MATERIALS AND METHODS

3.1 Bacterial strain

The bacterial strain, *Bacillus subtilis* SAV-21 used for production, purification, immobilization and industrial application of pectinase, was isolated in our laboratory from the soil samples collected from different regions of India. The stock cultures of this microbial strain were being maintained on nutrient agar (peptone 5.0 g/L, beef extract 3.0 g/L and agar 25.0 g/L, pH 7.0) at 4°C by periodic sub-culturing after every one week. For short-term preservation, the culture was streaked on nutrient agar slants, incubated at 37°C and stored at 4°C. For long-term preservation of the culture, glycerol stocks (30%) were prepared and stored at -20°C in a deep freezer.

3.2 Chemicals

Chemicals of high purity were used in the present investigation. The chemicals namely acrylamide, bis-acrylamide, tris base, sodium dodecyl sulphate (SDS), ammonium sulphate, glycine, bromophenol blue, N,N,N′,N′-tetramethylene-diamine (TEMED), Ammonium persulfate (APS), bovine serum albumin (BSA), β-mercaptoethanol, Coomassie brilliant blue R-250 and Coomassie brilliant blue G-250 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pectin, peptone, beef extract, tryptone, yeast extract, sodium chloride, yeast extract, sucrose, agar, sodium hydroxide, calcium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium acetate, ammonium sulfate and protein molecular weight markers for SDS-PAGE were procured from Himedia Laboratories (India). Methanol, glacial acetic acid hydrochloric acid, sulphuric acid and nitric acid were purchased from Ranbaxy Laboratories, India. Glutaraldehyde, silver nitrate, glycerol and Folin-Ciocalteau reagent were purchased from Merck Laboratories. Agro-residual wastes used in the present study were procured from the local market.

3.3 Isolation, screening and identification of a potent pectinase producing bacterial strain

The aim of this experiment was to isolate and identify an efficient pectinase producing bacterial strain. The methods employed to carry out this experiment are given below:
3.3.1 Growth media used for isolation and screening of pectinolytic bacterial strain

Nutrient agar (peptone 5.0 g/L, beef extract 3.0 g/L and agar 25.0 g/L, pH 7.0) was used for isolation of bacterial strains. Mosambi peel agar (peptone 5.0 g/L, beef extract 3.0 g/L, agar 25.0 g/L and mosambi peel 10 g/L), pectin agar (peptone 5.0 g/L, beef extract 3.0 g/L, agar 25.0 g/L and pectin-10 g/L) and pomegranate peel agar (peptone 5.0 g/L, beef extract 3.0 g/L, agar 25.0 g/L and pomegranate peel 10 g/L) were used for screening of pectinolytic bacteria.

3.3.2 Sample collection and processing

Pectinase producing microorganisms are likely to be found at places where pectin rich substances are present. A total of 12 samples comprising of fruit and vegetable wastes, decaying coconut husks and agricultural waste collected from six different places for the isolation of pectinase producing bacterial strains. One gram of soil sample was suspended in 10 ml of distilled water and serially diluted. An aliquot (100 µl) of each serially diluted sample was spread onto nutrient agar plates which were incubated at 37°C for 24 h. The colonies grown on the plates were streaked onto fresh nutrient agar plates. After 24 h incubation at 37°C, replica plates were made and incubated under the same conditions. The colonies developed on the plates were purified by repeated transfer of cultures.

3.3.3 Qualitative screening for pectinase producing bacteria

For qualitative screening of pectinase producing bacteria, the isolated bacterial colonies were streaked onto mosambi peel agar, pectin agar and pomegranate agar media plates. The plates were incubated at 37°C for 24 h and stained with I$_2$/KI solution (0.5% I$_2$ dissolved in 1% KI solution) for 30 min followed by destaining with distilled water. The pectinase producing bacteria were identified by the formation of yellow zone of hydrolysis against dark brown background. The colonies showing zone of hydrolysis were picked up from the replica plates, grown on the respective media and subjected to quantitative screening for pectinase production. The diameter of each zone of hydrolysis was recorded.

3.3.3 Quantitative screening for pectinase-producing bacteria

The bacterial isolates showing positive result in qualitative analysis were subjected to quantitative screening for pectinase production under submerged
fermentation (SmF) in 250 ml Erlenmeyer flasks containing 50 ml of modified Horikoshi media (Table 3.1). The flasks were inoculated with overnight grown cultures at 1% level and incubated at 37°C for 48 h in an orbital shaker at 200 rpm. After incubation, the flasks were taken out and the culture filtrates were centrifuged at 10,000 x g for 15 min at 4°C. The resulting clear supernatants were collected and assayed for pectinase activity.

Table 3.1 Composition of modified Horikoshi medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0 g/L</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0 g/L</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>5.0 g/L</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.0 g/L</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.1 g/L</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 L</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

3.3.4 Assay of pectinase activity

Pectinase activity was assayed by measuring the amount of D-galacturonic acid liberated from pectin by the enzyme. The reaction mixture containing 50 µl of appropriately diluted enzyme and 450 µl of 0.5% pectin (having 65-70% degree of esterification and dissolved in 50 mM glycine- NaOH buffer, pH 11.0) was incubated for 10 min at 60°C and the end products were quantified by using DNSA reagent (Miller, 1959). A standard curve of D-galacturonic acid (25 to 200 µg) concentration versus absorbance (at 540nm) was plotted. The concentration of D-galacturonic acid liberated in the test samples was estimated using standard curve and enzyme activity was calculated, where one unit of pectinase activity was defined as the amount of enzyme required to liberate 1 µmol of D-galacturonic acid/min under the assay conditions.

3.3.5 Protein estimation

Protein concentration in the chromatographic fractions was estimated by measuring absorbance at 280 nm. In the pooled purified fraction and other samples, protein content was estimated according to Bradford's method (Bradford, 1976) which is as follows:
Reagents:

1) Coomassie brilliant blue G-250 (100mg) (2.0 %, w/v, in 0.1 N NaOH)
2) 95% ethanol
3) 85% (w/v) phosphoric acid
4) Working reagent: It was prepared by dissolving 100 mg of Coomassie brilliant blue G-250 in 50 ml of 95% ethanol. To this solution, 100 ml of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter.

Procedure:

In triplicate sets of test tubes, varying amounts of BSA and distilled water were added to make final volume 0.1 ml. A blank was also prepared in which 0.1 ml of distilled water was added. In each tube, 3.0 ml of Bradford reagent was added (which led to development of intense blue color), followed by immediate recording of absorbance in test samples against blank at 595 nm. A standard graph was prepared by plotting BSA concentration (0-100 µg) versus $A_{595}$. The protein concentration in test samples was calculated from the standard graph constructed using BSA.

3.3.6 Identification of the selected pectinase-producing bacterial isolate

On the basis of qualitative and quantitative analysis of the isolates, a potent pectinase producing bacterial isolate (SAV-21) was selected for further studies.

