Chemicals

The chemicals and kits necessary for the present Research were obtained from the different standard companies. All the chemicals that were used for enrichment of the soil sample, selective media, staining reagents and biochemical characterization were procured from Himedia Laboratories Pvt. Ltd., Mumbai. The Scanning Electron Microscope photographs were obtained from Ruska labs, Sri Venkateswara Veterinary University, Rajendranagar, Hyderabad. The absolute ethyl alcohol was acquired from Hayman Ltd., England. The chemicals employed in DNA extraction protocols, amplification reactions and electrophoresis were procured from Merck India Pvt. Ltd., Mumbai and remaining chemicals which employed in further experiments were purchased from Fisher Inorganic and Aromatics Ltd., Chennai. The Universal primers for the ribo typing and Bacterial Genomic DNA Isolation kit and the necessary reagents for the PCR reaction were commercially procured from Helini Biomolecules. The primers synthesis and the sequencing of fragments were executed at Helini Biomolecules Pvt. Ltd., Chennai. The different Softwares that were employed for the Homology Modeling of proteins were from Bio Minds Pvt.Ltd., Hyderabad.

Isolation of Bacterial Strains

50 soil samples were collected from different areas of agricultural lands at Duggirala and Burripalem in Guntur district, Andhra Pradesh and sieved through a 2mm sieve, constituted the soil sample. The soil samples were collected from
1-15 inches depth below the soil where there was moisture which supports ideal environment for the growth of microbes. The samples were dispensed into bags and were brought to the laboratory.

**Enrichment of the soil sample**

One hundred grams of the soil sample was taken in a sterile beaker and to this soil sample, 1g of the pectin and a small quantity of water (Equivalent to 60% of the water holding capacity) was added and incubated for 30 days for enrichment.

**Enrichment Broth for Isolation of Pectinolytic Bacteria**

One gram of the soil sample of different samples that were enriched with 1% of pectin was taken in a conical flask containing 100 ml of sterilized enrichment broth which had the following composition (gms/L).

**Composition of the Enrichment Broth**

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectin (1%)</td>
<td>1 gram</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.1 gram</td>
</tr>
<tr>
<td>Peptone (Himedia)</td>
<td>0.5 grams</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.2 grams</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.2 grams</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Materials and Methods

The Pectin was supplemented as the carbon source and the peptone as the source of nitrogen. The enrichment broth was inoculated with the enriched soil sample and placed on the rotatary shaker at 200 rpm for 10 days.

Serial dilution technique

The bacteria were isolated from the collected soil samples by subjecting the soil for enrichment with Pectin. 10 fold serial dilutions ($10^{-1}$ to $10^{-8}$) of the enriched soil sample were prepared. 1ml portion of the dilution ($10^{-4}$ to $10^{-6}$) was inoculated into 100 ml of the selective broth (Vincent’s mineral salts broth) at pH 7.

Selective Media

A selective medium is the one that allows the growth of microorganisms of interest. Selective media are very important in primary isolation of a specific type of microorganism from soil sample that harbor millions of microbes of different types. They hasten isolation by suppressing the unwanted background organisms and favour the growth of desired ones. Thus, a selective medium i.e. Vincent’s mineral salts broth (Vincent, 1970) was employed here which allows only the desired bacteria to utilize pectin as the sole carbon source and inhibits the growth of the bacteria that cannot use pectin as the carbon source. The bacterial isolates were inoculated and grown in Vincent’s mineral salts broth at pH 7 (Culture broth) and incubated.
The composition of the medium (g/L) (Vincent’s mineral salts broth at pH 7)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>1</td>
</tr>
<tr>
<td>KNO₃</td>
<td>0.6</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1</td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.1</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>2</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1</td>
</tr>
<tr>
<td>Pectin (1%)</td>
<td>10</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

All the above ingredients were weighed to the desired volume and made up to 250 ml in Erlenmeyer flask and sterilized at 121°C for 15 minutes according to the standard procedure.

