3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Glassware: Borosil® and Schott Duran® glassware were used throughout the study, which were washed twice with the detergent with final rinsing in distilled water. It was then dried in hot air oven at 70°C and used.

3.1.2. Plasticware: Tarsons® and Laxbro® plasticware were also used during the study. It was also washed twice with the detergent, rinsed with distilled water, dried in hot air oven at 70°C and then used.

3.1.3. Chemicals and reagents: All the chemicals and reagents used in the study were of high purity obtained from Hi-Media, SD Fine Chemicals, E-Merck, Qualigens, and SRL, India.

3.1.4. Growth media

3.1.4.1. Solid media

- **Nutrient Agar – Raw starch medium**

  **Composition:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>10 g</td>
</tr>
<tr>
<td>Raw starch (potato, wheat, maize or rice)</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

  **Procedure**

  5g of raw starch was sterilized by mixing in absolute alcohol, which was then evaporated by keeping it overnight at 50°C in an oven. The suspension of the alcohol treated starch was made in sterile distilled water. Peptone and beef extract were separately added to distilled water and the agar agar was added after adjusting the pH to 7.0. The medium without starch was autoclaved at 121°C for 20 min and the sterile starch suspension was added to it at 50°C under sterile conditions and the medium was then poured in the petriplates.
3.1.4.2. Liquid media

- **Seed medium**
  
  **Composition:**
  
  Peptone 10 g  
  Beef extract 10 g  
  Distilled water 1000 ml  
  pH was adjusted to 7.0  

- **Production medium**
  
  **Composition:**
  
  Peptone 10 g  
  Beef extract 10 g  
  Starch (soluble) 10 g  
  Distilled water 1000 ml  
  pH was adjusted to 7.0  

3.1.5. Buffers used

- 0.1M Citrate phosphate buffer pH 4.0, 4.5, 5.0, 5.5  
- 0.1M Phosphate buffer pH 6.0, 6.5, 7.0, 7.5, 8.0  
- 0.1M Tris HCl buffer pH 7.0  

3.1.6. \(\alpha\)-Amylase assay reagents:

- **Iodine solution**
  
  0.2% Iodine crystals in 2% Potassium iodide solution.  
  
  **Procedure:** Dissolved 2 g potassium iodide in 100 ml of distilled water and to clear solution added 200 mg iodine crystals. The resulting solution was kept in brown bottle.  

- **Starch substrate**
  
  Soluble starch (E-Merck) 0.2g  
  0.1M Phosphate buffer (pH 7.0) 100 ml  
  Heated gently till a clear solution was formed with constant mixing.  

- **1N HCl**
  
  Concentrated HCl 8.5ml  
  Distilled water 100ml
3.1.7. **Standard curve of starch:**

Made a soluble starch stock solution having a concentration of 10mg/ml. A standard curve was plotted of this stock solution ranging 0.25 to 4mg. To the tubes was added 25, 50, 75, 100..........500µl of this stock and the final volume in each tube was made to 0.5ml with distilled water. Then 0.25 ml of HCl and 0.25ml of iodine was added. The contents of each tube were diluted with 4.0 ml distilled water and absorbance was read at 690 nm (see Appendix 1).

3.1.8. **DNSA (Dinitrosalicylic acid) Reagent:**

**Composition:**

- Sodium potassium tartarate 200g
- Sodium sulfite 0.5g
- Phenol (crystals) 2.0g
- Sodium hydroxide 10.0g
- Dinitrosalicylic acid (DNSA) 5.0g

**Procedure:**

Dissolved in 700 ml distilled water all the components, except DNSA, stepwise till the previous one gets completely dissolved on magnetic stirrer in a conical flask covered with black paper. Then added DNSA powder. This was mixed until completely dissolved. Final volume was made to 1000 ml. This was then filtered and stored in brown colored reagent bottle.

3.1.9. **Standard curve of glucose:**

Made a d-glucose stock solution having a concentration of 1mg/ml. A standard curve was plotted of this stock solution ranging from 100 - 1000µg. To the tubes was added 100, 200, 300........1000µl of this stock and the final volume in each tube was made to 1ml with distilled water. To all the tubes was added 1.5 ml of DNSA reagent. Kept all the tubes in boiling waterbath for 12-15 min. Diluted the contents of each tube with 4.0 ml distilled water to a final volume of 6.5ml in each tube. Absorbance was read at 570 nm (see Appendix 2).

3.1.10. **Protease assay reagents:**

- **Glycine – NaOH buffer**
  
  (0.1M, pH 9.0)
- **Substrate solution**
  0.6% Casein made in glycine- NaOH buffer (0.1M, pH 9.0)

- **Na$_2$CO$_3$ solution**
  (0.5M)

- **Trichloroacetic acid solution:**
  0.11M trichloroacetic acid solution, 0.22M sodium acetate and 0.33M acetic acid.

- **Folin-phenol ciocalteau’s reagent:**
  2N Folin Ciocalteau reagent diluted with distilled water to 1N.

### 3.1.11. Standard curve of tyrosine

Made a stock solution of tyrosine having a concentration of 2mg/ml. A standard curve was plotted of this stock solution ranging from 100-1000µg. To 2.5 ml of 0.5N Na$_2$CO$_3$ solution and 0.5ml of 1N Folin’s phenol ciocalteau’s reagent was added 50, 100, 150......500 µl of stock solution and final volume was made to 500µl with distilled water. The contents were thoroughly mixed and were incubated in dark at room temperature for 30 min. The absorbance was read at 660 nm (see Appendix 3).

### 3.1.12: Reagents for determination of N-acetyl glucosamine

- **Potassium tetraborate solution**
  0.8M in distilled water

- **p-Dimethyl aminobenzaldehyde reagent**
  10g of p-Dimethyl aminobenzaldehyde was dissolved in 100ml of glacial acetic acid which contained 12.5% 10N HCl. Shortly before use, it was diluted with 9 volumes of glacial acetic acid.

### 3.1.13. Standard curve of N-acetyl glucosamine

Made an N-acetyl glucosamine stock solution having a concentration of 2mg/ml. A standard curve was plotted of this stock solution ranging from 100-1000µg. To the tubes was added 50, 100, 150….500µl of this stock and the final volume in each tube was made to 500µl with distilled water. To all the tubes was added 0.1ml of potassium tetraborate solution. The tubes were heated in a boiling water bath for 3 min and were cooled under tap water. Thereafter, 3.0 ml of p-dimethyl aminobenzaldehyde reagent was added and immediately after mixing, the tubes were placed in water bath at 37°C for 20
The tubes were then cooled under tap water and absorbance was read at 544 nm (see Appendix 4).

3.1.14. Solutions for native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

- **Acrylamide solution (30%)**
  - Acrylamide 29g
  - N, N-methylenebisacrylamide 1g
  - Distilled water to make 100ml
  - Filtered through whatman filter paper and stored in brown colored bottle.

- **Separating gel buffer (pH 8.8)**
  - Tris 1.5M
  - Adjust pH 8.8 with HCl

- **Stacking gel buffer (pH 6.8)**
  - Tris 0.5M
  - Adjust pH 6.8 with HCl.

- **Fresh solution of 10% Ammonium persulphate (APS)**
  - Ammonium persulfate 0.5g
  - Distilled water 5.0 ml

- **Running gel/Electrode buffer**
  - Tris 3g
  - Glycine 14.4g
  - 10% SDS 10ml
  - Distilled water to make 1000ml

- **Solubilizing buffer**
  - Tris 0.625 M, pH 7.8
  - SDS 1g
  - Bromophenol blue 0.01%
  - Glycerol 5 ml
  - Distilled water to make 50 ml

- **Sample buffer (for SDS-PAGE)**
  - Solubilizing buffer 1ml
  - Mercaptoethanol 50 μl
  - SDS 0.001%

- **Tetramethyl ethylene diamine (TEMED)**

- **10% SDS (Sodium dodecyl sulfate) (For SDS-PAGE only)**
  - SDS 10g
  - Distilled water 100ml
3.1.15. Reagents for silver staining:

- **Fixing solution**
  Ethanol: glacial acetic acid: distilled water 3:1:6 (by volume)

- **Ethanol**
  30% in distilled water

- **Solution of silver nitrate**
  0.1% in distilled water

- **Developing solution**
  2.5% sodium carbonate in 0.02% formaldehyde

- **Acetic acid solution**
  1% in distilled water

3.1.16. Reagents for protein estimation by Lowry’s method:

- **Lowry A**
  2% Sodium carbonate in 0.1M Sodium hydroxide

- **Lowry B**
  1% CuSO₄·5H₂O in distilled water

- **Lowry C**
  2% Sodium potassium tartarate in distilled water

- **Working solution**
  98ml of Lowry A + 1ml of Lowry B +1ml of Lowry C.

