4. MATERIALS AND METHODS

PHASE - I

4.1 ISOLATION & IDENTIFICATION OF MICROORGANISM

4.1.1 Isolation of Microorganism

Soil samples were collected in sterile containers from dump yards of Thiruvananthapuram and transferred to the lab aseptically. The microorganisms present in the collected sample were isolated by serial dilution method. The isolated microorganism was screened for protease and chitinase activity (Appendix-I).

4.1.2 Screening of Protease Activity (Coolbear et al., 1991)

The microorganisms obtained from natural sources were grown on skimmed milk agar medium plates (Appendix-II) for 24hrs. Efficient proteolytic organisms which produced clear hydrolytic zone around the culture were cultured in protease production medium (Appendix-III) and tested for protease activity by Anson M.L. method.

4.1.3 Screening of Chitinase Activity (Roberts & Selitrennikoff, 1988)

The organisms producing clear hydrolytic zone on skimmed milk agar medium plates were then plated on to colloidal chitin agar plates (Appendix-IV) and were incubated at 30°C for up to 10 days. After 10 days 0.1% Congo red was added to the plate. Organisms having chitinase activity produced clear hydrolytic zone and the colonies that produced on holo zones were selected for further studies.
4.1.4 Identification of Microorganism

The organism was identified by primary staining method gram staining, and followed by biochemical tests (IMVC Tests) and Molecular identification by 16SrRNA analysis.

4.2 Biochemical Tests

4.2.1 Indole Test

A loopful of culture was taken and inoculated in the tubes of peptone broth (appendix-5.1). The tube was inoculated at 37 degree for 24 - 48hrs. At the end of the incubation period 0.2ml of kovac's reagent (appendix-V.2) was added and mixed thoroughly. The tube were set aside for 5 minutes and observed for dark red violet colour in the top layer.

4.2.2 Methyl Red Test

MR-VP broth (appendix-V.3) was prepared, dispersed into test tubes. The test tubes containing MR-VP broth were sterilized at 121 degree centigrade and 15 lb-sq.inch for 15 minutes. Then they were inoculated with organism and incubated at 37 degree for 24hrs. Methyl Red indicator (appendix-V.4) was added after incubation. a positive test is indicated by the development of red colour.

4.2.3 Voges - Proskauer Test

MR-VP broth (appendix-V.3) was prepared, dispersed into tubes and sterilized. This was then incubated with the test organism and incubated at 37 degree for 24hrs. After incubation 3ml of solution A (appendix-V.5) and
solution B (appendix-V.6) of barrits reagent were added. A positive reaction is indicated by development of pink colour becoming crimson in 30 minutes. The tube can be shaken at intervals to ensure maximum aeration.

4.2.4 Citrate Utilization Test

Simmon Citrate agar medium (appendix-V.7) was prepared, sterilized at 121 degree centigrade, 15 lb/sq.inch for 15 minutes, and dispersed into test tubes, and slants were made. The test culture was streaked on the slant and incubated for 48hrs at 37 degree and observed for growth. A positive test shows a blue colour. Retention of original green colour and no growth on streak line indicates a negative growth.

4.2.5 Urease Test

The base was prepared and sterilized by autoclaving at 121ºC for 15 minutes. It was cooled to 50ºC in water bath and then 5ml of filter sterilized 40% urea solution was added to it. The contents were then mixed and distributed in 2-4ml amounts in 12×100 mm such way to get % inch blunt and one inch slant.

Procedure:

The slants were incubated with a drop of 4-6 hrs growth of bacterium in broth and incubated at 30-37ºC for 18-24 hrs or longer. The taste positive and negative controls were maintained.
4.3 MOLECULAR CHARACTERIZATION OF SERRATIA MARCESCENS USING 16SrRNA

4.3.1 GENOMIC DNA ISOLATION

1.5ml of overnight culture was taken in an eppendorf tube and it was centrifuged at 12,000 rpm for 2 minutes to collect the cell pellet. Supernatant was discarded and to the pellet 467µl of TE buffer, 30µl of 10% SDS and 3µl of Proteinase K was added. The contents were incubated at 37°C for 1 hour, to this equal volume of Phenol: Chloroform - 24:1 was added. And the eppendorf tube was centrifuged at 12,000 rpm for 15 minutes at 4°C. Then the aqueous phase was transferred to fresh Eppendorf tube. 1/10th volume of 3M sodium acetate was added to this solution and twice the volume of 99.9% Ethanol was added to aqueous phase. Invert mixed slowly. Then it was centrifuged at 12,000 rpm for 15 minutes at 4°C. The pellet obtained was washed with 70% ethanol. The supernatant was discarded and the pellet was air dried and suspended in 20 µl of 1X Tris EDTA buffer. Agarose gel electrophoresis was performed using the isolated DNA sample. The quantitative and qualitative determination of DNA was done by spectrophotometric method. The ratio of A_{280} to A_{260} was calculated to check the purity of DNA sample.