Vincent’s Agar Medium

Vincent’s Agar Medium is a selective medium in which the pectin has been supplemented as a carbon source. All the collected soil samples were enriched and diluted. Aliquots of 0.5 ml diluted (10⁻⁴, 10⁻⁵) culture broth was inoculated under aseptic conditions into Petri plates containing the Vincent’s agar medium and the plates were incubated at 37°C for 48 hrs in inverted position. These plates were maintained in replicates. The secretion of pectinase was
identified by the clearing zones of the medium after the addition of Logule's iodine solution. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest pectinase activity producer. The largest ratio was assumed to contain the highest activity. The composition of the Vincent's agar medium is as follows:

**Composition of the Vincent's Agar Medium (g/L)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>1</td>
</tr>
<tr>
<td>KNO₃</td>
<td>0.6</td>
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<td>CaCl₂</td>
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<td>2</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1</td>
</tr>
<tr>
<td>Pectin (1%)</td>
<td>10</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>20</td>
</tr>
</tbody>
</table>

**Maintenance of pure Cultures**

The pure cultures of bacteria used in the present investigation were maintained by cross streak method and incubated at 37°C. When three isolated simple colonies appeared, it was transferred on to the slants of same
composition and incubated at the same optimized conditions. Periodic transfers were made at regular intervals and every time purity of the cultures was checked by microscopic observation using gram’s stain preparation.

**Identification of the Bacteria**

The isolated bacteria were identified using different staining techniques, different biochemical and molecular techniques.

**Simple Staining**

One drop of the bacterial suspension of the isolates was taken and a smear was prepared by heat fixation on a clean glass slide and the bacterial smear was flooded with basic stain and kept for 1 min, then washed and blot dried and observed under the microscope (100X magnification, Olympus). Bacteria are very small and transparent when observed with a wet mount preparation. In order to observe their cell characteristics, they need to be stained (Dyed). The simple stain consists of one dye. The dye adheres to the cell wall and colours the cell making it easier to see. Basic stains, such as methylene blue, Gram’s Saffranin or Gram’s Crystal Violet are used for staining most of the bacteria. These stains readily give up a hydroxide ion or accept a hydrogen ion, which leave the stain positively charged. Since the surface of most bacterial cells is negatively charged, these positively charged stains adhere readily to the cell surface.
Gram Staining

Several different kinds of bacteria can be examined by gram staining, a method developed by Christian Gram in 1884 for categorizing bacteria on the basis of differences in cell wall structure. Gram positive (+) bacteria stain dark purple color, while gram negative (-) bacteria stain light red. One drop of the bacterial suspension of the isolate was taken and a smear was prepared by heat fixation on a clean glass slide and the bacterial smear was flooded with basic stain i.e. crystal violet for 2 minutes, washed, blot dried and flooded with gram's iodine for 1 minute, then washed, blot dried and the slide was subjected to decolourization step with 95% ethanol. After adding 20 drops of 95% ethanol to the slide, the slide was left for 10 seconds and then rinsed with water from a squirt bottle and the slide was counterstained with Saffranin for 60 seconds, and then observed under the microscope (100X magnification, Olympus). Since the cell wall of the gram +ve bacteria constitutes a barrier to the decolorizing agent, the gram +ve bacteria remain purple, while the gram -ve bacteria are decolorized and appear light red.

Motility Test

This test is to determine the motility of the bacteria at a given temperature. The medium used was a semi-solid nutrient agar medium. The test organism was stabbed into the medium with the inoculating needle to approximately one-half deep and the tubes were kept for incubation at 37°C for 24 hrs. Motile
bacteria migrate from the stab line and diffuse into the medium causing turbidity (Motility of the bacteria is confirmed). While the non-motile bacteria show the growth confined to the stab line (Bacteria are non-motile).