3.3.6.1 Morphological and Biochemical tests

Morphological (cell shape, size, motility, surface and Gram staining) and biochemical tests (casein hydrolysis, starch hydrolysis, citrate utilization, catalase test, and gelatin hydrolysis) were performed on the selected bacterial isolate. The selected strain was further identified by using carbohydrate utilization test kit (HiCarboTM) purchased from HiMedia Laboratories (India), which are based on the principle of pH change and substrate utilization. On incubation, bacteria undergo metabolic changes which are indicated by a color change in the media that is either interpreted visually or after the addition of a reagent. The carbohydrate utilization ability of the selected isolate was tested for cellobiose, starch, inulin, inositol, glycerol, salicin, rhamnose, melezitose, xylitol, raffinose, trehalose, sorbitol, D-arabinose, melibiose, mannitol, sucrose, malonate, L-arabinose, sorbose, mannose, ribose, α-methyl-D-glucoside,
dulcitol, sodium-glucionate, maltose, fructose, lactose, galactose, glucosamine, dextrose and xylose.

3.3.6.2 Molecular identification by 16S rDNA sequencing

Molecular identification of the selected bacterial isolate involved DNA extraction, PCR amplification of 16S rDNA from the genomic DNA, sequencing of the PCR amplified product (from GCC Biotech limited, Kolkata) and homology search of the 16S rRNA gene sequence in NCBI database.

3.4 Pectinase production in SmF by *B. subtilis* SAV-21

Pectinase was produced in SmF from *B. subtilis* SAV-21, which was selected on the basis of qualitative and quantitative screening of the isolates. The SmF conditions were optimized using the conventional one factor at-a-time approach as well as statistical approach to obtain maximum yield of the enzyme. The procedures employed for this experiment are as follows:

3.4.1 Preparation of inoculum

First, a loop full of *B. subtilis* SAV-21 culture (from the plate culture) was inoculated in 50 ml of nutrient broth taken in 250 ml conical flask (pre autoclaved at 1.05 kg/cm$^2$ for 20 min) and grown overnight at 37°C in an orbital shaker at 200 rpm. One ml of this overnight grown culture was transferred to another 50 ml of autoclaved nutrient broth in conical flask and incubated overnight at 37°C in an orbital shaker at 200 rpm to allow the growth of bacterial cells.

3.4.2 Pectinase production in SmF using basal medium

Pectinase production in SmF was initially carried out in conical flasks (250 ml capacity) containing 50 ml of the basal medium comprising of peptone 5.0 g/L; yeast extract 5.0 g/L; potassium nitrate (KNO$_3$) 5.0 g/L; potassium dihydrogen sulphate (KH$_2$PO$_4$) 1.0 g/L; magnesium sulphate (MgSO$_4$) 0.1 g/L, mosambi peel 1.0 g/L. The flasks were autoclaved at 1.05 kg/cm$^2$ for 20 min and cooled. They were then inoculated with 1% (w/v) of overnight grown culture and incubated at 37°C for 48 h under shaking at 200 rpm in an orbital shaker. The contents of each flask were centrifuged at 10,000 x g for 20 min at 4°C, and the clear cell-free supernatant thus obtained was referred as the crude enzyme extract. It was evaluated for pectinase activity.
3.4.3 Optimization of pectinase production in SmF by one variable at-a-time approach

For enhancing the pectinase yield from *B. subtilis* SAV-21, the enzyme production in SmF was optimized with respect to various parameters using one variable at-a-time approach. The optimization was done by producing the enzyme in the basal medium (as described above in section 3.4.2) and varying the different parameters one at a time keeping all other parameters constant. In each case, the enzyme produced was extracted from the culture filtrate by centrifugation at 10,000 x g for 15 min at 4 °C. To maximize the pectinase production by SAV-21, the following parameters were optimized using one variable approach.

3.4.3.1 Inoculum age and inoculum size

To study the effect of inoculum age on pectinase production, 1.0 ml inoculum of different age (2, 4, 6, 8, 10 and 12 h) was added separately to 50 ml of production medium flask and the flasks were incubated at 37°C for 48 h under shaking at 200 rpm in an orbital shaker. The culture filtrates of the flasks were centrifuged at 10,000 x g for 15 min at 4 °C and used the clear supernatant for pectinase assay.

To study the effect of inoculum size, 50 ml of production medium was inoculated at a level of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 % (v/v) from 18 h old bacterial culture broth. After incubation at 37°C for 48 h, the culture filtrate was centrifuged at 10,000 x g for 15 min and pectinase activity was determined in the clear supernatant.

3.4.3.2 Optimization of medium pH and temperature

The effect of pH on pectinase production was investigated by varying the pH of the production medium in the range of 3.0 – 13.0. The production media (50 ml each) of different pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 and 13.0) were inoculated with 1 ml of 18 h old inoculum and incubated at 37°C for 48 h in an orbital shaker incubator at 200 rpm. The activity of pectinase was then assayed in the supernatant obtained after centrifugation of each culture filtrate.

Pectinase production was studied at temperatures ranging from 30-50°C. Each flask containing 50 ml of production medium was inoculated with 1.0 ml of 18 h old inoculum and incubated at different temperatures (30, 35, 40, 45 and 50°C) for 48 h in an orbital shaker incubator at 200 rpm. The pectinase activity was then determined in each case as described earlier.
3.4.3.3 Effect of Agitation rate

Each flask containing 50 ml of production medium was inoculated with 1.0 ml of 18 h old inoculum and incubated at 37°C for 48 h under shaking at different agitation rates (50, 100, 150, 200 and 250 rpm) in an orbital shaker incubator. One flask was also kept under stationary conditions. Then the enzyme was extracted from the culture filtrates and the pectinase activity was determined.

3.4.3.4 Optimization of incubation period

Each flask containing 50 ml of production medium was inoculated with 1.0 ml of 18 h old inoculum and incubated at 37°C for different incubation time (6 h to 108 h). The culture filtrates were centrifuged and the pectinase activity was determined.

3.4.3.5 Effect of additives

The effect of additives on pectinase production was investigated using detergents (SDS and Triton-X-100), non-polar (Tween 20) and polar (glycerol) solvents, metal salts (FeCl₃, MnCl₂, KCl and ZnCl₂) and EDTA. Each additive was added to the enzyme production medium at a concentration of 0.2 %, v/v (prior to autoclave). The production medium was then inoculated with 1.0 ml of 18 h old inoculums followed by incubation at 37°C for 48 h in an orbital shaker incubator at 200 rpm. A control devoid of any additive was also kept.

3.4.3.6 Optimization of carbon source

The enzyme production was carried out using various commercial carbon sources (pectin, fructose, galactose, glucose, sucrose, maltose, starch, cellulose, mannitol and lactose) and agro- wastes (mosambi peel, pomegranate peel, saw dust, wheat bran, wheat husk, rice bran, rice straw, sugarcane bagasse, bengal gram peel and urd dal peel) each at a concentration of 0.1 %, w/v. A control devoid of carbon source was also processed simultaneously.