- **Lowry D (Folin Ciocalteau reagent)**
  2N Folin Ciocalteau reagent diluted with distilled water to 1N.

3.1.17. Reagents for preparative gel electrophoresis:

- **Elution buffer:**
  Tris HCl  pH 7.9,(0.05M)
  EDTA  0.1mM
  NaCl  0.2M
  Distilled water  100ml
3.1.18. Reagents used for coomassie blue staining:

- **Staining solution**
  - Methanol 40 ml
  - Acetic acid 10 ml
  - Coomassie blue 0.05 g
  - Distilled water 50 ml

- **Destaining solution**
  - Methanol 400 ml
  - Glacial acetic acid 100 ml
  - Distilled water 500 ml

3.1.19. Standard curve of protein

Made a stock solution of bovine serum albumin (BSA) having a concentration of 2 mg/ml. A standard curve was plotted of this stock solution ranging from 100-1000 µg. To 2.5 ml of working solution (as discussed in section 3.1.16.) was added 50, 100, 150, ....... 500 µl of stock solution and final volume was made to 500 µl with distilled water. It was mixed thoroughly and allowed to stand at 37°C for 10 min. Added 0.25 ml of 1 N Folin’s reagent, mixed it rapidly, allowed it to stand at 37°C for 30 min and read absorbance at 660 nm (see Appendix 5).

3.1.20. Paper Chromatography:

- **Whatman paper no. 1**
- **Developing reagent:**
  - butanol:pyridine:water 6:4:3 (by volume)
- **Detection reagent:**
  - Acetone 100 ml
  - Phthalic acid 0.66 g
  - Aniline 93 ml

3.1.21. Commercial enzymes

Three different commercial enzymes were used in the study.

- Termamyl™ capable of complete liquefaction of the starchy substrates was obtained from Jagatjit Industries Ltd., Jalandhar, India.
AMG™ capable of complete saccharification of starchy substrates was obtained from Jagatjit Industries Ltd., Jalandhar, India.

Commercial α-amylase preparation (Palkoyme) capable of desizing was obtained from MAPS, India.

3.1.22. Microorganisms

- A fungal strain, Aspergillus sp., capable of producing glucoamylase was available in our own laboratory.
- A distiller’s yeast strain Saccharomyces cerevisae, capable of fermentation was obtained from Jagatjit Industries Ltd., Jalandhar, India.

3.2. METHODS

The present study was done after taking into consideration the need of reducing the cost of hydrolysis of starch to sugars and its major thrust has been aimed at obtaining a bacterial isolate for the production of direct raw starch liquefying α-amylase and its evaluation for the direct liquefaction of natural starch substrates for ethanol production.

3.2.1. Microorganism

An extensive screening procedure was done with the aim to obtain a bacterial isolate capable of producing direct raw starch liquefying α-amylase by both solid state and submerged fermentation techniques using simple and low cost media and its ability to hydrolyze different types of insoluble raw starches.

3.2.2. Primary screening

Soil samples collected from the vicinity of flourmills of Chandigarh city, hot springs of Manikaran, Kullu, and rotten potatoes collected from the local market, Chandigarh, were screened for the isolation of natural bacterial strains capable of producing direct raw starch liquefying α-amylase.

Screening of the efficient isolates was initially carried out on the basis of zone of clearance on nutrient raw starch agar plates after flooding them with the iodine solution followed by their ability to produce α-amylase when grown in submerged cultures.

The isolation procedure involved the enrichment of amylolytic organisms by dispensing 1.0 g samples in 9ml sterilized distilled water and then adding 0.1 ml of these
suspensions, separately, to 100ml Erlenmeyer flasks containing 20ml of nutrient broth, each supplemented with 1% raw insoluble maize, potato, rice and wheat starches (pH 7.0, section 3.1.4.1.) respectively. The raw starches were sterilized separately but without autoclaving. 1% of different insoluble raw starches were sterilized by washing with absolute alcohol and then keeping in an oven at 50°C overnight, followed by dispensing in sterile distilled water. The different starches were then added to sterile production medium in the respective flasks under sterile conditions.

The flasks were then incubated at 37°C for 48h under shake conditions (150 rpm) on New Brunswick Environmental shaker. The raw starch digesting amylolytic bacteria were screened by streaking the contents of the enriched flasks on nutrient agar plates having 0.5% raw maize, potato, rice and wheat, separately. The bacterial colonies showing zones of clearance against a blue background as a result of flooding the plates with iodine solution were picked up and evaluated on the basis of their degree of clearing. The pure line cultures of bacterial isolates were established by repeated streaking on the solid media.

Of a large number of raw starch degrading amylolytic colonies, isolated at 37°C, 53 were selected on the basis of their degree of clearing in raw starch plates. These were then subjected to liquid enzyme production in shake flasks and the enzyme yields were determined after 48h of incubation.

3.2.2.1. Liquid culture α-amylase production

Twenty milliliters of production medium (peptone 10 g, beef extract 10 g, starch (soluble) 10 g and distilled water 1000 ml, pH 7.0; section 3.1.4.2.), taken in different sets of 100 ml Erlenmeyer flasks (in duplicate), was autoclaved at 121°C for 20 min and used as basal medium for liquid culture α-amylase production.

The flasks were inoculated with 2 ml of the seed culture (A_{600; 0.8, 1.92x10^7 cells/ml}) prepared from 12 h old shake flask cultures of bacterial isolates in seed medium (peptone 10 g, beef extract 10 g, distilled water 1000 ml, pH 7.0; section 3.1.4.2.), and incubated at 37°C on a rotary shaker (150 rpm) for 48h. The contents of the flasks were then centrifuged (10,000rpm, 20 min, 4°C) and the cell free supernatants were used for determining the extracellular α-amylase yield.
3.2.2.2. α-Amylase assay

The enzyme activity was determined with slight modification of original method of Fuwa (1954). Appropriate dilutions of the enzyme preparations were made in 0.1M Phosphate buffer pH 7.0 and 0.25 ml of each dilution was incubated with 0.25 ml of starch substrate at 60°C in water bath for 10 min. The reaction was stopped using 0.25 ml of 1N HCl and the residual starch was measured by adding 0.25 ml of iodine solution, diluting it by adding 4.0 ml distilled water and taking absorbance at 690 nm. Simultaneously, blank which contained 0.5 ml of 0.1M Phosphate buffer, pH 7.0, 0.25 ml of 1N HCl, 0.25 ml iodine solution diluted to 5.0 ml and substrate control, which contained 0.25 ml of 0.1M Phosphate buffer, pH 7.0, 0.25 ml starch substrate, 0.25 ml of 1N HCl, 0.25 ml iodine solution diluted to 5.0 ml, were prepared.

In addition to these, enzyme control with 0.25 ml enzyme sample with 0.25 ml of 0.1M Phosphate buffer pH 7.0, 0.25 ml of 1N HCl, 0.25 ml iodine solution diluted to 5.0 ml was also read at 690 nm to detect any starch present in enzyme sample.

One unit of the α-amylase activity was expressed as equivalent to the enzyme causing 10% reduction in O.D. of test as compared to that of substrate control in 10 min under the assay conditions.

Calculations for the enzyme activity:

\[
\frac{\text{O.D. of substrate control} - \text{O.D. of test}}{\text{O.D. of substrate control}} = \text{factor A}
\]

For any dilution, if the factor A is between 0.2-0.4, only then that dilution was used for calculating activity.

Enzyme units/ml (U/ml) = factor A × 4 × \(\frac{100}{10}\) × dilution factor

Since 0.25 ml of the test was taken, so to express the enzyme units per ml, factor A is multiplied by 4. It is multiplied by \(\frac{100}{10}\), because one enzyme unit is defined as that causing 10% reduction in O.D. of test as compared to that of substrate control.