4.3.2 POLYMERASE CHAIN REACTION (PCR)

100ng of DNA is used for molecular characterization of Bacterial strain. The PCR reaction is performed for 20µl of sample. Reaction was performed for 16s rRNA gene. The PCR tubes were placed in thermocycler and the reaction was carried inside the thermocycle. A reaction mixture of 10.3µl Milli Q water,
2µl dNTP mix, 1.6µl Mgcl2, 2µl Taq buffer, 2µl forward primer, 2µl reverse primer, 1 µl DNA template, 0.5 µl of taq DNA polymerase was used for PCR amplification.

<table>
<thead>
<tr>
<th>STEPS INVOLVED</th>
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<tr>
<td>Denaturation</td>
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<td>Annealing</td>
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<tbody>
<tr>
<td>Total number of cycles</td>
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</tr>
</tbody>
</table>

Table 1 - PCR Conditions

4.4 Protease Assay (Anson M. L. et al., 1938)

4.4.1 Production of protease:

A loopful of proteolytic S. marcescens strain was inoculated into 250ml capacity conical flask containing 100ml of protease production medium and was incubated at 37°C for 24hrs in a shaking incubator. The cell supernatant after centrifugation at 4500rpm for 10minutes at 4°C was for assay.

The proteolytic activity of organism was measured by Anson M.L (1938). Casein was used as the substrate and amino acid tyrosine liberated on incubation with protease was assayed by colour development using Folin's Reagent.
Test sample containing 5ml of 0.65% (w/v) casein in 50mM potassium dihydrogen orthophosphate buffer, pH 7.5 with 1ml enzyme sample and the control with 1ml of enzyme were incubated at 37°C for 10 minutes. The enzyme substrate reaction was terminated by the addition of 5ml of 110mM trichloro acetic acid. In control substrate was added after the addition of TCA. All tubes were incubated at 37°C for 30 minutes. The reaction mixture was centrifuged at 4500 rpm for 10 minutes at 4°C. 2ml of supernatant was mixed with 5ml of 500mM sodium carbonate and 1ml of Folin's Reagent and the absorbance was measured at 660nm in spectrophotometer (Appendix-VI).

A set of tyrosine standard (0.4 to 2.0 µg) were also determined simultaneously. A graph was plotted with concentration of tyrosine along X-axis and optical density along Y-axis. Concentration of test samples were detected from the standard graph. One enzyme unit was defined as the amount of enzyme required to liberated 1Mumole tyrosine per minute under assay condition.

\[
\text{Units/ml enzyme} = \frac{\mu \text{mole of tyrosine equivalent released} \times \text{total Volume of assay}}{\text{Volume of enzyme (ml) } \times \text{time of assay} \times \text{volume used in colorimetric determination}}
\]

4.5 Chitinase Assay (Miller, 1959)

A chitinase assay was performed to nullify the chitinase producing ability of microorganisms. The crude enzyme from culture supernatant was incubated with 2ml of chitin substrate was kept for 2 hrs at 25°C with vigorous shaking. After incubation period, the reaction mixture was boiled for 5 minutes.
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After boiling 2ml substrate was added to the control without incubation, followed by centrifugation at 4500rpm for 10 minutes. To 1ml of supernatant, 1.5ml of DNSA was added and boiled for 5 minutes in a water bath. Cooled to room temperature and colour produced was read at 540nm in a spectrophotometer (Appendix-VII).

A set of N acetyl glucosamine standards (20 to 100µg) were also determined simultaneously. A graph was plotted with concentration along X-axis and optical density along Y-axis. Concentration of test sample was detected from the standard graph. One enzyme unit was defined as the amount of energy required to liberate 1µmole N-acetyl glucosamine/minute under assay condition.

\[
\text{Units/ml enzyme=} \frac{\text{µmole N-acetyl glucosamine released} \times \text{total volume of assay}}{\text{Volume of enzyme (ml)} \times \text{time of assay} \times \text{volume Used in colorimetric determination}}
\]

4.6 Deproteinization of shrimp shell wastes using *Serratia marcescens*

4.6.1 Collection of shrimp shell waste:

Shrimp shell waste was collected from food processing unit in Thiruvananthapuram.