**Biochemical Tests**

The strain isolated from soils of different villages of Guntur District (Duggirala and Burripalem) were identified by conventional biochemical tests in accordance with Bergey’s Manual of Systematic Bacteriology (Sneath, 1986; Holding and Colle, 1971; Buchanan and Gibbons, 1974; Taiwo and Oso, 2004). The isolate was subjected to different tests like Indole production test, Methyl red and Voges proskauer tests, Gelatin Hydrolysis (Production of Gelatinase), Starch Hydrolysis, Oxidase Production, Catalase Activity, Citrate utilization test, Nitrate reduction test, Caesin Hydrolysis etc.

**Identification of the Isolated Bacteria by Sequencing of the Amplified 16S rRNA Gene**

The most powerful tool to identify the unknown bacteria is to sequence the DNA coding for 16s rRNA, since the 16s rRNA is encoded by the gene in the chromosome of the bacteria. So the gene coding for the 16s rRNA is amplified using the Polymerase Chain Reaction (Mullis, 1990), and the amplified product has been subjected to sequencing and the sequence obtained has been compared with the sequence obtained from the Nucleotide Database of NCBI.
Genomic DNA Isolation of the bacterial Isolates

Genomic DNA was isolated from Bacillus according to the following procedure. 1L of LB broth (Luria-Bertani) was prepared with the following composition (Tryptone 10 gm, Yeast Extract 5 gm and NaCl 10 gm). From the above broth 50ml was taken, inoculated with the test isolate, incubated at 37°C and grown to an OD 600 of 0.5–1.0. After centrifugation at 5000 rpm, 4°C, for 10 min, cells were collected for further study. The genomic DNA of the Bacteria was isolated by using the Bacterial Genomic DNA isolation kit according to the manufacturer protocol (Helini Biomolecules).

Estimation of the Quantity and Quality of the DNA

The isolated DNA might contain impurities of RNA and protein that can interfere in the DNA tests. There are two methods by which the quantity and quality of the DNA can be determined and followed.

Agarose Gel Electrophoresis

Gel electrophoresis is a technique used for the separation of nucleic acids and proteins, where electrically charged molecules migrate at a rate proportional to their charge in mass ratio when placed under an electric field (Raj Kashyap et al., 2003). It is undoubtedly the routine technique to monitor the success of the nucleic acid isolation procedures and analyze enzymatic manipulations such as
restriction enzyme digestion. About 0.8 g of agarose was weighed and taken into
100 ml reagent bottle. To it, 100 ml of 1X TBE buffer along with 0.5 µg/ml of
ethidium bromide (EtBr) was added for visualization and it was heated in a
microwave oven till all agarose gets melted up. The agarose solution was then
poured in to gel casting unit assembled with appropriate comb and it was allowed
to polymerize. After polymerization, the comb was removed and the gel was
placed in an electrophoretic tank consisting of 1X TBE buffer. About 2 µl of the
isolated genomic DNA was mixed with 2 µl of the gel loading dye (Bromophenol
blue) and it was loaded in 0.8 % agarose gel. The gel was then electrophoresed
at 90 volts for about 30 minutes and it was observed in a gel documentation
system.

**Spectrophotometry**

The most comprehensive way to evaluate DNA concentration is to use the
UV spectrophotometric measurements. DNA purity can be checked by UV
absorption at wave lengths of 260 to 280nm. The basic principle is that the DNA
absorbs the ultra violet light (UV region) between 260 to 280nm owing to the
spectral characteristics of the four bases. Measurement of DNA purity can be
determined by the OD260: OD280. This ratio indicates the protein contamination
in the sample tested. Based on the absorbance of the light, the concentration of
the DNA can also be calculated. About 2 µl of the DNA was added to 500 µl of
the distilled water, mixed well and the diluted DNA was taken in a 0.5 ml quartz
cuvette. In another cuvette 0.5 ml of the distilled water was taken and the spectrophotometer was calibrated by reading the blank at 260nm and 280nm respectively. Later the samples were also read at 260nm and 280nm respectively. The OD value at 260nm provides a measure of concentration (roughly 1.0 reading at OD 260nm is equivalent to 50 µg/ml). The absorbance obtained was then used for calculating the quality and quantity respectively.