The same procedure was adopted to assay α-amylase activity during rest of the study.
3.2.3. Secondary screening

Based on primary screening, 27 isolates were selected and these were further screened on the basis of their ability to grow as solid state cultures. The enzyme preparations from these isolates were also evaluated for their capabilities in the direct liquefaction of these starches.

3.2.3.1. Production of α-amylase by solid state fermentation (SSF)

Five grams of wheat bran moistened with 5.0 ml of distilled water was taken as the basal medium for SSF. It was taken in different sets of 250 ml Erlenmeyer flasks, autoclaved, cooled and then inoculated with 2.5 ml of the inoculum, \( A_600 \), 0.8 obtained from 12h old shake cultures of different isolates in seed medium (peptone 10 g, beef extract 10 g, distilled water 1000 ml, pH 7.0; section 3.1.4.2). Flasks, in duplicates, were withdrawn after 48h of incubation at 37°C and analyzed for α-amylase.

3.2.3.2. Extraction and assay of α-amylase

The enzyme was extracted by adding 50 ml of tap water to the flask of solid state culture and keeping on a New Brunswick water bath shaker at 37°C and 150 rpm for 30-45 min. After shaking, the contents of the flask were filtered through metallic sieve and the solid residue was pressed to release any remaining liquid. The final volume of this released liquid was noted down after centrifuging it at 10,000 rpm for 10 min and cell free supernatant was then used for assaying the α-amylase.

The α-amylase was assayed similarly (section 3.2.2.2). The enzyme yield in SSF was expressed as U/g of original substrate used in the production medium.

3.2.3.3. Raw starch hydrolysis

To the different sets of 100 ml Erlenmeyer flasks each containing 100mg of different raw starches, was added 500 units (5U/mg of raw starch) of α-amylase preparation, separately, from different isolates. The final volume of each flask was made to 10 ml with distilled water. This was incubated at 60°C in a water bath shaker at 150 rpm. The extent of liquefaction was estimated after 6h by measuring the decrease in starch content with iodine method (Gogoi et al., 1987). To determine the extent of saccharification, the reducing sugar content was measured by withdrawing the samples.
of the reaction mixture (test) after 6h by measuring glucose content with DNSA method (Miller, 1959).

Simultaneously, substrate control (To 100 ml flasks, 100 mg of different raw starches were added and the volume was made to 10 ml with distilled water. This was incubated at 60°C in a water bath shaker at 150 rpm) and enzyme control (To 100 ml flasks, 500 units of α-amylase preparations from different isolates were added and the final volume was made to 10 ml. This was incubated at 60°C in a water bath shaker at 150 rpm) were also prepared along with. The samples were similarly withdrawn from substrate controls and enzyme controls after 6h and were checked for the extent of liquefaction and saccharification to sugars.

To determine the extent of direct liquefaction of raw starches, the amount of residual starch in the sample was determined. To 0.25 ml of the supernatant of each sample was added 0.25 ml of 0.1M Phosphate buffer (pH 7.0), 0.25 ml of 1N HCl and 0.25 ml of iodine solution, diluting it by adding 4.0 ml distilled water. For enzyme control, to 0.25 ml of enzyme preparation from each isolate was added 0.25 ml of 0.1N Phosphate buffer (pH 7.0), 0.25 ml of 1N HCl and 0.25 ml of iodine solution, diluting it by adding 4.0 ml distilled water. For blank, 0.25ml of distilled water was added to 0.25 ml of Phosphate buffer (pH 7.0), 0.25 ml of 1N HCl and 0.25 ml of iodine solution, diluting it by adding 4.0 ml distilled water and taking absorbance of all the tubes at 690 nm. The amount of residual starch present in the samples was calculated from the standard curve of starch (Appendix 1; section 3.1.7.). The extent of direct liquefaction of raw starch was then determined from the following calculations:

**Calculations:**

From the O.D. of test compared to Standard curve, let the starch content

\[ A \mu g \]

From the O.D. of enzyme control compared to Standard curve, let the starch content

\[ B \mu g \]

Total residual starch (C)

\[ C = A - B \mu g \]

Total mg of starch/ml (D)

\[ D = \frac{C \times 4 \times \text{dilution factor}}{1000} \]

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% Liquefaction of raw starch = \frac{\text{Total starch/ml (mg) - D}}{\text{Total starch/ml (mg)}} \times 100

To determine the extent of saccharification, of every centrifuged sample, 0.1 ml of the supernatant was taken and the final volume was made to 1.0 ml with distilled water. For blank, 1.0 ml of distilled water was taken. To all the tubes was added 1.5 ml of DNSA reagent. Kept all the tubes in boiling water bath for 10 min. Diluted the contents of each tube with 4.0 ml of distilled water to a final volume of 6.5 ml in each tube. Absorbance was read at 570 nm and the amount of glucose present was calculated from the standard curve of glucose (Appendix 2; section 3.1.9). The extent of starch hydrolysis was then determined from the following calculations:

**Calculations:**

From the O.D. of test compared to Standard curve, let the glucose content

\[ A \mu g \text{ (in 0.1 ml of sample)} = A \times 100 \mu g \text{ (in 10 ml of sample)} = B \mu g \]

From the O.D. of substrate control compared to Standard curve, let the glucose content

\[ C \mu g \text{ (in 0.1 ml of sample)} = C \times 100 \mu g \text{ (in 10 ml of sample)} = D \mu g \]

From the O.D. of enzyme control compared to Standard curve, let the glucose content

\[ E \mu g \text{ (in 0.1 ml of sample)} = E \times 100 \mu g \text{ (in 10 ml sample)} = F \mu g \]

Total amount of glucose formed by the enzyme preparation from an isolate (G)

\[ = B - (D + F) \mu g \]

Maximum amount of glucose that can be formed from 100 mg of \text{starch} (H)

\[ = 110 \text{ mg} \]
On the basis of ability of isolates to grow as liquid and solid state cultures, and raw starch hydrolysis, one bacterial isolate, designated as P₁, was selected for further studies.

3.2.4. Identification of the strain

The taxonomic status of the selected strain (P₁) was identified following the criteria laid down by Bergey’s Manual of systematic Bacteriology (Claus and Brekeley, 1986). On the basis of various morphological, biochemical and physiological characteristics, the strain was identified as *Bacillus subtilis*.

Complete identification of the bacterial strain was done by 16s rRNA sequencing at Microbial Type Culture Collection and Gene Bank, IMTECH, India and was identified as *Bacillus subtilis* subsp. *spizizenii*.

3.2.5. Maintenance of the strain

The organism was maintained on nutrient agar, supplemented with 0.5% raw starch and stored at 4°C after overlaying with sterile mineral oil.

3.2.6. α-Amylase production by the selected strain of *Bacillus subtilis* subsp. *spizizenii*

Solid state (substrate) fermentation (SSF) has been used successfully for the production of enzymes and secondary metabolites. These products are associated with the stationary phase of microbial growth and are produced on an industrial scale for use in agriculture and the treatment of disease. Many of these secondary metabolites are still produced by submerged liquid fermentations (SmF) even though production by this method has been shown to be less efficient than SSF. As large-scale production increases further, so do the costs and energy demands. SSF has been shown to produce a more stable product, requiring less energy, in smaller fermentors, with easier downstream processing measures.

So, the α-amylase production by selected bacterial strain was studied both by solid-state fermentation (SSF) and by submerged fermentation (SmF) techniques.
3.2.7. α-Amylase production by solid state fermentation technique

3.2.7.1. Pattern of α-amylase production

Different sets of 250 ml Erlenmeyer flasks, each containing 5g of wheat bran moistened with 5 ml of distilled water were inoculated with 2.5 ml of the inoculum (A_{600}, 0.8) obtained from 12 h old shake culture of *Bacillus subtilis* subsp. *spizizenii* to obtain the final ratio of 1:1.5 (wheat bran: moisture content). The flasks were incubated at 37°C under stationary conditions. Flasks, in duplicate, were withdrawn at regular intervals of time. Every time, the contents of each flask were extracted and the cell free supernatant was analysed for pH and was used for determining the extracellular α-amylase and protease activity.