4.6.2 Partial purification of shrimp shell waste (Muralidhara, 1981)

The exoskeleton, which have been peeled from shrimp, and the exoskeleton materials was then mixed with 70% saline solution. This was then filtered nursing muslin cloth and was kept for drying under vacuum condition. The dried shrimp shells were then powdered using blender.
4.6.3 Deproteinization of partially purified shrimp shell waste (Hall, G.M., C. L. Reid and Zakaria, 1994)

To 100ml protease production media (Appendix-VIII), 3g shrimp shell powder was added as the chitin source and was inoculated with the culture of isolate. Fermentation was carried out at 37°C at pH-7. The Protease enzyme activity, amount of chitin and protein in the sample were analyzed in alternative days (3rd, 5th, 7th and 9th day).

4.6.4 Demineralization (Percot et al.,2003)

1g of microbially deproteinized shell powder (MDS) was mixed with 50ml 6N HCL in a tightly closed conical flask and placed in a water bath for 2-3hrs at 100°C followed by keeping for evaporation at 40-50°C in hot air oven, untill it was fully evaporated and precipitate is formed in the bottom of conical flask. The precipitate was diluted in distilled water. The amount of chitin in the sample was analysed.

4.6.5 Estimation of chitin (George. C. Chen and Bruce. R. Johnson. 1983)

The chitin was assayed colorimetrically by George. C. Chen and Bruce. R. Johnson method. To 1ml of diluted hydrolysate 0.25ml of 4% acetyl acetone was added and heated at 90°C for 1hr in a test tube with its mouth covered with aluminium foil. After cooling, 2ml of ethanol was added, with shaking to dissolve precipitate. 0.25ml of Ehrlich reagent was added to the above mixture. The colour developed in the solution was measured at 530nm in a spectrophotometer (Appendix-IX). A set of N-acetyl glucosamine standards (0.1g in 100ml) were also
determined simultaneously. A standard graph was drawn and the amount of chitin in the sample was calculated.

### 4.6.6 Estimation of protein content from microbially and chemically deproteinized sample (Lowry et al., 1951)

Samples were treated for the estimation of protein content by using Lowry's method. For the chemical deproteinisation of the sample the dried material were hydrolyzed with 1M NaOH at 55°C for 24 hrs. Concentration of the protein was calculated by plotting in graph of standard Bovine serum albumin in varying concentration (40 to 100µg). The color developed using Folin's Reagent was used in a spectrophotometer at 670nm (Appendix-X).

### 4.7 Enzyme kinetics of purified protease from *Serratia marcescens* and its characterization

#### 4.7.1 Purification and Molecular Weight Determination of Protease Enzyme (Nilson. B.H.K et al., 1993)

The culture supernatant of the organism was collected from 24 hours old culture and was subjected to salting out with ammonium sulphate (enzyme grade, SRL) at a final saturation of 80% (w/v). The precipitate was recovered by centrifugation at 10,000 rpm for 15 minutes at 4°C. The pelleted proteins were collected. The pellet was dialyzed against phosphate buffer (pH 7.4, 0.02M) using dialysis membrane (Dialysis membrane 60, Himedia).

#### 4.7.2 Purification of Protease by Column Chromatography

DEAE Cellulose was used as the column material which was suspended in a large volume of phosphate buffer (pH 7.4). The gel was swollen by heating in a
water bath for 2 to 4 hours. Good slurry of the gel was filled in the column without the trapping of any bubble. The gel was settled down to desired height by gravitational force.

The column was allowed to equilibrate thoroughly by passing through the column buffer, applied the dialyzed sample on the top of the column bed. The sample volume was preferably limited to 1-3% of the total bed volume. The sample was applied by careful pipetting and was kept at -4°C for 2-3 hours for incubation. After incubation the unbound proteins were eluted out with 10ml of 0.02M phosphate buffer.

The bound proteins were eluted with 10ml of increasing concentration of sodium chloride (0.1 to 1M, 2M, 5M) followed by a final washing with phosphate buffer (pH 7.4, 0.02M). The fractions were collected and tested for protease activity. The active fractions were stored at -4°C. The whole process was carried out at -4°C.

A graph was plotted with concentration of sodium chloride along x-axis and optical density along y-axis. From the graph, which fraction have maximum protease activity was obtained (Appendix-XI).

4.7.3 Molecular Weight Determination of Protease by SDS-Polyacrylamide Gel Electrophoresis (SDS-Page) (Sambrook et al., 1989)

The purified protein were mixed with equal volume of SDS-Gel loading buffer and loaded to on to a 12.5% Polyacrylamide gel. The electrophoresis was developed at the protein band were analyzed by Coomassive brilliant blue staining.
The molecular weight was determined by using **GelEval 1.22 software** (Appendix-XII).