Calculating the purity and yield

One absorbance unit at 260 nm of the double stranded DNA is equal to 50 µg/ml of the double stranded DNA. One absorbance unit at 260 nm of the single stranded DNA is equal to 40 µg/ml of the single stranded DNA.

- Total $A_{260}$ Units = $(A_{260}) \times$ dilution factor

- Concentration (µg/ml) = total $A_{260}$ units $\times$ (50 µg/ml)

- Yield (µg) = Volume $\times$ Concentration

- Pure DNA exhibits an absorbance ratio $(A_{260}/A_{280})$ of 1.8 to 2.0

- If the DNA exhibits an absorbance ratio $(A_{260}/A_{280})$ of less than 1.7, the Sample is contaminated by protein.

Amplification of the 16s rRNA Gene of Bacterial Chromosome

The polymerase chain reaction is an enzyme catalyzed biochemical reaction in which small amount of the specific DNA sequences are amplified into large amounts of linear double stranded DNA (Mullis, 1990). PCR is used to
amplify the DNA sequence in between two known sequences. The 16s rRNA gene of the bacteria was carried out in our laboratory in the Thermocycler (Eppendorf). Specific primers (Forward and Reverse primers) complementary to the known sequences, master mix (HELINI Biomolecules, Chennai) were added to the template DNA and the mixture was placed in a thermocycler, heated at 94°C for denaturation of the DNA. The mixture was then allowed to cool enabling the primers to anneal to the complementary sequences. A heat stable DNA polymerase was used to make the copies of the DNA from the original DNA sequence. About 30 cycles of the DNA amplification were performed that resulted in a very large amplification of the DNA. The 1542 bp rRNA gene was amplified using two primers. Two primers annealing at the 5’ and 3’ end of the 16S rDNA were (Forward Primer) 5’ - GAGTTTGATCCTGGCTAG-3’ [positions 9–27 (Escherichia coli 16S rDNA numbering)] and (Reverse primer) 5’-AGAAA GGAGG TGATC CAGCC-3’ [positions 1542–1525 (E. coli 16S rDNA numbering)] were used. The master mix containing 10X Taq buffer, 10 mM dNTPs, 25 mM of MgCl$_2$, 1 U of Taq DNA polymerase, 1.5 µl of forward primer, 1.5 µl of Reverse primer, 100 ng of Genomic DNA and PCR grade molecular water to make the final volume to 20 µl was used. Taq DNA polymerase initiates the replication of DNA fragments by using nucleotide base from dNTP mixture (A, T, G, and C).
Setting up the PCR reaction

A fresh master mix tube which contains the dNTP and Taq DNA Polymerase was taken and the following components were added

Master Mix vial 10 µl
Forward Primer 1.5 µl
Reverse primer 1.5 µl
Template DNA 2 µl
Nuclease free water 5 µl

Total volume 20 µl

(Master Mix contains 1U Taq DNA polymerase, 10X Taq buffer, 10 mM dNTPs and PCR grade water. Primer dye mix contains 10 µM forward primers, 10 µM Reverse primer and PCR compatible dye with glycerol).

After addition of all the components, the PCR tube was gently spun down in centrifuge briefly and was placed in the Thermal cycler. The thermal cycler was programmed as follows.

Program 1 (one cycle) (Initial denaturation)
94 °C for 2 minutes

Program 2 (30 cycles) (Amplification)
Step one (denaturation) 94 °C for 45 seconds
Step two (annealing) 56 °C for 1 minute
Step three (extension) 72 °C for 1 minute 30 seconds
Program 3 (one cycle - final extension)

72°C for 5 minutes, then hold at 4°C

The amplified DNA was subjected to Agarose gel electrophoresis along with the marker DNA (DNA Ladder) and basing on the size of the amplified 16S rRNA (DNA) fragment, it was confirmed that the 16S rRNA gene was amplified. The DNA band of the amplified product was cut from gel, eluted and subjected for sequencing (Helini Biomolecules). The sequence so obtained was compared with the reported results present in the public databases (NCBI) and the sequence of the unknown bacteria was determined. The objective of this rDNA sequencing is to determine a new bacterial strain that has been isolated basing on its taxonomy: whether it belongs to a known genus, and if not, to identify the new species within known genera and also to determine (as much as possible) if it is a new species or a new strain of an already known species.