3.2.7.2. Assay of protease

The protease activity was measured in terms of amino acids released by enzyme. The amino acids were estimated according to the method of Anson (Takami *et al.*, 1989). To 0.5ml of the appropriately diluted crude preparation was added 2.5ml of substrate solution (0.6% casein made in glycine- NaOH buffer, 0.1M, pH 9.0; as discussed in section 3.1.10.). The tubes were incubated at 60°C in a water bath for 10 min. The reaction was stopped by the addition of 2.5 ml of trichloroacetic acid solution. The mixture was again incubated at 60°C in a water bath for 10 min. The reaction mixture was then centrifuged at 10000 rpm for 5 min to remove all the unreacted and precipitated proteins. To 0.5ml of the supernatant was added 2.5 ml of 0.5N Na_{2}CO_{3} solution and 0.5ml of 1N Folin’s phenol ciocalteau’s reagent (2N Folin Ciocalteau reagent diluted with distilled water to 1N; section 3.1.10.). The contents were thoroughly mixed and were incubated in dark at room temperature for 30 min. The absorbance was read at 660 nm. The protease activity in terms of protease units in the supernatant was measured by referring to the standard curve ranging from 100-1000μg tyrosine as discussed in section 3.1.11.(Appendix 3). One international unit of protease activity per ml is defined as the amount of enzyme required to release 1 micromole of tyrosine under the assay conditions.
3.2.7.3. Optimization of α-amylase production by varying ‘one variable at one time’ approach

α-Amylase production by bacterial strain was optimized in SSF (using wheat bran as substrate) by studying the effect of various environmental and cultural variables, using 250 ml Erlenmeyer flasks after 48h of incubation at 37°C unless otherwise stated. Production media containing 5g of wheat bran moistened with 5.0 ml of distilled water was taken as control.

Solid substrate and support material

This was studied by using a variety of solid substrates and their combinations (listed below), separately, for the enzyme production.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple pomace</td>
<td>5</td>
</tr>
<tr>
<td>Brewer’s spent grain (BSG)</td>
<td>5</td>
</tr>
<tr>
<td>Polyurethane foam (PUF) with nutrient broth</td>
<td>5</td>
</tr>
<tr>
<td>Rice Bran</td>
<td>5</td>
</tr>
<tr>
<td>Rice Husk</td>
<td>5</td>
</tr>
<tr>
<td>Sugarcane Baggasse</td>
<td>5</td>
</tr>
<tr>
<td>Wheat Bran</td>
<td>5</td>
</tr>
<tr>
<td>Wheat Husk</td>
<td>5</td>
</tr>
<tr>
<td>Rice Bran + BSG</td>
<td>2.5 + 2.5</td>
</tr>
<tr>
<td>Rice Bran + Rice Husk</td>
<td>2.5 + 2.5</td>
</tr>
<tr>
<td>Wheat Bran + BSG</td>
<td>2.5 + 2.5</td>
</tr>
</tbody>
</table>
Since wheat bran proved to be the best substrate, it was used in all subsequent studies.

**Particle size**

This was studied by using wheat bran of different particles mesh sizes including 10, 20, 30 and 40 in solid-state fermentation cultures.

Since wheat bran without sieving (i.e. containing a mixture of different mesh sizes) as a substrate for solid state fermentation, gave comparable α-amylase yields when compared to that of different mesh sizes, it was used in all subsequent studies.

**Temperature**

This was studied by incubating the flasks containing solid state cultures having wheat bran (5g) as the basal medium and distilled water in the ratio of 1:1.5, at different temperatures including 25, 30, 37, 42, 45 and 50°C.

Since the cultures incubated at 37°C proved to be favorable for producing maximum amount of the enzyme, all the subsequent studies were carried out at this temperature.

**Moisture content**

This was studied by altering the level of moisture content in the standard basal medium consisting of wheat bran (5 g) and using distilled water as the moistening agent. The various ratios of substrate (wheat bran) to water used in the study included 1:1.0, 1:1.5, 1:2.0, 1:2.5, 1:3.0, 1:3.5 and 1:4.0.

Since 1:1.5 ratio of wheat bran: moisture content gave the best α- amylase yield as compared to other ratios, it was used in all the subsequent studies.
Nature of moistening agent and its pH

This was studied by replacing distilled water (pH 7.0) in basal medium as moistening agent with buffers of varying pH (as listed below), and tap water (pH 6.7).

<table>
<thead>
<tr>
<th>Buffers</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M Citrate phosphate buffer (CPB)</td>
<td>4.0, 4.5, 5.0, 5.5</td>
</tr>
<tr>
<td>0.1M Phosphate buffer (PB)</td>
<td>6.0, 6.5, 7.0, 7.5, 8.0</td>
</tr>
<tr>
<td>0.1M Tris HCl buffer</td>
<td>7.0, 7.5</td>
</tr>
</tbody>
</table>

Since tap water as moistening agent gave the best activity, this was further used in the subsequent studies.

Effect of Inoculum size

The effect of inoculum size was studied by incorporating different volumes of inoculum (A660: 0.8; 1.76x10⁷ cells/ml) including 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 ml separately in the basal medium. Corresponding to the inoculum size, the moisture content in the standard basal media was also adjusted to give a final moisture concentration of 1:1.5.

Since 2.5 ml of inoculum gave the maximum α-amylase production, it was used in the subsequent studies.

3.2.7.3.1. Effect of exogenous supplementation of various nutrients in the basal medium on α-amylase production in SSF

Carbon

This was studied by supplementing different carbohydrates, including amylopectin, arabinose, cellobiose, carboxy methyl cellulose (CMC), dulcitol, erythritol, fructose, galactose, glucose, glycerol, inositol, inulin, lactose, maltose, mannitol, mannose, raffinose, rhamnose, soluble starch, sorbitol, sucrose, trehalose and xylose (1% w/w), purified gelatinized and raw insoluble starches including maize, potato, rice and wheat starch (1% w/w), in the basal media. In case of gelatinized starches the starches were autoclaved with the rest of the production medium whereas in case of raw insoluble starches, the raw starchy sources were sterilized separately but without
autoclaving with rest of basal medium. Different raw starches (0.05g) were sterilized by mixing in absolute alcohol, which were then evaporated by keeping it overnight at 50°C in an oven. The suspension of the alcohol treated starches were then made in sterile distilled water and added to the basal medium.

Since glycerol induced the highest enzyme production, the effect of different concentration (0.5, 0.75, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75 and 3.0%) of glycerol was studied by varying the levels of this carbon source in the basal medium. Basal medium supplemented with 1% (w/w) glycerol was taken as control.

Nitrogen

This was studied by supplementing different organic and inorganic nitrogenous compounds including beef extract, corn steep liquor, peptone, protease peptone, soyabean meal, soyapeptone, tryptone, yeast autolysate, yeast extract, ammonium chloride, ammonium citrate, ammonium molybdate, ammonium sulphate and urea at the level of 1% (w/w), separately in the basal medium.

Since soyabean meal induced the maximum enzyme production, different concentrations (0.5, 0.75, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75 and 3.0% w/w) of the same were also evaluated in the basal medium. Basal medium supplemented with 1% soyabean meal (w/w) was taken as control.

Metal ion

This was studied by supplementing various metal salts, separately, including aluminium chloride, barium chloride, calcium chloride, cobalt chloride, copper sulphate, iron chloride, iron nitrate, iron oxide, lead acetate, lithium sulphate, magnesium sulphate, mercury chloride, potassium chloride, silver nitrate, sodium chloride, sodium molybdate, sodium tungstate, zinc sulphate and EDTA (ethylenediaminetetraacetic acid) at the level of 1mM (v/w) in the tap water used as the moistening agent in the basal medium.

Since sodium chloride induced the maximum enzyme production, the effect of different concentrations (0.5, 0.75, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 mM) of the same was also studied in the basal medium. Basal medium supplemented with 1mM (v/w) sodium chloride was taken as control.
Vitamin

Effect of vitamins on α-amylase production was studied by incorporating various vitamins like ascorbic acid (Vitamin C), B-Complex (combination of B₁, B₆ and B₁₂), biotin (Vitamin H or B₇), calcium pantothenate (Vitamin B₅), folic acid (PGA, pyroglutamic acid), nicotinic acid (niacin), pyridoxine (Vitamin B₆), riboflavin (Vitamin B₂), thiamine (Vitamin B₁), and tocoferol (Vitamin A), separately, in the basal medium at a concentration of 0.01% (w/w).