### 4.8 Study of Enzyme Kinetics of Proteolytic Enzymes from *Serratia marcescens*

The enzyme kinetics was studied to optimize pH, temperature required for the maximum activity of the enzyme.

#### 4.8.1 Optimum pH for enzyme activity:

The effect of pH was determined by varying the pH 3, 4, 5, 6, 7, 8, 9, 10 and 11 of phosphate buffers, by the modified method of Anson M.L et al., 1938.

A graph was plotted with different pH values along X-axis and activity along Y-axis. From the graph optimum pH of protease was obtained.

#### 4.8.2 Optimum temperature for enzyme activity:

The effect of temperature on enzyme activity was determined by incubating the medium at different temperatures (30°C, 45°C, 50°C, 55°C & 60°C) by the modified method of Anson M.L et al., 1938. A graph was plotted with different temperature values along X-axis and activity along Y-axis. From the graph optimum temperature of protease having peak value was obtained.

#### 4.8.3 Optimum Substrate Concentration for Enzyme Activity:

The effect of substrate concentration on enzyme activity was determined by using different substrate concentration (0.45, 0.55, 0.65, 0.75, and 0.85) by the modified method of Anson M.L et al., 1938. The Km and Vmax values were calculated by computation, using software Graphpad prism.
4.8.4 Optimization of Deproteinization Media (Hall, G.M, Reid & Z.Zakaria, 1994)

The effect of media components on deproteinization of *Serratia marcescens* was determined by deproteinizing different medias. The enzyme activity, amount of chitin and total protein were assayed and the results were compared with the basic deproteinization medium. The modified medias are

1. Glucose media (Appendix-XIII, 1).
2. EDTA media (Appendix-XIII, 2).
3. Tween 80 media (Appendix-XIII, 3).

4.8.5 Effect of pH and Temperature on Microbial Deproteinization:

To optimize the incubation temperature of the glucose media in which the enzyme shown maximum activity and deproteinization was studied in detail. The temperature and pH of the fermentation was optimized was deproteinizing the shrimp shells in different pH (4, 6, 7 &10) and temperatures (20°C, 37°C & 50°C). The total proteolytic activity and chitin yield was studied using standard procedure described above.

A standard graph was plotted with different pH and temperature values along x axis and optical density at 660nm at y axis. From the graph optimum pH and temperature of deproteinization was obtained.
PHASE - II

4.9 Deacetylation of shrimp shell wastes using *Lactobacillus. spp*

4.9.1 Isolation of protease producing and chitinase deficient microorganism

The organisms were already isolated and preserved in the Laboratory. That was streaked on the nutrient agar plate (Appendix-I) and screened for protease and chitinase activity.

4.9.2 Isolation of Deacetylating Bacteria

Curd samples were collected in sterile containers and transferred to lab aseptically. The sample was inoculated on *lactobacillus* selective medium (Appendix-XVI) and incubated for 24 hours. The isolated colonies were inoculated on CDA medium (Appendix-XVII). This was inoculated for 2 more days at 30°C. Colonies that produced Chitin deacetylase were screened by color reactions of 4-nitroacetamide.

The highest CDA activity was determined. The colonies of chitin deacetylase producing colonies were identified by colony morphology, gram staining and biochemical tests. (as performed by previous methods)

4.9.3 Study of Enzyme Kinetics of Chitin deacetylase Enzyme from *Lactobacillus spp*

The enzyme kinetics was studied to optimize pH, temperature required for the maximum activity of the crude enzyme.

4.9.4 Optimum pH for enzyme activity:

The effect of pH was determined by varying the pH 4, 5, 6, 7, 8, 9 and 10 of phosphate buffers, by the modified method of Beyer and Fridovich, 1987.
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A graph was plotted with different pH values along X-axis and activity along Y-axis. From the graph optimum pH of Chitin deacetylase was obtained.

4.9.5 Optimum temperature for enzyme activity:

The effect of temperature on enzyme activity was determined by incubating the medium at different temperatures (25°C, 35°C, 45°C, 50°C, 55°C) by the modified method of Beyer and Fridovich, 1987. A graph was plotted with different temperature values along X-axis and activity along Y-axis. From the graph optimum temperature of Chitin deacetylase having peak value was obtained.

4.9.6 Preparation of chitosan

Chitosan membranes are finding widespread applications in medical fields. The membrane forming ability of chitosan derived from chitin produced by deacetylating shrimp shells through biological method were compared with that of chitosan formed from chitin produced by deacetylating shrimp shells through Chemical Method (Muzzarelli, 1977). The thickness and area of membrane formed the two chitosans were compared by analysis of image of the chitosan membrane using Pixcavator IA- Image Analysis software.