Scanning Electron Microscopy of *Bacillus subtilis*

Scanning Electron microscopic studies was carried out by taking 24 hrs old cultures of *Bacillus subtilis* and fixed in 6% buffered glutaraldehyde followed by post fixation in Osmium tetroxide and then dehydrated in increasing concentration of ethyl alcohol. The samples were mounted on copper stubs with double sided adhesive tape, coated with gold polaron, AU/PD sputter-coater and scanned in SEM (Jeol JSM 5600, Japan) and photographed. The SEM studies
were conducted at Ruska labs, Sri Venkateswara Veterinary University, Rajendranagar, Hyderabad.

Stock seed preparation

About 5 ml of sterile distilled water was added on to the well grown culture and vortexed for few minutes to make uniform bacterial suspension. One ml of this suspension was used to incubate 50 ml of sterile seed medium present in 250 ml Erlenmeyer flask. These inoculated flasks were kept on a shaker and maintained at 37° C for 24 to 36 hours and this seed was used for all the other experiments.

Production of Pectinase

For production of pectinase, 2.0 ml of the seed was taken and inoculated in to 250 ml Erlenmeyer flasks containing 50 ml sterile productive medium (Vincent Broth). The inoculated flasks were incubated at 37° C for 96 hours using aerated and agitated condition on a rotary shaker with 250 rpm for 96 hours. At the end of the fermentation cycle, 5.0 ml of the fermented broth was aseptically removed and centrifuged at 2000 rpm for 10 minutes. The clear supernatant containing the enzyme was used in the enzyme assay. For all the experiments, triplicates were maintained for consistent observations.

Enzyme Assay

Enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNS) method (Miller, 1959). For this, to 0.2 ml of 1% pectin solution, 2.0
ml of sodium citrate buffer of pH 7.0 and 1.0 ml of enzyme extract were added. The reaction mixture was incubated at 37°C±1°C for 25 min. After 25 min, 1.0 ml of this reaction mixture was withdrawn and added to test-tubes containing 0.5 ml of 1M sodium carbonate solution. To each test-tube, 3.0 ml of DNS reagent was added and the test-tubes were shaken to mix the contents. The test-tubes were heated to boiling on the boiling water-bath for 10–15 min. The tubes were cooled and 4 ml of the reaction mixture was measured at 550 nm. The enzyme and substrate blanks were run parallel to one another and the standard curve was prepared for reducing sugars with glucose. One enzyme unit of endopolygalacturonase is the number of µM of reducing sugars measured in terms of glucose, produced as a result of the action of 1.0 ml of enzyme extract in 1 minute at 37°C ± 1°C.

The OD of test was subtracted from the OD unknown

= OD of Test – OD of Unknown = to the OD of the Colour intensity of the liberated product.

From graph, the concentration of the reducing sugar liberated by the action of the enzyme is determined and the Enzyme activity is expressed.

Enzyme Activity = µ moles of the product liberated per mole of enzyme per ml per Minute of Glucose liberated.

\[
\text{IU/ml/min enzyme} = \frac{(\mu\text{mol of glucose equivalent released})}{(1) (10) (2)}
\]
Materials and Methods