Vitamin B-complex, whose supplementation caused the highest improvement in the enzyme yield, was also evaluated at different concentrations (0.005, 0.0075, 0.0125, 0.015, 0.0175, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045 and 0.05% w/w) in the basal medium. Basal medium supplemented with 0.01% (w/w) Vitamin B complex was taken as control.

Amino acid

Amino acids including alanine, arginine, aspartic acid, cysteine, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan and valine were supplemented, separately, in the basal medium at a concentration of 0.01% (w/w).

Amino acid threonine, whose supplementation caused the highest improvement in the enzyme yield, was then evaluated at different concentrations (0.005, 0.0075, 0.0125, 0.015, 0.0175, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, and 0.05% w/w) in the basal medium. Basal medium supplemented with 0.01% (w/w) threonine was taken as control.

Surfactants and polyethylene glycol (PEG) 3000

This was studied by supplementing different surfactants including Triton X100, Tween 20, Tween 40, Tween 60, Tween 80 and SDS (v/w) and PEG (w/w) separately in the basal medium at the level of 0.01%.

Surfactant Tween 40, whose supplementation caused the highest improvement in the enzyme yield, was then evaluated at different concentrations (0.005, 0.0075, 0.0125, 0.015, 0.0175, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, and 0.05 % v/w) in the basal medium. Basal medium supplemented with 0.01% (v/w) Tween 40 was taken as control.
Optimization of enzyme recovery from the fermented bran

Enzyme recovery from the fermented bran was optimized by using different buffers (as listed below), surfactants (Triton X100, Tween 20, Tween 40, Tween 60, Tween 80, SDS, 0.01%, v/w and PEG, 0.01%, w/w) and tap water as extractants in place of distilled water (which was taken as control).

<table>
<thead>
<tr>
<th>Buffers as extractants</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M Citrate phosphate buffer</td>
<td>4.0, 4.5, 5.0, 5.5</td>
</tr>
<tr>
<td>0.1M Phosphate buffer</td>
<td>6.0, 6.5, 7.0, 7.5, 8.0</td>
</tr>
<tr>
<td>0.1M Tris HCl buffer</td>
<td>7.0, 7.5</td>
</tr>
</tbody>
</table>

Surfactant Tween 40, whose supplementation caused the highest enzyme recovery, was then evaluated at different concentrations (0.005, 0.0075, 0.0125, 0.015, 0.0175, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, and 0.05 % v/w) for the extraction of the enzyme from the fermented bran. Also, Tween 40 at 0.01 % (v/w) as extractant was taken as control.

Production in optimized medium

The production of α-amylase was carried out in optimized medium consisting of 5g wheat bran, moistened with 7.5 ml of moistening agent (5ml tap water and 2.5 ml inoculum) and supplemented with glycerol (2.0 % w/w) as carbon source, soyabean meal (2% w/w) as nitrogen source, sodium chloride (2mM) as the metal ion source, B-complex (0.015% w/w) as vitamin source, threonine (0.0125% w/w) as amino acid source and Tween 40 (0.0125%v/w) as surfactant. This was incubated at 37°C for 48h. Enzyme recovery was done by using Tween 40 (0.0175% v/w) as extractant. Basal medium without any supplementation was taken as control.
3.2.8. a-Amylase production by submerged fermentation (SmF) technique

3.2.8.1. Pattern of growth and α-amylase production

Different sets of 100 ml Erlenmeyer flasks, each containing 20 ml of production medium (containing nutrient broth supplemented with 1% soluble starch, section 3.1.4.2.), were inoculated with 2 ml of the inoculum (A_600, 0.8) obtained from 12h old shake culture of Bacillus subtilis subsp. spizizenii in seed medium (section 3.1.4.2). The flasks were incubated in New Brunswick Environmental shaker (150 rpm) for 120h at 37°C. Flasks, in duplicate, were withdrawn at regular intervals of time and analyzed for growth (A_600) and pH. Every time, the contents of each flask were centrifuged (10,000 rpm, 20 min, 4°C) and the cell free supernatant was used for the extracellular α-amylase and protease activity.

3.2.8.2. Optimization of α-amylase production by Bacillus subtilis subsp. spizizenii employing submerged fermentation (SmF) technique by varying ‘one variable at one time’ approach

α-Amylase production by the selected bacterial strain was optimized in SmF by studying the effect of various environmental and cultural variables, using 100 ml Erlenmeyer flasks, containing 20 ml production medium after 48h of incubation at 37°C unless otherwise stated, in shake cultures (150 rpm). Production medium (peptone 10g, beef extract 10 g, starch (soluble) 10 g, distilled water 1000 ml, pH 7.0; section 3.1.4.2.) was taken as control.

Temperature

This was studied by incubating the flasks containing production medium at different temperatures including 25, 30, 37, 42, 45, 50, 55 and 60°C.

Since the cultures incubated at 50°C proved to be favorable for producing maximum amount of the enzyme, all the subsequent studies were carried out at this temperature.
pH

This was studied by altering the pH of the production medium with IN HCl and 1N NaOH in the range of 4.0-9.0 (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0).

Since pH 7.0 of the production medium gave the maximum α-amylase production, it was used in the subsequent studies.

Inoculum size

The effect of inoculum size was studied by incorporating 5, 6, 7, 8, 9, 10, 11, 12 and 13% of the seed culture (A600; 0.8; 1.87x10^7 cells/ml) in the production medium separately.

Since 10% of inoculum gave the maximum α-amylase production, it was used in the subsequent studies.

Carbon source

This was studied by replacing soluble starch in the production medium, separately, with different carbohydrates including amylopectin, arabinose, cellobiose, carboxy methyl cellulose (CMC), dulcitol, erythriotol, fructose, galactose, glucose, glycerol, inositol, inulin, lactose, maltose, mannotol, mannose, raffinose, rhamnose, sorbitol, sucrose, trehalose and xylose (1% w/v), purified gelatinized and raw insoluble starches including maize, potato, rice and wheat starch (1% w/v). In case of gelatinized starches the starches were autoclaved with the rest of the production medium whereas in case of raw starches, the raw starchy sources were sterilized separately but without autoclaving with rest of production medium (section 3.2.7.3.1.).

Since glycerol induced the highest enzyme production, the effect of different concentration (0.5, 0.75, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75 and 3.0% v/v) of glycerol was studied separately, by varying the levels of this carbon source in the production medium (without soluble starch). Production medium in which soluble starch was replaced by glycerol at 1% (v/v) concentration was taken as control.
Nitrogen source

This was studied by replacing beef extract and peptone in the production medium, separately, with different organic and inorganic nitrogenous compounds including beef extract, corn steep liquor, peptone, protease peptone, soyabean meal, soyapeptone, tryptone, yeast autolysate, yeast extract, ammonium chloride, ammonium citrate, ammonium molybdate, ammonium sulphate and urea at the level of 1% (w/v). Production medium containing yeast extract and peptone as the nitrogen source and soluble starch as carbon source was taken as control.

Since soyabean meal induced the maximum enzyme production, the effect of different concentrations (0.5, 0.75, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75 and 3.0% w/v) of the same was studied separately, by varying the levels of this nitrogen source in the production medium (without beef extract and peptone). Production medium in which beef extract and peptone were replaced by soyabean meal at 1% (w/v) concentration was taken as control.

Metal ions

This was studied by incorporating various metal ions in the production medium, separately, including aluminium chloride, barium chloride, calcium chloride, cobalt chloride, copper sulphate, EDTA, iron chloride, iron nitrate, iron oxide, lead acetate, lithium sulphate, magnesium sulphate, mercury chloride, potassium chloride, silver nitrate, sodium chloride, sodium molybdate, sodium tungstate, zinc sulphate and EDTA (ethylenediaminetetraacetic acid) at the level of 1mM (v/v).

Since sodium chloride induced the maximum enzyme production, it was also evaluated at different concentrations (0.5, 0.75, 1.25, 1.5, and 1.75, 2.0, 2.25 and 2.5 mM v/v) in the production medium. Production medium containing 1mM (v/v) sodium chloride was taken as control.