3.4.3.7 Optimization of nitrogen source(s)

The enzyme production was monitored using various inorganic nitrogen sources [KNO₃, NaNO₃, NH₄NO₃ and NH₄Cl, (NH₄)₂SO₄, (NH₄)₂HPO₄] and organic nitrogen sources [peptone, yeast extract (Y.E), beef extract (B.E) and tryptone] each at a concentration of 0.5% (w/v or v/v) individually and also in combinations (peptone+Y.E, peptone+B.E, peptone+KNO₃, peptone+B.E, B.E+Y.E, B.E+KNO₃,
B.E+tryptone, Y.E+KNO$_3$, Y.E+tryptone and tryptone+ KNO$_3$) of 0.5 % each. A control devoid of nitrogen source was also processed simultaneously.

### 3.4.3.8 Optimization of the nitrogen and carbon sources by RSM

Concentrations of the best nitrogen source i.e. combination of tryptone and peptone and the best carbon source i.e. wheat husk (identified by one variable approach at-a-time approach) were optimized via Response Surface Methodology (RSM). Using the statistical software package Design Expert 7.1.2, Stat-Ease, Inc, a $2^3$ small factorial Central Composite Design (CCD) with the above three factors and five replicates at the central point leading to a set of 15 experiments was used to optimize the nitrogen and the carbon sources for pectinase production from *B. subtilis* SAV-21. The range and levels of variables (-$\alpha$, -1, 0, 1, +$\alpha$) are given in Table 3.2. All the variables were taken at a central coded value considered as zero. The 15 experiments of CCD included four trials for factorial design, six trials for axial point and five trials for replication of central point. The response value from each experiment of CCD was the average of triplicates.

**Table 3.2** Experimental range of each selected variable using CCD in terms of actual factors for pectinase production from *B. subtilis* SAV-21

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Symbols</th>
<th>Coded levels of variables</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-$\alpha$</td>
</tr>
<tr>
<td>P Peptone (%)</td>
<td>A</td>
<td>0.1</td>
</tr>
<tr>
<td>T Tryptone (%)</td>
<td>B</td>
<td>0.1</td>
</tr>
<tr>
<td>Wheat husk (%)</td>
<td>C</td>
<td>0.01</td>
</tr>
</tbody>
</table>

### 3.5 Pectinase production in SSF

#### 3.5.1 Processing of agro-industrial residues for use as substrates

Various agro-residues (wheat bran, rice bran, paddy straw, corn cob, sugarcane bagasse, mustard oil cake, saw dust, mustard straw, cotton straw, groundnut peel, wheat straw and cottonseed oil cake) and fruit peel wastes (lemon peel, mosambi peel, pineapple peel, papaya peel, banana peel, mango peel, coconut fiber, pomengranate peel, orange peel, cheeku peel and kinnow peel) were procured from local market of Yamuna Nagar, Haryana, India. These agro-residues were washed
several times with tap water to remove all water-soluble compounds and sun dried until they were completely dehydrated. Dried agro-residues were grounded to about 2 to 3 mm particle size in a grinder and used for the production of pectinase and pectin lyase (PL).

3.5.2 SSF for the production of pectinase and pectin lyase

SSF was performed in Erlenmeyer flasks (250 ml) each carrying 5.0 g of one of the above mentioned substrates (alone or in combination) moistened with 70% distilled water before sterilization. The basal production medium in flasks was autoclaved, cooled and inoculated aseptically with 1.0 ml (20% v/w) of 18 h old inoculum of *B. subtilis* SAV-21 (CFU = 1 × 10⁹ ml⁻¹). Flasks were then incubated at 37°C for 48 h in a bacteriological incubator. Pectinase and pectin lyase (PL) were extracted from the fermented substrate twice with 50 ml of distilled water (pH 7.0) by constant shaking in an orbital shaker at 200 rpm for 10 min. The extract was squeezed through two layers of muslin cloth and centrifuged at 10,000 x g for 15 min at 4°C. The clear supernatant was used as a source of crude pectinase and PL.

3.5.3 Assay of PL activity

The assay of PL activity was based on measuring the absorption of colored derivatives obtained by the reaction of unsaturated uronic acid ester and thiobarbituric acid at 550 nm (Nedjma et al., 2001). A 250 µl aliquot of suitably diluted enzyme solution was incubated with 250 µl of 0.5 % (w/v) pectin (dissolved in 0.05M Tris-HCl buffer, pH 9.0) at 60 °C for 10 min and then 50 µl of 1.0 N NaOH was added. The mixture was shaken and heated at 80 °C in a water bath for 5 min. After cooling, 600 µl of 1.0 N HCl was added to the mixture and shaken. This resulted in the disappearance of the yellow color due to acidification of the medium. Then 500 µl of 0.04 M thiobarbituric acid solution was added and incubated at 80 °C for 5 min in a water bath. The solution was cooled and its absorbance was measured at 550 nm. A control was prepared by adding 1% NaCl solution instead of the enzyme in the reaction mixture. The enzyme activity was expressed in terms of μmoles of unsaturated galacturonide released per min, based on the molar extinction coefficient value of 5500 M⁻¹ cm⁻¹ of the unsaturated product.
3.5.4 Optimization of parameters affecting the enzyme production in SSF

Pectinase and PL were produced in SSF using basal medium containing a combination of orange peel and coconut fiber in 4:1 ratio (the best substrate selected after screening) as described above and various parameters affecting the enzyme production were optimized by one varying one factor at a time keeping all other factors constant. All the experiments were run in triplicates. The parameters optimized for enhancing enzyme yield are described below:

3.5.4.1 Effect of inoculum age and size

Flasks containing 5.0 g of orange peel and coconut fiber were inoculated with inoculum of different ages (2, 4, 6, 8, 10 and 12 h) and sizes (10, 20, 30, 40 and 50 %, v/w). The flasks were then incubated at 37°C for 48 h followed by extraction and assay of pectinase and PL.

3.5.4.2 Effect of temperature and pH

The effects of cultivation temperature and pH on enzyme production were studied by incubating the flasks containing 5.0 g of orange peel and coconut fiber (4:1) at different temperatures (30-50°C) and over a pH range of 3.0 to 10.0 for 48 h.

3.5.4.3 Effect of nitrogen source

The best nitrogen source for the production of pectinase and PL by *B. subtilis* SAV-21 was determined by supplementing different organic (yeast extract, beef extract, peptone, tryptone, glycine, urea, lysine, ornithine and arginine) and inorganic nitrogen sources (ammonium chloride, ammonium nitrate and ammonium sulphate) separately in the solid media to a final concentration of 1% (w/v) after dissolving them in distilled water used for adjusting the moisture content. In control, no exogenous nitrogen source was added.

