (1955) as mentioned as Dixon et al., 1964. The precipitated proteins were regimented by centrifugation for 15 min at 500 min-1. The resulted pellet was dissolved in 5 ml of Tris-Hcl buffer at (pH 8.5). The left supernatant was applied again with ammonium sulfate to achieve 20, 30, 40, 50, 60, 70, 80, 90 and 100% (w/v) saturation. Both enzyme activity and protein content were determined for each separate fraction. The 70 and 80% fraction precipitate was collected and pooled together. This precipitate was subjected to dialysis.

Step 3. Dialysis against distilled water and buffer

The obtained ammonium sulfate precipitate (in solution) was introduced into special plastic bag (dialysis membrane) for dialysis against distilled water for 3hrs, followed by dialysis against Tris-Hcl buffer at pH 8.5. The obtained protease enzyme preparation was concentrated against crystals of sucrose and kept in the refrigerator at 4°C for further purification.
**Step 4. Application on column chromatographic technique**

Preparation of the gel column and the fractionation procedures was determined as previously mentioned by Ammar (1975). For this purpose, a Pharmacia column (column length-56cm; column radius-1.5cm; column volume-98.91cm³) has been used. Sephadex G-200 (Pharmacia, Upsulla, Sweden) “practical size 200 μ” was also used. 20 mM Tris-Hcl buffer was used at pH 8.5 and the slurry was allowed to swell for 3 d at room temperature (approximately, 22 ±1°C). Sodium azide (0.02%) was added to prevent any microbial growth. Applying a mixture of blue dextran 2000 and bromophenol blue determined the void volume. One ml-1 of the enzyme preparation sample was applied carefully to the top of the gel. It was allowed to pass into the gel by running the column. Buffer was added without disturbing the gel surface and to the reservoir. 120 fractions were collected (each of 2 ml-1 at the flow rate of 1.2ml/min.).

Proteolytic activity and protein content assay (at 280nm) were carried out for each individual fraction. Sharp peaks of fractions obtained after applying Sephadex G 200 column were collected and investigated for the properties of the partially purified protease enzyme. The active fractions justified by high protease activity coupled with low protein content were pooled and concentrated by ultrafiltration (Centriprep YM-30, Millipore) and then stored at -20°C prior to further study on the enzyme properties.

**b. Enzyme activity**

The protease enzyme activity was determined as previously mentioned by El-Safey and Ammar, (2003).

**c. Protein determination**

The protein content of protease enzyme was determined by the method of Biuret as mentioned in Chykin, (1966).

**d. Determination of the specific activity of protease enzyme**

The specific activity of the protease enzyme protein was expressed in terms of units/mg protein/ml-1 according the following equation:
Specific activity = enzyme activity / protein content (mg/ml-1)

e. Molecular Weight Determination

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% acrylamide gel by the method of Laemmili (Laemmili, 1970; Yossana et al., 2006). Proteins were stained with Coomassie brilliant blue R-250 (Sigma). The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins consisting of Phosphorylase b, 97 kDa; Albumin, 66 kDa; Ovalbumin, 45 kDa; Carbonic anhydrase, 30 kDa; Trypsin inhibitor, 20.1 kDa; Alpha lactalbumin, 14.4 kDa.

4.13.7. Characterization of purified protease enzyme

The purified enzyme thus obtained was subjected to assay for its pH optimization, temperature optimization and determination of substrate specificity.

4.13.7.1. Study on Optimal pH for enzyme activity

The purified protease was determined for an enzyme activity by using 1% casein as a substrate. The pH-activity profile was studied in a pH range of 6.0–12.0 at 60°C using 50 mM buffers of different pHs [6.0–6.5, sodium acetate; 7.0–8.0, Tris–HCl and 8.5–12.0, NaOH:glycine] (Banerjee et al., 1999).

4.13.7.2. Study on Optimal temperature for enzyme activity

The purified protease was determined for an enzyme activity by using 1% casein as a substrate. The temperature-activity profile was studied by assaying protease activity in a temperature range of 25–75°C at pH 10.5 (Banerjee et al., 1999).

4.13.7.3. Study on substrate specificity of the enzyme

The purified protease was assayed at 60°C for substrate specificity by using different substrates dissolved in 50mM NaOH: glycine buffer (pH 10.5) at the concentration of 1 mg/ml. The substrates tested were casein, gelatin, albumin (egg), soybean protein isolate, gluten, albumin (bovine) and cytochrome C (Yossana et al., 2006).
4.14. Application of individual isolates and bacterial consortium in biodegradation of municipal solid waste

4.14.1. Inoculum Preparation

The bacterial strains used in this study were individual isolates and consortium (between isolates having no antagonism). 24 hours of old bacterial culture in tube 10 ml of autoclaved distilled water was added and then mixed well for making a suspension. After that the sterilized garbage was inoculated with 5ml of this bacterial suspension and mixed well. Control treatments were also performed with inoculation (Elango et al., 2009).

4.14.2. Biodegradation of municipal solid waste

The Waste Degradation Potential of Bacteria was studied by weight loss method. Litter sample was collected in sterile litter bags under aseptical condition. Collected samples were brought to the laboratory and then cut into small pieces, and 5 g of each was aliquoted into petri plates, which were then wrapped by using polythene bags. The plates containing municipal solid waste were then autoclaved at 121°C for 15 min. After sterilization, the inoculum was inoculated. Moisture content was maintained at 50–60% throughout the active biodegradation in the plates. The pH and temperature were also measured periodically after 10 days intervals. Turning of the organic waste was provided once in every week to ensure aerobic condition in the plates. Changes in odour and weight loss of the decomposed organic solid waste were observed at 10-day intervals upto 30 days (Gautam et al., 2011). For measurement of weight loss (%), the following formula was used:

\[
\text{Weight loss (\%) = } \frac{W_1}{W} \times 100
\]

Where W is initial weight, and W1 is final weight.
4.15. Molecular characterization of potent bacterial strains

The molecular characterization was done on the basis of 16S rDNA sequence analysis. This analysis was performed by Chromous Biotech Pvt. Ltd. (Bangalore - 92, India). DNA was isolated using the Bacterial Genomic DNA Isolation Kit (RKT09) and evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16s Forward (5'-AGAGTRTGATCMTYGCTWAC-3') and 16s Reverse (5'-CGYTAMCTTWTTACGRCT-3') primers using Big Dye Terminator version 3.1” Cycle sequencing kit on ABI 3500 XL Genetic Analyzer. Consensus sequence of 1398 bp rDNA gene was generated from forward and reverse sequence data using Seq Scape v 5.2 software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using Jukes-Cantor corrected distance model and the phylogenetic tree was constructed using neighbor with alphabet size 4 and length size 1000 (William et al., 2000; Wiley et al., 1991). Among the sequence obtained, the sequence of the most potent isolate was submitted to MTCC GenBank.

4.16. Application of protease from Paenibacillus mucilaginosus; s-4, in removing stains

A clean piece of cloth was soaked in tea and blood stains. The cloth was then dried and soaked in 2% formaldehyde and washed with water to remove the excess formaldehyde. The partially purified protease was dropped on the cloth and incubated at 40°C. After incubation, each piece of cloth was washed and dried. Controls were put up without enzyme (Rai and Mukherjee, 2010).
4.17. Statical procedures

In all experiments, the measurements were carried out with duplicated parallel cultures. The values reported are means ± S.D. calculated as described by Snedecor and Cochran (1980).