4.9.7 Deacetylation by biological method

In biological method Chitosan was produced from chitin by using the enzyme chitin deacetylase. The deproteinized sample was prepared by *serratia* culture, which was inoculated and incubated for 7 days in the protease production medium. Then it was decontaminated and the collected particles were dried under sun light and powdered.
1.5g of prepared chitin and 1ml of chitin deacetylase producing culture in *Lactobacilli* MRS broth 7406 (Man *et al.*, 1960) (Appendix-XV) were inoculated in the 50ml of nutrient broth and incubated for 5 days. Then the medium was collected and centrifuged. The pellet was dried under sun light and Chitosan was prepared in powder form.

4.9.8 Deacetylation by chemical method (Muzzarelli, 1977)

In chemical method, the shrimp shell waste was demineralized with 0.68M HCL solution at temperature 30°C for 12 hours. The residue was washed and soaked in tap water for 6-8 hours. Then dewatered and deproteinized with 0.62M NaOH solution at 30°C for 20 hours.

The chitin obtained from the above process was deacetylated in 12.5M NaOH solution at 65°C for 20 hours. After deacetylation, the Chitosan was washed and dried in sunlight and assayed. The degree of deacetylation was analyzed by spectrophotometer.

4.10 QUALITATIVE ANALYSIS OF CHITOSAN

4.10.1 Degree of deacetylation

**Hydrogen bromide titrimetric analysis (Roberts, 1992)**

0.5g of Chitosan was dissolved in 100 ml of freshly prepared 0.2M hydrobromic acid. 9M hydrobromic acid (50ml) was then added to the Chitosan solution with vigorous stirring to precipitate the hydro bromide salt. The resultant slurry was centrifuged at 2000rpm for 30 minutes and the supernatant was discarded. The Chitosan hydro bromide salt was then filtered off. And washed
several times with mixture of methanol and diethyl ether (1:1 v/v) until the filtrate was neutral to litmus. Residual moisture in the Chitosan hydro bromide salt was removed by stirring for 6 hours in anhydrous diethyl ether. After final filtration the precipitate was dried in vacuum desiccator for 12 hours to yield a white Chitosan hydro bromide salt.

An accurately weighed (0.2g) Chitosan hydro bromide salt was dissolved in 100ml of distilled water. The resulting solution (20ml) was titrated against a standard 0.1M NaOH solution using phenolphthalein as an indicator. The moles of neutralized alkali corresponded to the moles of hydrobromic acid present, which corresponded to the mole of glucosamine units of Chitosan initially present in the solution, thus facilitating the calculation of degree of Chitosan samples.

4.10.2 Scanning Electron Microscopy

Chemically deproteinsed and biologically deproteinised Chitin Samples were analysed using Philips 505 Microscope for Scanning Electron Microscopy (SEM).

Sample Preparation

The samples were made to be free from moisture and other contaminants as possible.

The samples were then pinned down to a sample holder using a conductive carbon tape that contains adhesive on both sides. With the sample conductively tied to the holder. Although, several possible metals used in this step, it is general practice to coat the sample surface with gold. This choice is primarily due to gold's
superior electrical conductivity. The use of sputter technology was employed in this part of sample preparation.

4.10.3 Fourier Transform Infrared Spectroscopy (FT-IR)

Both the Sample Chemically deproteinsed and biologically deproteinised Chitin Samples were analysed using Fourier Transformed Infrared Spectroscopy transmission spectra were acquired in the range of 4600 - 650 cm\(^{-1}\).

Sample Preparation

Before the analysis samples were soaked for 2 hours in ultrapure water and all the slides were dried for 24 hours at 60°C to avoid water related bands Interference.

4.11 Metal Binding Efficiency Using Chitin Samples

4.11.1 Adsorption of Chromium (American water works association, 1971)

Another important application of chitin and chitosan is in the removal of toxic chemicals from water and other effluent wastes. In the current study a comparative analysis was performed on the efficiency of chitin to remove chromium from synthetic waste water by adsorption.

The effect of retention time of chitin in solution for the removal of chromium was estimated using the diphenylcarbazide method from standard methods for the examination of water and waste water (American water works association, 1971).
In this method the remaining chromium content was assayed after the adsorption of chromium by chitin and percentage of chromium removal due to bioadsorption was calculated as

\[
\% \text{chromium removal} = \left( \frac{C_0 - C_1}{C_0} \right) \times 100
\]

Where,

- \(C_0\) - initial concentration of chromium
- \(C_1\) - Concentration of chromium in test sample obtained from standard graph of chromium.