3.5.4.4 Effect of carbon source

The effect of different carbon sources (glucose, mannitol, pectin, galactose, sucrose, lactose, maltose, sodium acetate and xylose) were evaluated by supplementing these in the solid media separately to a final concentration of 1% (w/v) after dissolving them in distilled water used for adjusting the moisture content. In control, no exogenous carbon source was added.
3.5.4.5 Incubation period

To determine the optimum fermentation time, enzyme production was monitored over a time period of 2-8 days for pectinase and 2-10 days for PL.

3.5.4.6 Effect of different metal salts and moisture content

The effect of different metal salts (CaCl$_2$.2H$_2$O, MgSO$_4$.7H$_2$O, CoCl$_2$.2H$_2$O, H$_3$BO$_3$, ZnCl$_2$, KCl and NaCl) at a final concentration of 1 mM was studied by adding these to the solid media after dissolving in the distilled water used to adjust the moisture level of the substrate followed by monitoring the production of enzymes.

The effect of moisture level on pectinase and PL production was investigated by adding different volumes of distilled water (moistening agent) to the solid substrate to give a final moisture content of 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%.

3.6 Temperature and pH profiles of crude pectinase secreted by *B. subtilis* SAV-21

The optimum temperature of extracellular pectinase produced by *B. subtilis* SAV-21 was determined by performing the enzyme assay at different temperatures ranging from 30ºC to 70ºC. The observed enzyme activity at various temperatures was calculated as relative activity (%) by considering the maximum activity as 100%. Thermal stability of the crude pectinase was determined by pre-incubating it at different temperatures (30-70ºC) for 30 min and 120 min. After the desired incubation at each temperature and time, the enzyme activity was measured at the optimum temperature under the defined assay conditions.

The optimal pH of extracellular pectinase produced by *B. subtilis* SAV-21 was determined by performing the enzyme assay at different pH values using acetate (pH 4.0-6.0), sodium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.0-9.0) and glycine-NaOH (pH 9.0-12.0) buffers of 0.05M concentration. The observed enzyme activity at various pH values was calculated as relative activity (%) by considering the maximum activity as 100%. The pH stability of pectinase was examined by pre-incubating 0.50 ml of enzyme and 0.50 ml of the above mentioned buffers (pH range 3-12) for 0.5 h, 1 h and 2 h at room temperature followed by measurement of residual pectinase activity under the optimal assay conditions. The residual activity (%) was calculated with reference to the zero time activity taken as 100%.
3.7 Purification of extracellular pectinase produced by *B. subtilis* SAV-21

Pectinase was produced from *B. subtilis* SAV-21 was grown in modified Horikoshi medium under optimal SmF conditions in an orbital shaker incubator. After 24 h of incubation, the culture filtrate was collected and centrifuged at 10,000 x g for 15 min at 4°C. The clear supernatant (crude enzyme extract) obtained after centrifugation of the culture filtrate was used for purification of extracellular pectinase in two steps by ammonium sulphate precipitation and gel filtration chromatography through G-100. The enzyme purification was carried out at 4 ºC.

3.7.1 Ammonium sulphate fractionation

The crude enzyme extract from *B. subtilis* SAV-21 was subjected to (NH$_4$)$_2$SO$_4$ fractionation by gradually adding solid (NH$_4$)$_2$SO$_4$ with constant stirring to bring it to 80 % saturation and kept it for overnight in a refrigerator to allow the protein to precipitate. The suspension was centrifuged at 10,000 x g for 20 min to collect the supernatant and the pellet. The pellet was dissolved in a minimum volume of 0.05M Tris-HCl buffer (pH 8.0) and dialyzed against Tris-HCl buffer (pH 8.0) overnight with change of buffer twice. The resulting dialyzate was analyzed for pectinase activity and protein content. Specific activity, % recovery and fold purification were then calculated. The above mentioned dialyzate was loaded on gel filtration column chromatography for further purification.

3.7.2 Sephadex G-100 column chromatography

Sephadex G-100 (15.0 g) was slowly added to 0.05M Tris-HCl buffer (pH 8.0) and was allowed to swell at room temperature for 72 h. The supernatant was decanted until a rapidly sedimenting gel slurry was obtained, which was gently poured down into a glass column with the help of a glass rod and the gel was allowed to settle under gravity. Equilibration buffer (0.05M Tris-HCl buffer, pH 8.0) was then passed through the column at a rate of 15ml/h for 24 h. The gel finally settled to give a gel bed height of 82.6 cm. Therefore, the size of gel bed in the column was 82.6 cm x 1.6 cm. The void volume of the above column was determined by applying 1.0 ml of 0.2 % (w/v) blue dextran-2000$^{®}$ (Mr ~2x10$^6$ Da) at the top of the gel in the column followed by its elution at the rate of 15 ml/h. The blue dextran-2000$^{®}$ traveled as a uniform band and yielded a symmetrical elution profile. The void volume of the gel filtration column was 37.5 ml. After collecting the void volume upto 13 fractions, the
fractions of 3 ml volume were collected and analyzed for protein (absorbance at 280 nm) and pectinase activity. Active fractions were pooled and the pooled fraction was concentrated using Millipore Amicon Ultra-15 centrifugal filter unit (10 kDa cut off) and its purity was checked by Native-PAGE and SDS-PAGE using a 12% polyacrylamide gel.

3.7.3 Native polyacrylamide gel electrophoresis (Native-PAGE)

Native-PAGE was performed using a 12% acrylamide gel according to anionic system of Davis (1964).

Reagents

Acrylamide/bis-acrylamide solution (30:0.8): Dissolved 30.0 g acrylamide and 0.8 g methylene-bis-acrylamide in distilled water and made up the final volume to 100 ml. Filtered the solution through Whatman No.1 filter paper and stored in a brown bottle at 4 °C.

Resolving gel buffer (1.5 M Tris-HCl, pH 8.8): Dissolved 18.15 g of Tris base in 50 ml distilled water and the pH of the solution were adjusted to pH 8.8 with 1 N HCl and the final volume was made 100 ml with distilled water. The solution was filtered through Whatman No.1 filter paper and stored at 4 °C.

Stacking gel buffer (0.5M Tris buffer, pH 6.8): Dissolved 6.05 g of Tris base in 50 ml distilled water and adjusted the pH to 6.8 with 1N HCl and made up its volume to 100 ml with distilled water. The solution was filtered through Whatman No.1 filter paper and stored at 4 °C.

Ammonium persulphate solution (1.5%, w/v): Dissolved 15.0 mg of ammonium persulphate in 1.0 ml distilled water and mixed it gently. This solution obtain was unstable and prepared fresh before use.

Reservoir buffer: Dissolved 3.0 g Tris base and 14.4 g glycine in 1 L distilled water, adjusted its pH to 8.3 and stored at 4 °C. Its working concentration was 25 mM Tris and 192 mM glycine.