Vitamins

Effect of vitamins on α-amylase production was studied by incorporating various vitamins like ascorbic acid (Vitamin C), B-Complex (combination of B1, B6 and B12), biotin (Vitamin H or B7), calcium pantothenate (Vitamin B5), folic acid (PGA, ptyroglutamic acid), nicotinic acid (niacin), pyridoxine (Vitamin B6), riboflavin (Vitamin
B<sub>2</sub>), thiamine (Vitamin B<sub>1</sub>), and tocoferol (Vitamin A), separately, in the production medium at a concentration of 0.01% (w/v).

Vitamin B-complex, whose supplementation caused the highest improvement in the enzyme yield, was also evaluated at different concentrations (0.005, 0.0075, 0.0125, 0.015, 0.0175, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, and 0.05% w/v) in the production medium. Production medium supplemented with 0.01% (w/v) Vitamin B complex was taken as control.

Amino acids

Amino acids including alanine, arginine, aspartic acid, cysteine, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan and valine were supplemented, separately, in the production medium at a concentration of 0.01% (w/v).

Amino acid threonine, whose supplementation caused the highest improvement in the enzyme yield, was then evaluated at different concentrations (0.005, 0.0075, 0.0125, 0.015, 0.0175, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, and 0.05% w/v) in the production medium. Production medium supplemented with 0.01% (w/v) threonine was taken as control.

Production in optimized medium

α- Amylase production was finally studied in the optimized medium at pH 7.0 and temperature 50°C. The optimized medium included glycerol (2% w/v) as carbon source, soyabean meal (2.5% w/v) as nitrogen source, sodium chloride (2.25mM v/v) as the metal ion source, B- complex (0.0175% w/v) as vitamin source and threonine (0.01% w/v) as amino acid source. The inoculation of this medium was done by 10% inoculum of seed culture. The optimized medium was incubated for 48h and was checked for α-amylase yield.

3.2.9. Comparison of α-amylase production by solid state fermentation and submerged fermentation

The organism was grown both as solid state culture and as shake flask cultures. To obtain solid cultures, different sets of 250 ml Erlenmeyer flasks, each containing
sterilized 5g of wheat bran moistened with 5 ml of tap water were inoculated with 2.5 ml of the inoculum ($A_{600}, 0.8$) obtained from 12h old shake culture of *Bacillus subtilis* subsp. *spizizenii* to obtain the final ratio of 1:1.5 (wheat bran: moisture content). Also, one flask containing sterilized 5g of wheat bran moistened with 7.5ml of sterilized tap water (uninnoculated with organism) was taken and was set as control. The flasks were incubated at 37°C under stationary conditions for a total time of 96h. To obtain the shake cultures, different sets of 100 ml Erlenmeyer flasks, each containing 20 ml of production medium (containing nutrient broth supplemented with 1% soluble starch, section 3.1.4.2.), were inoculated with 2 ml of the inoculum ($A_{600}, 0.8$) obtained from 12h old shake culture of *Bacillus subtilis* subsp. *spizizenii* in seed medium (section 3.1.4.2.). The flasks were incubated in New Brunswick Environmental shaker (150 rpm) for 96 h at 37°C. Flasks, in duplicate, were withdrawn at regular intervals of time from both the culture conditions and were analyzed for N-acetyl glucosamine content to obtain the biomass content. Also, the flasks withdrawn after 48h of incubation from both the cultural conditions were analysed for $\alpha$-amylase yields. The volume of the contents of flask in case of SmF was noted down.

To obtain the biomass, the contents of the flasks were extracted (in case of SSF) and were centrifuged at 10000 rpm for 20 min. The residue obtained from both the submerged and solid state cultures was dried by keeping overnight at 55°C. Biomass in case of submerged fermentation was obtained by direct weighing of the dried pellet (X g). To obtain the biomass in case of SSF, the dried fermented bran was crushed with a pestle motor. A known amount (T g) of the dried fermented bran was taken in a 100ml flask and 10ml of 7N HCl was added. The flasks were kept in a water bath at 70°C for 4h. After 4h of incubation, the pH of this acid hydrolysed culture was neutralized by using 10% NaOH. The final volume of the contents of the flask was noted down. Similar treatment was given to culture (preweighed) obtained from submerged cultures.

The free N- acetyl glucosamine so released from the above treatment was assayed by the colorimetric method of Ressing et al. (1955) using N- acetyl D- glucosamine as the standard (section 3.1.13). To 0.5ml of the supernatant, 0.1ml of Potassium Tetraborate solution (0.8M; section 3.1.12.) was added. The tubes were heated in a boiling water bath for 3 min and were cooled under tap water. Thereafter, 3.0 ml of p-
dimethyl aminobenzaldehyde reagent was added and immediately after mixing, the tubes were placed in water bath at 37°C for 20 min. The tubes were then cooled under tap water and absorbance was read at 544 nm. The amount of N-acetyl glucosamine present was calculated from the standard curve of N-acetyl glucosamine as shown in Appendix 4.

Calculations:

**SmF:**

Let the weight of the pellet = Xg

From the O.D. of test compared to Standard curve, let the N-acetyl glucosamine content

= A µg (in 0.5 ml of sample)

= A x 2 x volume of sample / 1000 mg of N-acetyl glucosamine (in total ml of sample)

= B mg

So, X g of pellet releases = B mg of N-acetyl glucosamine

Therefore, 1 g of pellet will release

= B/X mg of N-acetyl glucosamine/g of cells

= C mg of N-acetyl glucosamine/g of cells

When the flask after 48h of incubation was withdrawn it was also assayed for total α-amylase yield.

Let the total α-amylase yield obtained

= Y units of α-amylase

Also, X g of cells releases = Y units of α-amylase

So, 1 g of cell will release = Y/X units of α-amylase

= Z units of α-amylase

Therefore, 1g of cells in case of SmF releases Z units of α-amylase and C mg of N-acetyl glucosamine.

**SSF:**

From the O.D. of test compared to Standard curve, let the N-acetyl glucosamine content

= D µg (in 0.5 ml of sample)

= D x 2 x vol of sample / 1000 mg of N-acetyl glucosamine (in total ml of sample)

= E mg of N-acetyl glucosamine
From the O.D. of the uninoculated wheat bran compared to Standard curve, let the N-acetyl glucosamine content

\[ = F \mu g \text{ (in 0.5 ml of sample)} \]
\[ = F \times 2 \times \text{vol of sample} / 1000 \text{mg of N-acetyl glucosamine (in total ml of sample)} \]
\[ = G \text{ mg} \]

So, the total amount of N-acetyl glucosamine liberated by complete volume of sample

\[ = E - G \text{ mg} \]
\[ = H \text{ mg of N-acetyl glucosamine} \]

Let the weight of the sample taken

\[ = T \text{ g} \]

So, \( T \text{ g of sample releases} \)

\[ = H \text{ mg of N-acetyl glucosamine} \]

Therefore, \( 1 \text{ g of sample will release} \)

\[ = H / T \text{ mg of N-acetyl glucosamine/g of sample} \]
\[ = I \text{ mg of N-acetyl glucosamine/g of sample} \]

We know that \( C \text{ mg of N-acetyl glucosamine is released by 1 g of cells} \)
Therefore, weight of the cells in \( T \text{ g of sample} \)

\[ = I / C \]
\[ = J \text{ g of cells} \]

Therefore, in \( T \text{ g of sample there are} \)

\[ = J \text{ g of cells} \]

When the flask after 48h of incubation was withdrawn it was also assayed for total \( \alpha \)-amylase yield.
Let the total \( \alpha \)-amylase yield obtained

\[ = S \text{ units.} \]

So, \( J \text{ g of cells gives} \)

\[ = S \text{ units} \]

Therefore, \( 1 \text{ g of cell will give} \)

\[ = S / J \text{ units.} \]
\[ = K \text{ units} \]

Also, \( T \text{ g of sample will release} \)

\[ = H \text{ mg of N-acetyl glucosamine} \]

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Therefore, J g of cell will release $H$ mg of N-acetyl glucosamine.

So, 1 g of cells will release $H/J$ mg of N-acetyl glucosamine.