### 4.11.2 Removal of Chromium from Waste Water Using Chitin Samples

(American water works association, 1971)

10gm of chitin was dissolved in 1L \(K_2Cr_2O_7\) stock solution (1000mg/L) with continuous stirring in a shaker for 1hr, followed by incubation at 37°C for 24hr. The same was repeated for non deproteinized shrimp, commercial chitin, and chitosan and chemically deproteinized chitosan and chitin.

### 4.11.3 Spectrophotometric Determination of Chromium Removal (American water works association, 1971).

10\(\mu l\) of sample was made up to 1ml with distilled water and acidified with 0.25ml \(H_3PO_4\). At pH 2.0± 0.05, add 2.0ml of 1, 5- diphenyl carbazide and were incubated at room temperature for 5 to 10 minutes. Measure the absorbance at 540nm, using reagent water as reference. Correct absorbance of a blank carried through the method. From the corrected absorbance, determined microgram chromium present by referring to the calibration curve (Appendix-XIV).
A standard graph was plotted with K$_2$Cr$_2$O$_7$ stock solution simultaneously. A graph was plotted with concentration of K$_2$Cr$_2$O$_7$ on x-axis and optical density on y-axis.

4.11.4 Effect of Retention Time in Chromium Removal by Chitin Samples 
(American water works association, 1971)

The effect of retention time in chromium removal by chitin was determined by modified method at 37° C for different time intervals (24hr, 48hr, 72hr & 96hr).


Beads were prepared by coagulating a mixture of 4wt%cellulose solution and 2wt% chitin solution in a solution of NaOH in the ratio 3:1 (w/w) by continuous stirring and filtered, degassed and injected into a solution of 5% H$_2$SO$_4$ as coagulant. The beads having a diameter of 0.3mm so formed were washed with distilled water for 15 minutes and stored in refrigerator prior to adsorption experiments. The bead so formed used for the purification of waste water by immersing in water for 24hr and the chromate was determined by Dao. Zhou et al., method.

PHASE - III

4.12 Transformation and Expression of Protease of Plasmid from Serratia marcescens in E.Coli DH5a

4.12.1 Plasmid Isolation of Serratia marcescens

Plasmid was isolated using standard methods recommended by Birnboin and Dolly. Single colony of each isolate was inoculated into 20 ml LB medium
separately and incubated overnight at 37°C under microaerophilic condition. The cells was harvested by centrifugation at 5000rpm for 5 minutes at 4°C. The pellet was resuspended in 1 ml of solution I then 2 ml of solution II was added and suspension was mixed gently and incubated on ice for 10 minutes. The mixer centrifuged at 11,500rpm for 15 minutes at 4°C and the supernatant from each tube was transferred to fresh eppendorf tubes. The mixer of phenol-chloroform in 1:1 proportion was added in the tube and mixed properly in each case, The phase was separated by centrifuged at 11,500 rpm for 15 minutes at 4°C. Aqueous layer from each tube was transferred to fresh set of eppendorff tubes and 250µl of 70% ethanol was added into each tube and incubated at room temperature for 10 minutes. The suspensions were centrifuged at 11,500rpm for 15 minutes at 4°C. The supernatant was removed aspiration and discarded. The precipitated plasmid DNA dried at room temperature for 30 minutes and 50 µl of TE buffer was added.

4.12.2 Agarose Gel Electrophoresis of Isolated Plasmid

0.5% agarose gel was prepared in TE, melted and cooled to bearable warm and 3 µl of ethidium bromide was added as the DNA interacting dye. The platform with gel was placed in the electrophoretic tank with 1X TE buffer so as to immerse the gel. DNA samples were prepared with an appropriate amount of loading buffer, and were loaded into the wells and run at 50 Volt until the tracking dye reaches the end of gel. The bands were visualized in a UV transilluminator.

4.12.3 Preparation of competent cells of *E. Coli* DH5α

*Ecoli DH5α* (Genei, Bangalore) was used as host for transformation studies. The cells were made competent by calcium chloride treatment method.
Pick 10-12 moderate sized colonies from the LB plate and inoculate into 100ml of LB broth (in a 1 litre conical flask). Incubate at 37°C in a shaker, grow until OD at 600 nm reaches 0.3, this takes about 2-3 hours. Transfer the culture aseptically into sterile centrifuge tubes and spin down at 6000 rpm for 8 minutes, preferably in a refrigerator centrifuge at 4°C or spin at room temperature. Supernatant was discarded and to the cell pellet add 3 ml of ice cold 0.1M NaCl₂ solution aseptically into all tubes. Suspended the cell pellets gently in the solution using a pre chilled pipettes. Place the tubes on ice for 30 minutes. Centrifuge at 6000 rpm for 8 minutes either at 4°C or room temperature. The supernatant was discarded and resuspended gently in 0.12ml of ice cold 0.1M CaCl₂ solution to all the tubes. Aseptically aliquot 100µl of competent cells into 4 pre chilled vials from ice. Competent cells were now ready and should be used immediately for the transformation experiment, as the efficiency of transformation drops on storage at higher than 70°C. The competent cell were transformed by the following procedure. Care should be taken not to remove the tubes from ice during resuspension.