Staining solution: Dissolved 0.25 g Coomassie brilliant blue R-250 dye in 250 ml of a mixture of water, methanol and glacial acetic acid (105: 105: 40). The solution was filtered through Whatman No.1 filter paper and stored at room temperature.
**Destaining solution:** Mixed 30 ml methanol with 10 ml glacial acetic acid and made up its volume to 100 ml with distilled water.

**Sample buffer (2X):** Tris-HCl buffer (1.0 M, pH 6.8) containing 5 % glycerol and 0.02 % bromophenol blue.

**Gel polymerization**

Properly cleaned and dried glass plates (8 cm x 7 cm) were assembled on gel caster of vertical slab gel assembly using 1 mm spacers. The resolving gel mixture was prepared just before use as described in Table 3.3 and poured down into the space between the gel plates. Few drops of distilled water were layered over the resolving gel with the help of a syringe to ensure the production of a flat gel surface and to exclude oxygen. The gel was allowed to polymerize completely. After polymerization of the resolving gel, the water layer was removed and soaked off with a filter paper. The stacking gel solution was then prepared according to its composition given in Table 3.3 and poured over the polymerized separating gel. A comb was placed immediately at the top of the stacking gel solution so as to make wells for sample loading. The stacking gel was allowed to polymerize. After the stacking gel was polymerized, the comb was removed and the wells were cleaned thoroughly with reservoir buffer using a syringe so that no unpolymerized acrylamide was left in the wells. The gel plates were transferred to the gel electrophoresis unit and the reservoir buffer was poured in the lower and upper chambers of the apparatus in such a manner that no air bubble was trapped between the gel and the buffer.

**Table 3.3 Composition of resolving and stacking gels for Native-PAGE**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Resolving gel (12%)</th>
<th>Stacking gel (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bis-acrylamide</td>
<td>12.0 ml</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>Resolving gel buffer (1.5 M Tris-HCl, pH 8.8)</td>
<td>7.50 ml</td>
<td>--</td>
</tr>
<tr>
<td>Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)</td>
<td>3.00 ml</td>
<td>--</td>
</tr>
<tr>
<td>APS (10 %)</td>
<td>1.90 ml</td>
<td>0.12 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.015 ml</td>
<td>0.01 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>8.585 ml</td>
<td>6.87 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>30 ml</td>
<td>12 ml</td>
</tr>
</tbody>
</table>
Sample preparation

An aliquot of the protein sample to be analyzed by polyacrylamide gel electrophoresis was mixed with equal volume of the sample buffer for loading.

Electrophoresis, staining and destaining of gels

Sample was loaded into the wells of the polyacrylamide gel using a Hamilton syringe. The electrodes were connected to an electrophoretic power supply unit and the electrophoresis was carried out initially at 10 mA to allow the sample to enter the gel and then the current was increased to 20 mA till the tracking dye reached close to the bottom of the resolving gel. After electrophoresis, the polyacrylamide gel was taken out from the electrophoresis unit and stained with Coomassie brilliant blue R-250 staining solution for 8 h while shaking to visualize the protein bands. The staining solution was then removed and the gel was transferred to destaining solution and kept on a gel rocker with gentle shaking. The destaining solution was changed several times till the gel background was clear. The gel was then photographed and preserved in distilled water containing 10% glycerol in a dark and cool place.

3.7.4 SDS-PAGE

SDS-PAGE was carried out by the method of Laemmli (1970) using 12% gel.

Reagents

Acrylamide/bis-acrylamide solution, resolving gel and stacking gel buffers, APS, staining and destaining solutions, and bromophenol blue were same as for Native-PAGE. The remaining solutions required for SDS-PAGE were as follows:

**SDS (10%, w/v):** It was prepared by dissolving 1.0 g SDS in 10 ml of distilled water.

**Reservoir buffer:** Dissolved 3.0 g Tris base, 14.4 g glycine and 1.0 g SDS in about 600 ml of distilled water and adjusted its pH to pH 8.3. Its final volume was made 1.0 l with distilled water and stored at 4 °C till further use. Its working concentration was 25 mM Tris, 192 mM glycine, and 0.1% SDS.

**Sample buffer (5X):** Mixed 1.25 ml of Tris-HCl buffer (2.0 M, pH 6.8), 3.0 ml glycerol (30%), 1.0 ml of SDS (10 %), 0.1 ml β- mercaptoethanol and 0.5 ml of 0.02% bromophenol blue and the final volume was made to 10.0 ml with distilled water.
Gel polymerization

SDS-PAGE was performed using 12% resolving and 5% stacking gel, the compositions of which are given in Table 3.4. The procedure for gel polymerization was similar to Native-PAGE except that the resolving and stacking gels were prepared as described in the above.

Table 3.4 Composition of resolving and stacking gels for SDS-PAGE

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Resolving gel (12%)</th>
<th>Stacking gel (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/bis-acrylamide solution</td>
<td>12.0 ml</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>Resolving gel buffer (1.5M, pH8.8)</td>
<td>3.75 ml</td>
<td>--</td>
</tr>
<tr>
<td>Stacking gel buffer (0.5M, pH6.8)</td>
<td>--</td>
<td>3.00 ml</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>0.40 ml</td>
<td>0.12 ml</td>
</tr>
<tr>
<td>APS (1.5%)</td>
<td>1.50 ml</td>
<td>0.12 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.015 ml</td>
<td>0.01 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>12.335 ml</td>
<td>6.75 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>30 ml</td>
<td>12 ml</td>
</tr>
</tbody>
</table>

Sample preparation

Samples were prepared by mixing protein sample and sample buffer (5X) in the ratio of 1:1 and boiled for 5 min in a water bath followed by cooling.

Molecular weight markers

A pre-stained SDS-PAGE protein ladder (Himedia, MBT092-10LN) was run simultaneously with the protein sample.

Electrophoresis, staining and destaining of gels

The procedure for electrophoresis and processing of the gel for visualization of protein bands was same as described for native-PAGE.

3.7.5 Activity staining (Zymogram analysis) for pectinase

Pectinase zymography was performed according to Ghazala et al. (2015) with slight modifications. For zymogram study of pectinase, the sample was not heated before electrophoresis. SDS gel containing 1% pectin was prepared and loaded with purified pectinase sample. After electrophoresis, the gel was submerged in containing
2.5 % Triton X-100 for 30 min at 60°C with constant agitation to remove SDS. Triton X-100 was then removed by washing the gel three times with 50 mM glycine - NaOH buffer (pH 11.0). After incubation, the gel was stained with 0.03% ruthenium red dye for 15 min. The pectinase activity appeared as clear hydrolysis area against red gel background.