11 = Total volume (in milliliters) of assay

10 = Time of assay (in minutes) as per the Unit Definition

1 = Volume of enzyme (in milliliter) used

2 = Volume (in milliliters) used in Colorimetric Determination

**Adjusting the pH of the Medium**

The media although contains buffering agents that keep the pH of the medium in a desired range, the pH of the medium may change. So before the medium is sterilized, one should check the pH and make any necessary adjustments. The pH of the medium is checked with the pH meter (ELICO, Hyderabad). The changes in the pH of the medium was made based on the change of the pH of the medium, if the pH of medium is too high (Alkaline) and above the desired pH range, then the pH of the medium was lowered by adding 1N HCl drop by drop until the pH reaches to the desired range, and if the pH of the medium is slightly above 0, then the pH of the medium was lowered by adding 1N HCl drop by drop and when the pH of the medium was too low (Acidic) the pH of the medium was increased by adding 1N NaOH drop by drop until the pH reached to the desired range and if the pH is slightly lowered, then the pH of the medium was increased by adding 0.1 N NaOH drop by drop until the pH reached to the desired range.
Optimization of Physico-chemical parameters for Pectinase Production by *Bacillus subtilis*

Pectinase enzyme production in any species such as bacteria can be varied and affected by different physico–chemical parameters (Suneetha and Khan, 2010). These parameters could affect the secretion of the enzyme in different ratios. Therefore, in our present investigation the pectinase enzyme production was optimized at various temperature ranges, pH, substrate concentration (natural carbon sources) and at various concentrations of nitrogen sources (Organic and inorganic). The experiments were conducted in 250 ml Erlenmeyer flasks containing the production medium (Vincent's mineral salts broth medium). After sterilization by autoclaving, the flasks were cooled and inoculated with pure cultures that have been selectively isolated using selective media.

Optimization of the pH for Pectinase Production by *Bacillus subtilis*

Optimization of the pH of basal medium and production media (Vincent's mineral salts agar medium) was adjusted to 4, 5, 6, 7, 8 and 9 by using 1N HCL or 1N NaOH. All the experiments were carried out in triplicates at 37°C and average values were noted. The optimum pH achieved by this process was followed for subsequent experiments (Patil and Dayanand, 2006).
Materials and Methods

**Optimum temperature for Pectinase Production by Bacillus subtilis**

The production process was carried out by shake flask method by placing the flasks on a rotary shaker. The production process was carried out at various temperatures such as 25°C, 30°C, 35°C, 40°C, 45°C, 50°C and 55°C, to study the effect of temperature on enzyme production (Soriano Diaz and Pastor, 2005). All the experiments were carried out in triplicates and the average values were recorded. The optimum range of temperature achieved by this step was followed for all subsequent experiments.

**Effect of Different substrates on Pectinase Production by Bacillus subtilis**

Since all the plant agro-industrial waste material contains pectin as the cell biomass which can be used as substrates for the production of the pectinase (Koki Horikoshi, 1995; Aparna and Gupta, 2001; Patil and Dayanand, 2006; Nitinkumar et al., 2010). Two different natural substrates such as dried orange peel powder and dried banana peel waste powder (0.5%, 1%, 1.5%, 2% and 2.5%) were used in the present study. The production medium was supplemented with the substrates i.e. dry orange peel powder and banana peel waste powder as the source of carbon and inoculum of Bacillus was added into 250 ml production medium for pectinase production. The study was carried out in triplicates by maintaining temperature, pH at their optimum level and the average values of pectinase production were recorded. The best substrate which gave
highest enzyme activity was identified and that substrate was used for all other parameters.

**Effect of different nitrogen additives on Pectinase Production by *Bacillus subtilis***

Nitrogen is needed for the synthesis of amino acids, purines, pyrimidines, some carbohydrates and lipids, enzyme cofactors, and other substances. Many microorganisms can use the nitrogen in amino acids, and ammonia often is directly incorporated by the action of enzymes such as glutamate dehydrogenase, glutamine synthetase and glutamate synthase. The nitrogen has a profound influence on the growth of the bacteria. The influence of different nitrogen sources such as ammonium chloride, sodium nitrate and ammonium sulphate (Inorganic), beef extract, peptone and tryptophan (Organic) on pectinase production was studied. All the experiments were carried out in triplicates and average values were recorded. The suitable nitrogen source identified and achieved by this step was followed for all the subsequent experiments.