4.12.4 Transformation

5µl of the plasmid DNA was added to aliquots of 100µl of the competent cells. Gently tap and incubated on ice for 20 minutes. The remaining one aliquot will not be transformed. Heat shock was given to cells by replacing the vials in 42°C water bath for 2 minutes, then return the vials to ice to chill for 5 minutes. Add 1ml of LB broth aseptically to the vials and incubated at 37 °C on a shaker for an hour. This is to allow bacteria to recover and express the antibiotic resistant.
PHASE - IV

4.13 Application of Chitin

The application of Deproteinised chitin as a growth promoting agent was determined in *Pisum sativum* plant in vivo. The root Length and shoot length was taken as growth indices and antioxidants as stress factors.

4.13.1 Seed collection

The *Pisum sativum* plant seed was collected from Haritha Agrofirm Mannuthy, Kerala.

4.13.2 Enzyme and non enzymic antioxidants

First set having five pots and one pot marked as control and the remaining pots having Chitin and soil with different concentration of chitin was prepared (50mg, 100mg, 150mg, 200mg). The chitin was added to the soil except control pot. The seeds are sown in it for 7 days. After 7 days the root length and shoot length was measured using a precision scale and the different enzyme assays were performed with the help of the plant extracts. Repeated the enzyme assay for this plant after 14 days.

Cell lysis buffer:

After 7 days treatment & 14 days treatment, Shoots and leaves tissue were cut in to small pieces using sterilized blade. Two plants were taken in each type of treatments. 2ml of lysis buffer added which containing 1ml of EDTA, 1ml of Tris buffer and 0.1ml of Triton which make up with 10ml distilled water. After
homogenized plants sample was centrifuged at 6000rpm for 10min in 4°C. The pellet were discarded, the supernatant was used for the assay extract respectively.

4.13.3 Catalase

About 0.5ml of (salt + water) treated sample, (salt + soil) treated samples and control was taken. Then added 300:1 potassium phosphate buffer, 1:35:1 methionine, 1:59:1 riboflavin and 2:52:1NBT, colour developed was read at 600nm (Beyer and Fridovich, 1987).

Reagents

- 50Mm Potassium phosphate buffer, pH 7.8
- 2.45um methionine
- 3.5.3mM riboflavin
- 4.84um NBT

4.13.4 Peroxidase

About 0.5ml (salt + water) treated sample, (salt + soil) treated sample and control was pipette out in to a series of test tubes containing 1ml phosphate buffer, 0.4ml distilled water and 0.5ml hydrogen peroxide. Tubes were incubated for 60 min. Then 2ml dichromate solution was added and the colour developed was read at 620nm (Chance and Mahely, 1955).

Reagents

- 0.067M Phosphate buffer.
- H₂O₂
- Dichromate.
About 0.3ml phosphate buffer, 0.05ml guaiacol solution, 0.1ml (salt + water) treated sample, (salt + soil) treated sample and control was taken then 0.03ml hydrogen peroxide solution were pipette out in to series of tubes. Mixed well and OD was read at 436nm (Chance and Maehly, 1955).

4.13.5 Super Oxide Dismutase

Reagents

- Phosphate buffer.
- Guaiacol solution.
- H$_2$O$_2$ solution.

About 1ml of supernatant was taken from (salt + water) treated sample, (salt + soil) treated sample and control. The volume was made up to 3ml by adding 0.5ml phosphate buffer, 1.3ml distilled water and 0.2ml of DTNB. The contents were mixed well and the absorbance was read at 420nm against a reagent blank (Smith et al., 1988).

Reagents:

- 0.6mM, 5, 5’-Dithio -2-nitro benzoic acid (DTNB).
- 0.2 M Phosphate buffer, pH-8.0.

4.13.6 Reduced Glutathione

About 0.1ml of supernatant was taken from (salt + water) treated sample, (salt + soil) treated sample and control was taken. Then added 0.05ml Reduced glutathione, 0.02ml CDNB, 0.68ml distilled water, 0.2ml phosphate buffer. The
contents were mixed well and the absorbance was read at 340nm against a reagent blank (Aebi and Bergmeyer, 1983).