3.7.6 High performance liquid chromatography (HPLC)

HPLC was also performed to test the enzyme purity. The HPLC system (Shimadzu Corporation) was equipped with dual lamp binary system, UV detector and Shimadzu LC6AD-C18 reverse phase column (4.6 mm x 150 mm, 5µm). The solvents were filtered through 0.22µm filter (Millipore) and degassed before use. The column was washed with acetonitrile and equilibrated with acetonitrile-water (80:20). The purified protein sample (10.0 µl) obtained from gel filtration chromatography was loaded was injected into the loop of column with the help of HPLC loading syringe and eluted with acetonitrile-water at a flow rate of 0.5 ml/min. The detector was set to read the absorbance at 280 nm and HPLC chromatogram was obtained by printing the data.

3.8 Characteristics of purified pectinase from B. subtilis SAV-21

Purified pectinase was characterized with respect to its molecular weight, pH optimum, pH stability, temperature optimum, thermostability, effect of metal ions, substrate specificity, effect of additives, storage stability, and kinetic parameters (Km and Vmax).

3.8.1 Determination of molecular weight

Relative molecular mass of purified pectinase was determined by gel filtration using Sephadex G-100 column chromatography and by SDS-PAGE.

3.8.1.1 Molecular weight determination by gel filtration chromatography

Molecular weight of pectinase was determined by gel filtration chromatography. For this, Sephadex G-100 column was equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. The eluent exhibited no absorbance at 280 nm. A mixture of gel filtration protein molecular weight markers (Sigma) containing albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa) was loaded on this column and these were eluted with 0.05 M Tris-HCl buffer, pH 8.0 at a flow rate of
15 ml/h. Fractions of 3.0 ml each were collected and their absorbance was recorded at 280 nm. A graph was drawn between A280 versus fraction number and elution volume (Ve) of each protein was determined from this plot. A graph was then plotted between Ve/Vo on x-axis and log MW of the marker proteins on y-axis. Similarly, the purified enzyme was passed through the column under identical conditions and its Ve was determined. Then a graph was was plotted between Ve/Vo and log MW for determination of the molecular weight of purified pectinase.

3.8.1.2 Molecular weight determination by SDS-PAGE

SDS-PAGE was performed as described under the section 3.7.3. Purified pectinase was co-electrophoresed with pre-stained ladder of standard molecular weight markers. After staining and destaining of the polyacrylamide gel, the distance travelled by the tracking dye as well as protein bands were measured. Relative mobility of each protein band in both the lanes was calculated in the following manner:

\[
\text{Relative mobility (Rm)} = \frac{\text{Distance moved by the protein band}}{\text{Distance moved by the tracking dye}}
\]

A logarithmic plot between molecular weight of the marker proteins versus the corresponding relative mobility was plotted. The relative mobility of purified pectinase band was extrapolated to the above standard graph and the relative molecular mass of pectinase was calculated.

3.8.2 Effect of temperature on purified pectinase

The optimum temperature of purified pectinase was determined by carrying out the enzyme assay at different temperatures ranging from 30-80 °C. The enzyme activity at each temperature was calculated as percent of the maximum activity. A temperature profile was drawn between temperature on x-axis and relative pectinase activity (%) on y-axis. The temperature showing maximum pectinase activity was taken as the optimum temperature.

Thermal stability of purified pectinase was examined by pre-incubating the enzyme at different temperatures ranging from 30–80 ºC for 4 h in the absence of the substrate. Samples were taken at 60 min, 120 min and 240 min interval for
measurement of pectinase activity under optimal assay conditions. The residual enzyme activity at each temperature was calculated as percent of the control in which the enzyme was not pre-incubated. A profile for enzyme thermostability was drawn between temperature on x-axis and residual pectinase activity (%) on y-axis.

### 3.8.3 Effect of pH on purified pectinase

The pH optimum of purified pectinase was determined by carrying out the enzyme assay at different pH values using acetate buffer (pH 4.0 - 6.0), potassium phosphate (pH 6.0 - 8.0), Tris-HCl (pH 8.0 - 9.0) and glycine-NaOH (pH 9.0 - 12.0) buffers.

Effect of pH on the stability of purified pectinase was examined by pre-incubating 0.50 ml of enzyme and 0.50 ml of the above mentioned buffers in the pH range 4.0 - 12.0 in the absence of substrate for 4 h at room temperature followed by measurement of pectinase activity at 60 min, 120 min and 240 min interval under optimal assay conditions. A control was run simultaneously in which the enzyme was incubated with distilled water instead of a buffer. The residual enzyme activity at each pH value was calculated as percent of the control. A profile for pH stability of the enzyme was drawn between pH on x-axis and residual pectinase activity (%) on y-axis.

### 3.8.4 Effect of metal ions

The effect of various metal ions (NaCl, KCl, MgCl2, CaCl2, CoCl2, MnCl2, HgCl2, FeCl3, ZnSO4, and CuSO4) on purified pectinase from culture filtrates of *B. subtilis* SAV-21 was studied at a final concentration of 1 mM in the reaction mixture. The relative activity (%) was calculated with reference to the control in which enzyme was not incubated with any metal ion.

### 3.8.5 Effect of different substrates

The effect of substrate such as pectin, polygalacturonic acid, xylan and carboxymethyl cellulose was investigated on purified pectinase activity. The relative activity (%) was calculated with reference to the pectin as substrate.

### 3.8.6 Effect of additives

The effect of various additives (SDS, Tween 20, Tween 80 and Triton X 100) at 1% (w/v) was investigated on purified pectinase activity. The relative activity (%)
was calculated with reference to the control in which enzyme was not incubated with any additive.

3.8.7 Determination of Km and Vmax

The activity of purified *B. subtilis SAV-21* extracellular pectinase was measured at different concentrations (0.1 to 5 mg/ml) of the substrate pectin. A Lineweaver-Burk plot was then drawn using the reciprocal of the substrate concentrations on x-axis and reciprocal of the corresponding pectinase activity values on y-axis to calculate Km and Vmax of pectinase.

3.8.8 Storage stability of purified pectinase

Purified pectinase was stored in a refrigerator at 4°C and samples were drawn at various time intervals for measurement of pectinase activity using standard enzyme assay. The enzyme activity at different storage periods was calculated as percent of the initial enzyme activity.

3.9 Immobilization of pectinase

Purified pectinase was immobilized on clay-chitosan composite beads, which were prepared in the laboratory. The factors for the immobilization of pectinase were optimized by one variable at-a-time approach.