**Statistical analysis**

Results on the optimization of the pectinase production by the isolate under different Physico-Chemical conditions were statistically analyzed. Statistical analysis of the model was performed to evaluate the analysis of
Materials and Methods

variance (ANOVA) (Zar, 1974). The mean values were compared by the least significant difference (LSD) test at 5% level of confidence.

Computational Study: Polygalacturonase of Bacillus subtilis

The structural studies of Pectinase enzymes were not reported to the protein database (PDB). So, the present study was undertaken to study the in Silico model of the Pectinase enzyme of Bacillus subtilis.

Retrieval of Amino Acid Sequence of the Protein Sequence from Swiss-Prot

Swiss-Prot was created in 1986 by Amos Bairoch during his PhD and developed by the Swiss Institute of Bioinformatics and the European Bioinformatics Institute (Bairoch Amos, 2000). Swiss-Prot is a manually curated biological database of protein sequences. Swiss-Prot strives to provide reliable protein sequences associated with a high level of annotation (such as the description of the function of a protein, its domains structure, post-translational modifications, variants, etc.), a minimal level of redundancy and high level of integration with other databases. In 2002, the UniProt consortium was created: it is collaboration between the Swiss Institute of Bioinformatics, the European Bioinformatics Institute and the Protein Information Resource (PIR), funded by the National Institutes of Health. Swiss-Prot and its automatically curated supplement TrEMBL, have joined with the Protein Information Resource protein database to produce the UniProt Knowledgebase, the world's most
comprehensive catalogue of information on proteins (Rodrigo et al., 2004; Wu et al., 2006). As of 3 April 2007, UniProtKB/Swiss-Prot release 52.2 contains 263,525 entries. As of 3 April 2007, the UniProtKB/TrEMBL release 35.2 contains 4,232,122 entries. The UniProt consortium produced 3 database components, each optimized for different uses. The UniProt Knowledgebase [UniProtKB (Swiss-Prot + TrEMBL)], the UniProt Non-redundant Reference (UniRef) databases, which combine closely related sequences into a single record to speed similarity searches and the UniProt Archive (UniParc), which is a comprehensive repository of protein sequences, reflecting the history of all protein sequences.

**Prediction of Amino Acid composition of the protein by Bioedit**

BioEdit is a biological sequence alignment editor written for Windows 95/98/NT/2000/XP (Hall, 1999). An intuitive multiple document interface with convenient features makes alignment and manipulation of sequences relatively easy. Several sequence manipulation and analysis options and links to external analysis programs facilitate a working environment which allows to view and manipulate sequences with simple point-and-click operations. (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html).
**Protein primary structure prediction by ProtParam**

ProtParam is a tool which allows the computation of various physical and chemical parameters for a given protein stored in Swiss-Prot or TrEMBL or for a user entered sequence. The computed parameters include the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (Gasteiger *et al.*, 2003).

**Enzyme secondary structure prediction using PDB sum**

PDBsum is a web-based database providing a largely pictorial summary of the key information on each macromolecular structure deposited at the Protein Data Bank (PDB). It includes images of the structure, annotated plots of each protein chain’s secondary structure, detailed structural analyses generated by the PROMOTIF program, summary PROCHECK results and schematic diagrams of protein–ligand and protein–DNA interactions. RasMol scripts highlight key aspects of the structure, such as the protein’s domains, PROSITE patterns and protein–ligand interactions for interactive viewing in 3D. Numerous links take the user to related sites. PDBsum is updated whenever any new structures are released by the PDB and is freely accessible via [http://www.biochem.ucl.ac.uk/bsm/pdbsum](http://www.biochem.ucl.ac.uk/bsm/pdbsum) (Laskowski, 2001).