Therefore, 1g of cells in case of SSF releases $K$ units of $\alpha$-amylase and $L$ mg of N-acetyl glucosamine.

Comparison of the productivity of enzyme by 1g of cell in SSF and SmF

\[
\text{Units of } \alpha\text{-amylase released/g of cells by SSF} = \frac{\text{Units of } \alpha\text{-amylase released/g of cells by SmF}}{\text{Units of } \alpha\text{-amylase released/g of cells by SSF}}
\]

3.2.10. Optimization of $\alpha$-amylase production by *Bacillus subtilis* subsp. *spizizenii* employing solid-state fermentation (SSF) technique by using Response surface Methodology (RSM)

After optimizing the values of variables by ‘one variable at one time method’ five most important variables namely, glycerol as carbon source, soyabean meal as nitrogen source, sodium chloride as the metal ion source, B- complex as vitamin source and threonine as amino acid source were selected and response surface methodology (RSM) was applied for studying combined interactions among various physiological parameters which were not possible in conventional ‘one – at – a time approach’. Levels of these factors were optimized for maximum amylase production using one of the RSM, the CCD (Central Composite experimental Design). A $2^5$ factorial central composite experimental design with nine start points and seven replicates at the central point, resulting in 33 experiments [generated by Design Expert, Version 6.0, Stat-Ease Inc., Minneapolis, MN] statistical software and the response surface generated using STATISTICA (StatSoft Inc., Tulsa, USA) was used to optimize the screened variables grouped as glycerol ($X_1$), soyabean meal ($X_2$), NaCl ($X_3$), Threonine ($X_4$) and B-complex ($X_5$). All the variables were taken at 5 different coded levels: -2, -1, 0, +1 and +2.

Upon completion of the experiments, the average maximum $\alpha$-amylase yield was taken as dependent variable or response ($Y$). A second order polynomial equation was then fitted to the data by the multiple regression procedure.
\[ Y = b_0 + \sum_{i} b_i x_i + \sum_{ij} b_{ij} x_i x_j + \sum_{i} b_{ii} x_i^2 + e. \]  

where \( Y \) = predicted response, \( b_0 \) = offset term, \( b_i \) = linear effect, \( b_{ij} \) = squared effect, \( b_{ij} \) = interaction effect.

This resulted in an empirical model that related the response measured to the independent variables of the experiment. For a five factor system, the model equation is:

\[ Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_4 X_4 + b_5 X_5 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{14} X_1 X_4 + b_{15} X_1 X_5 + b_{23} X_2 X_3 + b_{24} X_2 X_4 + b_{25} X_2 X_5 + b_{34} X_3 X_4 + b_{35} X_3 X_5 + b_{45} X_4 X_5 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{44} X_4^2 + b_{55} X_5^2 + \]

With \( Y \), predicted response; \( b_0 \), intercept; \( b_1 \), \( b_2 \), \( b_3 \), \( b_4 \), \( b_5 \) linear coefficients; \( b_{12}, b_{13}, b_{14}, b_{15}, b_{23}, b_{24}, b_{25}, b_{34}, b_{35}, b_{45} \) interaction coefficient; \( b_{11}, b_{22}, b_{33}, b_{44}, b_{55} \) squared coefficients.

The three dimensional response surface were then plotted to find out the optimum concentration of each factor for maximum \( \alpha \)-amylase production. The response surface plots were plotted for the variation in \( \alpha \)-amylase yield as a function of concentration of two variables when all the other factors were kept at their central levels. The optimum concentration of each nutrient was identified based on the hump in the three dimensional plot of the corresponding plot.

For all experimental designs fermentation was carried out in 250ml Erlenmeyer flasks having 5g of wheat bran as solid substrate, moistened with 7.5 ml of moistening agent (5ml tap water and 2.5 ml inoculum).

The experimental design is shown in Table 3.1. and the coded variables are reflected in Table 3.2.

**Table: 3.1. Central composite design consisting of 33 experiments for the study of five experimental factors in coded units**

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<thead>
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<th>( X_1 )</th>
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134
Table: 3.2. Boundaries of experimental domain and spacing of levels expressed in coded natural units

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<tr>
<th>Code Unit</th>
<th>Glycerol (%) (X₁)</th>
<th>Soyabean meal (%) (X₂)</th>
<th>Sodium chloride (mM) (X₃)</th>
<th>Threonine (%) (X₄)</th>
<th>B-Complex (%) (X₅)</th>
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3.2.11. Laboratory scale up of α-amylase production

Scale up studies were carried out both by solid state fermentation and by submerged fermentation for α-amylase production.

3.2.12. Scale up of enzyme production by solid state fermentation

Scale up studies were carried out with basal media as wheat bran for α-amylase production. These studies were conducted under different parameters as follows:

3.2.12.1. Varying the amount of substrate in 250ml Erlenmeyer flask

This trial was carried out to determine the maximum amount of wheat bran that can be used in 250ml Erlenmeyer flask SSF without significant decrease in the enzyme yields. Optimized medium (section 3.2.7.3.1.) containing 5.0, 10.0, 20.0 and 40.0g of wheat bran was taken and the corresponding level of moisture (1:1.0 of substrate: tap water) and inoculum (1:0.5 of substrate: inoculum) for the bacterial growth was incorporated to obtain a final ratio of 1:1.5 of substrate: total moisture.
3.2.12.2. Varying the volume of Erlenmeyer flask

Scale up studies were attempted in the optimized medium by increasing the size of Erlenmeyer flasks (250ml, 500ml and 1000ml) and by keeping the ratio of wheat bran with respect to flask to 1:50 and 1:25. For each ratio, the required wheat bran and the flask size is given below:

<table>
<thead>
<tr>
<th>Flask size</th>
<th>Amount of solid substrate used (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250ml</td>
<td>5</td>
</tr>
<tr>
<td>500ml</td>
<td>10</td>
</tr>
<tr>
<td>1000ml</td>
<td>20</td>
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<table>
<thead>
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<th>Flask size</th>
<th>Amount of solid substrate used (g)</th>
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</thead>
<tbody>
<tr>
<td>1:25</td>
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<tr>
<td>250ml</td>
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<tr>
<td>500ml</td>
<td>20</td>
</tr>
<tr>
<td>1000ml</td>
<td>40</td>
</tr>
</tbody>
</table>

The amount of water and inoculum was adjusted accordingly to maintain the ratio of solid: total moisture as 1:1.5.

3.2.12.3. Scale up in enamel coated metallic trays

The scale up study at flask level was followed by scale up in enamel coated metallic trays of dimension 38.5cmx30cmx4.2cm. Different quantities of wheat bran (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500g) were taken in enamel coated metallic trays and were supplemented with optimized medium (section 3.2.7.3.1.) with the bed depth ranging from 1-3 cm to study the pattern of production of α-amylase. These were moistened with tap water in such a way that the total substrate: moisture level was maintained at 1:1.5 after addition of inoculum. These were covered with aluminum foil, autoclaved at 121°C for 30 minutes, cooled and inoculated with seed culture and mixed thoroughly with a sterilized spatula. The first set of tray (in duplicate) was kept at the bottom of the fermentor followed by a set of multiple trays lying over one another in which the bottom tray was covered with a wire mesh fitted with one inch high tray sized
wooden frame having two inch high wooden supports on the four corners for supporting the enamel coated metallic tray over it. The upper tray was again covered with a mesh fitted with a wooden frame having two-inch high supports on the corners for placing another tray over it and in this way a total of ten trays were placed in a stack. The trays were incubated at 37°C for 96h. The samples were withdrawn after regular intervals of time of 24h from each tray to study the pattern of α-amylase production.

Since the enzyme yield obtained with 200g was quite appreciable as compared to higher quantities of wheat bran, so a batch of 2 kg was put in 10 enamel coated trays each containing 200g of wheat bran as a substrate and supplemented with optimized medium. These were moistened with tap water in such a way that the total substrate: moisture level was maintained at 1:1.5 after addition of inoculum. These were covered with aluminum foil, autoclaved, cooled and inoculated with seed culture and mixed thoroughly with a sterilized spatula. The trays were incubated similarly, in vertical stacks for 48h. Owing to heat liberation problem with big size batch, incubation was done at 32°C rather than 37°C. The samples were withdrawn after 48h and pH profile was noted down from each tray. To note the pH of the fermented bran, 5g of the fermented bran was extracted by 50ml of tap water. The content of the flask was filtered and the filtrate was centrifuged at 10,000 rpm for 10 min. The supernatant thus obtained was checked for pH. Also, while sampling the temperature of the tray was taken at its center.