3.9.1 Preparation and activation of clay-chitosan beads

The clay-chitosan beads were prepared according to method of Jiang et al. (2005) by dissolving 1.0 g chitosan and 1.0 clay powdered in 100 ml of 1% acetic acid (v/v) and kept overnight. The clay-chitosan solution was added drop-wise into gently stirred coagulating mixture containing 15% NaOH and 95% ethanol in 4:1 ratio through nozzle (1-2 mm diameter) with constant shaking of the solution. The formation of beads occurred when the drop of clay-chitosan mixture entered into a NaOH solution because the chitosan became insoluble under alkaline conditions. The beads were incubated in coagulating mixture at room temperature and kept overnight. The swollen clay-chitosan beads were washed with deionized water until the solution became neutral. The beads were activated by treatment with 1% aqueous glutaraldehyde solution for 1h at room temperature. The activated beads were washed with deionized water to remove the excess cross-linking agent.
3.9.2 Immobilization of purified pectinase on activated clay-chitosan beads

The glutaraldehyde-activated clay-chitosan beads were used for immobilization of purified pectinase. The glutaraldehyde-activated clay-chitosan beads were incubated with purified pectinase (33.88 IU) at room temperature for 30 min followed by washing to remove the unbound enzyme. The activity of pectinase was determined in the supernatant as well as in the enzyme bound beads. Immobilization yield was calculated by the following manner:

\[
\text{Immobilization Yield (\% acid)} = \frac{\text{Total activity recovered on pellets}}{\text{Total activity recovered for immobilization}} \times 100
\]

3.9.3 Assay of immobilized pectinase

Pectinase activity of the immobilized pectinase was measured by adding 450 µl of 0.5% pectin to the immobilized enzyme followed by incubation 60°C for 10 min. The beads were separated from the reaction mixture by transferring the latter to another test tube. Then 1.5 ml of 3, 5-dinitrosalicylic acid reagent was added to the reaction mixture and the test tubes were placed in a boiling water bath for 10 min. After cooling, absorbance of the resulting color was read at 540 nm in a spectrophotometer. A control was run simultaneously that contained all of the reagents but 3, 5-dinitrosalicylic acid reagent was added prior to the addition of enzyme. The amount of galacturonic acid produced was measured from its standard curve.

3.9.4 Effect of glutaraldehyde concentration on the immobilization of pectinase

The influence of crosslinker concentration on the immobilization of pectinase was determined by using different concentrations of glutaraldehyde (1.0–5.0%) in the immobilization procedure keeping the other parameters such as reaction time (1.0 h) and temperature (30°C) constant.

3.9.5 Effect of crosslinking time on the immobilization of pectinase

The effect of crosslinking time between clay-chitosan composite beads and glutaraldehyde on the immobilization of enzyme was analyzed by incubating the clay-chitosan composite beads in 1% glutaraldehyde solution for different time intervals ranging from 0.5 to 3.0 h at 30°C.
3.9.6  Effect of enzyme dose on immobilization

The effect of enzyme dose on immobilization was studied by incubating different doses of pectinase (22.59, 33.88, 45.18, 56.47, 67.70 and 79.00 IU) with glutaraldehyde-activated clay-chitosan composite beads keeping the other parameters such as reaction time (1.0 h) and temperature (30°C) constant.

3.9.7  Effect of pH on immobilized pectinase

The pH optima of soluble and immobilized pectinase were determined by measuring their activities according to their respective standard assay procedures at different pH values (4.0-12.0) using acetate (pH 4.0-6.0), phosphate (pH 6.0-8.0), Tris-HCl (pH 8.0-9.0) and glycine-NaOH (pH 10.0-11.0) buffers, each at a concentration of 0.05M. Relative activity (%) was calculated with reference to the maximum activity which was taken as 100%.

The pH stability of soluble and immobilized pectinase was investigated by pre-incubating these enzymes separately with the above mentioned buffers for 2 h at room temperature followed by measurement of residual activity under standard assay conditions at the pH optimum. Residual activity at different pH was calculated by with reference to the enzyme sample pre-incubated with distilled water, instead of buffers, taking it as 100%.

3.9.8  Effect of temperature on immobilized pectinase

Temperature optima of free and immobilized pectinase were determined by measuring their activities according to their respective standard assay procedures at different temperatures ranging from 30°C to 70°C. Relative enzyme activity at different temperatures was calculated by considering the highest activity as 100%. The temperature at which the enzyme showed maximum activity was taken as the optimum. Thermal stabilities of free and immobilized pectinase were studied by separately pre-incubating these enzymes at different temperatures ranging from 30°C to 70°C for 2h followed by determination of enzyme activity using standard assays at the optimum temperature. The residual activity was calculated by taking the enzyme activity at zero time pre-incubation as 100%.
3.9.9 Reusability of clay-chitosan composite beads immobilized pectinase

The recycling efficiency of chitosan beads immobilized pectinase was determined by repeatedly reusing the beads in batch of 10 reactions. After each batch, the beads were washed with deionized water and buffer, and reintroduced in fresh substrate solution for next batch.

3.10 Applications of B. subtilis SAV-21 pectinase

Pectinase is an enzyme having a wide range of industrial applications. In this study, the potential of B. subtilis SAV-21, an alkali-tolerant pectinase, was investigated in fruit juice enrichment and bioscouring of cotton. The methods employed to carry out these experiments are given below.

3.10.1 Applications of B. subtilis SAV-21 pectinase in fruit juice enrichment

Purified pectinase from the culture filtrates of B. subtilis SAV-21 was used for enrichment of juice from apple and orange fruit pulps. The methods employed to carry out this experiment are as follows:

3.10.1.1 Optimization of pectinase treatment conditions for fruit juice enrichment

Pulps prepared from apple and orange were treated with purified B. subtilis SAV-21 pectinase (free form) under different reaction conditions for enhancing the yield and quality of juices. Untreated controls were also run simultaneously. The fruit juice extraction process was optimized with respect to enzyme dose and incubation time by varying these parameters one at-a-time keeping all other factors constant. The response was analyzed in terms of juice yield, reducing sugar content and clarity, which are expressed as percent relative to control.

To optimize the enzyme dose, 5 g of each fruit pulp was treated with 5, 10, 15 and 20 IU of pectinase/g fruit pulp (gfp) at 30°C for 30 min in a rotary shaker at 50 rpm for slight mixing of the contents. The untreated pulp sample was taken as control. At the end of desired incubation period, the enzyme was inactivated by heating the suspension in a boiling water bath for 5 min. After cooling, the fruit pulps were filtered through two layers of muslin cloth and the filtrate was centrifuged at 10,000 x g for 15 min to separate the juice. The supernatant (juice) was analyzed for yield, reducing sugars and clarity.

The optimum time of enzyme treatment was determined by incubating 5 g of
each fruit pulp with 10 IU pectinase/gfp at 30°C for a period of 30, 60, 90 and 120 min keeping zero time pulp as control in a rotary shaker at 50 rpm. This was followed by separation and analysis of the juice for yield, reducing sugars and clarity.

3.10.1.2 Treatment of fruit pulp with purified pectinase under optimized conditions

The potential of purified *B. subtilis* SAV-21 pectinase in enhancing the yield and quality of fruit juices was evaluated by treating the apple and orange fruit pulps (150 g each) under the optimized conditions of enzyme dose (15 IU/gfp for apple pulp and 10 IU/gfp for orange pulp), incubation time (60 min) at temperature 30°C followed by extraction of juice from these pulp samples as described above. The resulting juices were analyzed for yield, clarity, reducing sugars, viscosity, acidity and filterability, turbidity, total soluble solids, total dissolved solids, and conductivity.