The PDBsum database ([http://www.biochem.ucl.ac.uk/bsm/pdbsum](http://www.biochem.ucl.ac.uk/bsm/pdbsum)) was created in 1995 (Laskowski *et al.*, 1997). Its aim was to provide an at-a-glance
Materials and Methods

summary of the molecules contained in each PDB entry (i.e. protein and DNA/RNA chains, small-molecule ligands, metal ions and waters) together with annotations and analyses of their key structural features. Thus, for each PDB entry there is a corresponding summary web page in PDBsum, accessible by the four-character PDB identifier. The original PDBsum paper (Laskowski et al., 1997) described the basic contents of each entry namely, a block of ‘header’ information relating to the entry as a whole followed by a list of the molecules making up the structure together with any relevant structural analyses of each. The header details start with a thumbnail image of the molecule(s) in question plus buttons for viewing the whole structure in 3D using RasMol (Sayle and Milner, 1995) or Virtual Reality Modeling Language (VRML). These are followed by information extracted directly from the header records of the PDB file, summary PROCHECK (Laskowski et al., 1993) analyses (including a Ramachandran plot) giving an indication of the stereochemical ‘quality’ of all the protein chains in the structure, and links to related databases. In the list of molecules, each protein chain is shown schematically by a ‘wiring diagram’ depicting its secondary structural motifs, primary sequence, structural domains and highlighting active site residues and residues that interact with ligands, metals or DNA/RNA molecules. The secondary structural motifs are computed by the PROMOTIF (Hutchinson and Thornton, 1996) program whose detailed outputs are available via hyperlinks while the domain definitions come from the CATH protein structural classification database ( Orengo et al., 1997). For each
ligand molecule a LIGPLOT diagram gives a schematic depiction of the hydrogen bonds and non-bonded interactions between it and the residues of the protein with which it interacts (Wallace et al., 1995).

Phyre Server

The Phyre-Server is a stand-alone in-house web-server system based upon the world-class Imperial College, London. Phyre (the Protein Homology/analogy Recognition Engine) server has been serving the academic community since 2005 and regularly receives between 600 and 700 submissions per week. Originally developed in 2004, Phyre was one of the top performing stand-alone protein structure recognition systems at Critical Assessment of Techniques for Protein Structure Prediction (CASP6) and was highlighted as having the best domain boundary prediction algorithm for multi-domain proteins. It combines primary and secondary structure profile information using highly optimized profile-profile comparison algorithms. The Equinox Phyre-Server is ideally suited to accommodate the needs of companies and research institutes with large number of potential users, or who regularly perform high-throughput structure prediction analyses. The Phyre-Server is designed to run on Linux-based desktop and server systems (Lawrence and Michael, 2009).
Structure validation by PROCHECK

It Checks the stereochemical quality of a protein structure, producing a number of PostScript plots analyzing its overall and residue-by-residue geometry. These Operating Instructions describe how to run the PROCHECK suite of programs for assessing the "stereochemical quality" of a given protein structure. The aim of PROCHECK is to assess how normal, or conversely how unusual, the geometry of the residues in a given protein structure is, as compared with stereochemical parameters derived from well-refined, high-resolution structures. Unusual regions highlighted by PROCHECK are not necessarily errors as such, but may be unusual features for which there is a reasonable explanation (e.g. distortions due to ligand-binding in the protein's active site). Nevertheless they are regions that should be checked carefully (Ramachandran and Sasisekharan, 1968; Richardson, 1981).

Input

The input to PROCHECK is a single file containing the coordinates of our protein structure. This must be in Brookhaven file format. One of the by-products of running PROCHECK is that your coordinates file will be "cleaned up" by the first of the programs. The cleaning-up process corrects any mislabeled atoms and creates a new coordinates file which has a file-extension of new. The new file will have the atoms labelled in accordance with the IUPAC naming conventions (IUPAC-IUB Commission of Biochemical Nomenclature, 1970).
Output

The output comprises a number of plots, together with a detailed residue-by-residue listing. The plots are output in PostScript format (Adobe Systems Inc., 1985), and so can be printed off on a PostScript laser printer, or displayed on a graphics screen using suitable software (e.g., GHOSTSCRIPT or GHOSTVIEW on Sun or Silicon Graphics workstations or PSVIEW on Silicon Graphics IRIS-4 D systems). The plots can be in colour or black-and-white and are described in Sample plots.

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