Reagents

- 100 Mm Potassium phosphate buffer, pH-6.5.
- 1mM EDTA.
- 0.5 mM, 1 -choro 2, 4 dinitro benzene (CDNB).
- 1 mM, Reduced glutathione.

4.13.7 Vitamin C

The reaction mixture consists of 1gm sample with 5ml 10% TCA were mixed. Then centrifuge at 40°C 3000rpm for 20 minutes. Supernatant were discarded and pellet extracted twice with 10% TCA. 0.5ml extract will taken and 1ml of DTL added and mixed. These mixtures were incubated at 37°C for 30min. 0.75 ice cold 65% H₂SO₄ added. Again it would be incubated at 37°C for 30mins. Measure the optical density at 520nm (MC De Pinto and Gara, 1999).

Reagents:

- 1.5ml10%TCA.
- 2.1ml DTL.
- 3.65% H₂SO₄.

4.14 Antibacterial activity

Antibacterial Assay (Mima et al., 1983)

In order to access the biological significance, antibacterial activity of microbial deproteinized chitin was compared with that of chitosan derived from
commercial chitin. It was obtained by Mima et al., 1983 method. Broth having *staphylococcus aureus* as test strain was added with chitosan from both Microbially Deproteinized and commercial chitin and inoculated for about 24 hours. Serially diluted samples from grown cultures were plated on to nutrient agar plates and incubated overnight. The colony forming units were counted (CFUs) on a colony counter.

4.15 Removal of Heavy Metals from synthetic tannery effluent

4.15.1 Spectrophotometric determination of Arsenic (Pasha C, Narayana B, 2008)

Standard arsenic solution was prepared by dissolving 0.1734g of sodium arsenate in 1000ml of distilled water. Toluidine blue solution (0.01%) or safranine solution (0.02%) was prepared by dissolving 0.01g of toluidine blue or safranine in distilled water and made upto 10ml each with distilled water. Hydrochloric acid (1M), potassium iodide (2%) and acetate buffer (pH 4.0) was prepared by dissolving 13.6g of sodium-trihydrate in solution of water. Solution pH was adjusted to 4 with acetic acid and the mixture was diluted to 100ml with distilled water.

Sample solution containing 1.2-10.5 or 4-11.5 µg of arsenic (VI) was transferred into a series of 10ml calibrated flasks. Potassium iodide (2% 1ml) and HCL (1M, 1ml) were added and mixture was gently Sample solution containing 1.2-10.5 or 4-11µg/ml of arsenic (VI) was transferred into a series of shaken until the appearance of yellow colour indicating the liberation of iodine. Toluidine blue (0.01%, 0.5ml) or safranine (0.02%, 0.5ml) was then added and the reaction mixture was shaken for 2min for maintaining pH 4.2ml of acetate buffer was
added. The contents were diluted to 10ml with distilled water and mixed well. The absorbance of the resulting solution was measured at 628 or 532nm against reagent blank.

4.15.2 Spectrophotometric determination of mercury (Jayateerth R Mudakavi, 1984)

Mercury solution (1mg/ml) was prepared by dissolving 0.3385g of mercuric chloride in water and dilute to 250ml. Dilute an appropriate volume of this solution to provide a 10ppm solution of mercury (II). Phenantroline solution (0.05%) was prepared by dissolving 0.05g of 1,10 phenentroline monohydrate in 100ml distilled water and was stored in an amber coloured bottle. Acetate Buffer (pH 4.5) was prepared by mixing 124ml of 1N acetic acid with 50ml of 1N sodium to 4.5. Eosin solution (0.05%) was prepared by dissolving 0.125g of eosin (1.45380) in water and dilute to 250ml. EDTA Solution (0.05M) was prepared dissolving 1.8615g of EDTA in 100ml of distilled water. Gelatin solution (0.05%) was freshly prepared by dissolving 0.025g of gelatin in 50ml of hot water.

To a series of solutions containing 5-30µg of mercury (II), 1ml of 0.05M EDTA solution, 5ml of acetate buffer, pH 4.5, 2ml of 0.05% of 1,10 phenantroline monohydrate, 1ml of 0.05% gelatin, 5ml of 0.05% eosin were added and mixed well. It was then diluted to 25ml using distilled water and the absorbance was measured at 552nm against reagent blank. Phenantroline coordinates readily with mercury (II) to form a mono, bis and tris complexes. Bis phenantroline mercury (II) complexes of the type Hg-X2B2 precipitates on mixing ethanolic solutions of phenantroline and mercury (II) halide.