The potential of purified *B. subtilis* SAV-21 pectinase, in both soluble (free) and immobilized forms, was also evaluated in the clarification of fruit juices. The potential of soluble pectinase was evaluated by treating the apple and orange juices (10 ml each) with enzyme (15 IU/gfp) for 120 min (incubation time) at 30°C followed by analysis of both the juices at different parameters such as clarity, reducing sugars, viscosity, acidity, filterability, total soluble sugars, total dissolved solids, turbidity and conductivity. The potential of immobilized pectinase in the clarification of fruit juices was evaluated by using the purified pectinase immobilized on clay-chitosan composite beads following the same procedure as for soluble enzyme. To immobilize the enzyme, seven clay chitosan beads were activated by dipping in 3% glutaraldehyde solution at room temperature for 90 min. The excess of glutaraldehyde was removed by washing the pellets with distilled water. The glutaraldehyde-activated beads were incubated with 56.47 IU of purified *B. subtilis* SAV-21 pectinase at room temperature for 30 min followed by washing to remove the unbound enzyme. The activity of pectinase was determined in the supernatant as well as in the enzyme bound beads. Immobilization yield was calculated in the following manner:

\[
\text{Immobilization Yield (% acid)} = \frac{\text{Total activity recovered on pellets}}{\text{Total activity recovered for immobilization}} \times 100
\]

Total activity immobilized on beads refers to the difference in enzyme activity offered
3.10.1.3 Determination of physico-chemical characteristics of fruit juices

The juices extracted from the control and pectinase-treated pulps of apple and orange fruits were analyzed for clarity, reducing sugars, viscosity, total acidity, total soluble solids, conductivity, turbidity, total dissolved solids and filterability. The clarity of juice was determined by measuring the percent transmittance (%T) at 650 nm against distilled water using a double beam UV-Visible spectrophotometer (Systronics-2203, India). The %T was considered a measure of juice clarity. Reducing sugars in the juice were estimated by Miller’s method (Miller, 1959) using 3, 5-dinitrosalicylic acid reagent and quantified from a standard curve of galacturonic acid. Filterability (per sec) was determined from the reverse of the time taken to filter 50 ml of pectinase treated juice through Whatman No. 1 filter under vacuum. Total titrable acidity (% citric acid in orange and % malic acid in apples) of the juice was determined by titrating 10 ml of diluted juice (20:80; juice: distilled water) against 0.1N NaOH using phenolphthalein as indicator. The appearance of light pink color indicates the end point. Titer value (volume of 0.1N NaOH used) was noted at the end point. Total acidity was calculated as citric acid (acid factor = 0.0064) in oranges and malic acid (acid factor = 0.0067) in apples equivalent using the following formula:

\[
\text{Total acidity (% acid)} = \frac{\text{Titer value} \times \text{acid factor} \times 100}{\text{Volume of NaOH used (ml)}}
\]

Viscosity (cP) of the juice was measured with a capillary Ostwald viscometer at constant room temperature and calculated using the following formula;

\[
\eta = \frac{\text{Time of flow of liquid}}{\text{Time of flow of water}}
\]

Total soluble sugars were measured by Erma Hand Refractrometer. Total dissolved solids were measured by TDS meter while conductivity was measured by conductivity meter. Turbidity was measured by turbidimeter.
3.10.2 Applications of *B. subtilis SAV-21* pectinase in bioscouring of cotton

### 3.10.2.1 Enzymatic bioscouring of cotton

For efficient removal of non-cellulosic impurities from cotton and to improve the absorbancy of the fabric for textile utilities, different variable conditions were optimized. Cotton fabric was procured from Khadi, Kurukshetra. The most effective reaction conditions were obtained by employing one variable at a time approach. Fabrics samples (6 x 9 cm) were treated at different pH values (8.0-11.5), pectinase doses (2.5-25.0 IU), treatment time period (15-120 min) and temperatures 40-65°C. To improve the water absorbency, different wetting agents were used such as, tween-20, tween-80, and triton-X-100. After each treatment, the fabric was washed in hot distilled water in order to deactivate the enzymes, following washing in cold distilled water and then oven dried at 45°C. For control treatments, fabric was treated under the same reaction conditions but in the presence of inactivated enzymes.

### 3.10.2.2 Alkaline scouring

Alkaline scouring was carried out according to Tzanov et al. (2001) with slight modifications using a solution containing sodium hydroxide (10g/l) and non ionic wetting agent triton X-100 (2g/l). The reaction was carried out at 90°C for 45 min. After scouring, the fabric samples were washed in hot distilled water following washing in cold distilled water and then oven dried at 45°C.

### 3.10.2.3 Analysis of fabric treated extract

#### 3.10.2.3.1 Determination of reducing sugars

To evaluate the effective removal of pectin, reducing sugars were measured after each treatment, using 3, 5- dinitrosalicylic acid (Miller, 1959).

### 3.10.2.4 Analysis of Bioscourd and Scoured fabric

#### 3.10.2.4.1 Weight loss

Weight loss of fibre/fabric samples due to specific treatment was measured after drying the sample in the oven at room temperature before and after a particular treatment. The following formula was used to calculate the % weight loss of the different fibre/fabric samples.
\[
\% \text{ Weight loss} = \frac{W_1 - W_2}{W_1} \times 100
\]

Where, \( W_1 \) and \( W_2 \) are the dry weights of the fabrics samples before and after treatment, respectively (Kalantzi et al., 2008).

**3.10.2.4.2 Drop test (Water absorbency test)**

Water absorbancy (wettability) of the fabrics was measured by water drop test, counting the elapsed time between the contact of the water drop with the fabrics and the disappearance of the drop into the fabric. Three readings were taken from different points of the fabric sample and the average was reported. Water absorbancy of the samples was tested using AATCC Test methods 39-1980 (Evaluation of wettability) (AATCC Technical Manual 1980). Wetting time of less than 1 sec was considered as an indication of adequate water absorbancy of the fabrics (Li and Hardin, 1998).

**3.10.2.4.3 Tensile strength**

Tensile strength of the raw, bioscoured and scoured fibres/ fabric samples was determined using digital tensile strength tester Paramount.

**3.10.2.4.4 Fiber surface analysis**

Microscopic characterisation of raw, bioscoured and scoured fibres was examined using inverted microscope at 40X.

**3.10.2.4.5 Ruthenium red dyeing**

The Raw, bioscoured and scoured fibres/ fabric was analyzed by staining with ruthenium red dye (Klug-Santner et al., 2006). The fabric was dyed with ruthenium red solution for 15 min at room temperature and rinsed with 1 liter of de-mineralized water. Then fabric was washed with demineralized water for 10 min at 60°C and then dried overnight. Then the dyeing pattern of fabric was examined.