3.2.12.4. Scale up in sieve trays

Initially the pattern of production of α-amylase with different quantities of wheat bran taken in sieve trays was studied.

The enamel tray studies were followed by the use of specially designed wooden trays (30cm x 45cm x 5cm), having sides made up of 1cm thick wooden frame and base fitted with the wire mesh, containing different quantities of the wheat bran (400 - 1000g) as the substrate in the optimized production media having tap water as the moistening agent, with a bed depth ranging from 2-4cm. These were covered with aluminum foil, autoclaved, cooled and inoculated with seed culture and mixed thoroughly with a sterilized spatula. The first set of tray (in duplicate) was kept at the bottom of the fermentor followed by a set of multiple trays lying over one another in which the base of each tray was fitted with three-inch high wooden strips on the four corners for keeping a
gap between the perforated tray base and the bottom and in this way a total of four trays (in duplicate) were placed in a stack. The trays were incubated at 37°C for 96h. The samples were withdrawn after regular intervals of time of 24h from each tray to study the pattern of α-amylase production.

Since the enzyme yield obtained with 750g was quite appreciable as compared to higher quantities of wheat bran, a batch of 18 kg was put in 24 sieve trays in two stacks (12 trays in each stack) each containing 750g of wheat bran as a substrate and supplemented with optimized medium. These were moistened with tap water in such a way that the total substrate: moisture level was maintained at 1:1.5 after addition of inoculum. These were covered with aluminum foil, autoclaved at 121°C for 30 min, cooled and inoculated with seed culture and mixed thoroughly with a sterilized spatula. To prevent the contamination by mold growth, greisofulvin (0.01% v/w) was added to the trays. So, a production system was worked out with several stacks lying adjacent to each other with a gap of 10 cm and each having a total of 24 trays (each containing 750g solid wheat bran moistened with 750ml of tap water and 375 ml inoculum) placed over one another. The stacks were incubated in BOD incubator, at 30°C for 48h. The samples were withdrawn after 48h and pH profile was noted down from each tray. Also, while sampling the temperature of the tray was taken at its center.

3.2.13. Scale up studies by submerged fermentation technique

Scale up studies was also carried out by submerged fermentation technique. These studies were conducted at different levels as follows:

3.2.13.1. Varying the volume of Erlenmeyer flask

Scale up studies were attempted in the optimized medium by increasing the size of Erlenmeyer flasks (100, 250, 500 and 1000ml) and by keeping the ratio of production volume with respect to flask to 1:5, 1:4 and 3:10. For each ratio, the required optimized production medium and the flask size is given below:

The flasks were incubated in New Brunswick Environmental shaker (150 rpm) for 48h at 50°C. Flasks were withdrawn after 48h of incubation. Every time, the contents of each flask were centrifuged (10,000 rpm, 20 min, 4°C) and the cell free supernatant was used for determining the extracellular α-amylase activity.
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<thead>
<tr>
<th>Flask size</th>
<th>Amount of optimized production medium (ml)</th>
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</thead>
<tbody>
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<tr>
<td>500ml</td>
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<tr>
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<td>300</td>
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3.2.13.2. α-Amylase production in laboratory fermentor

α-Amylase study was scaled up from shake flask to fermentor level. α-Amylase production by *Bacillus subtilis* subsp. *spizizenii* was studied in 7.5L fermentor (Chemap AG, Switzerland). 4.5L of the optimized medium (section 3.2.8.2.), was used as the production medium with pH 7.0 and 0.5L of the seed medium (section 3.1.4.2.), was inoculated aseptically into the production medium. Foam formation was controlled with polypropylene glycol (PPG). Incubation was carried out at 50°C at 150 rpm. Sterilized
air was supplied at 0.6vvm. Samples were withdrawn after every 12h till 72h of incubation and were checked for pH and α-amylase production. Dissolved oxygen in the medium was monitored throughout the experiment.

3.2.14. Localization of α-amylase production

This study was done to locate α-amylase in *Bacillus subtilis* subsp. *spizizenii* whether it was produced extracellularly, intracellularly or was cell associated under both types of fermentation conditions including solid state fermentation and submerged fermentation conditions.

3.2.15. Localization of α-amylase production in solid state cultures

Different sets of 250 ml Erlenmeyer flasks, each containing 5g of sterilized wheat bran moistened with sterilized tap water, were inoculated with 2.5 ml of the inoculum ($A_{600}$, 0.8) obtained from 12 h old shake culture of *Bacillus subtilis* subsp. *spizizenii* to obtain the final ratio of 1:1.5 (wheat bran: moisture content). The flasks were incubated at 37°C under stationary conditions. After 48h of growth, the contents of each flask were extracted and the filtrate was centrifuged at 10,000g for 20 min. The filtrate obtained was used to estimate the extracellular activity.

**Intracellular α-amylase**

The cell biomass obtained from above flasks was suspended in 0.1M Phosphate buffer, pH 6.5. The contents were mixed gently, centrifuged and the residue obtained was again given washing in a similar way. The process was repeated five times. The washed cell biomass thus obtained was suspended in the same buffer and was lysed by sonication in a sonicator (Labsonic 2000, B.Braun). The cell debris was removed by centrifugation at 12,000 rpm for 30 min and the supernatant was checked for the α-amylase activity.

**Cell associated α-amylase**

The cell debris obtained from above flasks was suspended in a minimum amount 0.1M Phosphate buffer, pH 6.5. and was checked for α-amylase activity.
Localization of \( \alpha \)-amylase production in liquid cultures

Different sets of 100 ml Erlenmeyer flasks, each containing 20ml of production medium, were inoculated with 2ml of the inoculum \((A_{600}, 0.8)\) obtained from 12 h old shake culture of *Bacillus subtilis* subsp. *spizizenii* in seed medium. The flasks were incubated in New Brunswick Environmental shaker (150 rpm) at 50°C. After 48h of incubation, the contents of each flask were extracted and the cell free supernatant was used to estimate the extracellular \( \alpha \)-amylase. 

**Intracellular \( \alpha \)-amylase**

The cell biomass obtained from above flasks was suspended in 0.1M Phosphate buffer, pH 7.0. The contents were mixed gently, centrifuged and the residue obtained was again given washing in a similar way. The process was repeated five times. The washed cell biomass thus obtained was suspended in the same buffer and was lysed by sonication in a sonicator (Labsonic 2000, B.Braun). The cell debris was removed by centrifugation at 12,000 rpm for 30 min and the supernatant was checked for the \( \alpha \)-amylase activity. 

**Cell associated \( \alpha \)-amylase**

The cell debris obtained from above flasks was suspended in a minimum amount 0.1M Phosphate buffer, pH 7.0. and was checked for \( \alpha \)-amylase activity.

Expression of \( \alpha \)-amylase during development of the bacterial strain

The pattern of \( \alpha \)-amylase production in the culture filtrates during the development of bacterial strain in both solid state cultures and liquid cultures was studied, by the analysis of the changes taking place in electrophoretic pattern during the growth of the bacterial strain (Sandhu and Puri, 1989; Soni et al., 1999).

Determination of the electrophoretic pattern during the growth of the bacterium in both solid and liquid cultures by native-polyacrylamide gel electrophoresis

The organism was grown as both solid state cultures and liquid cultures for 84h under stationary and shaking conditions respectively. Flasks, in duplicate, were withdrawn at regular intervals of time of 12h from both the conditions of growth and...
were analysed electrophoretically for the production of α-amylase. To study the production of α-amylase, initially the banding pattern of the α-amylase was seen electrophoretically by native - PAGE. 10% Native-PAGE (composition of resolving and stacking gel given below) was run following the procedure as laid down by Laemmli, (1970) to locate the presence of the α-amylase band in the crude extracellular filtrate of both the cultures. A zymogram was also run along to confirm the exact position of the α-amylase band from the crude culture filtrates. Stacking gel was run at 50V and resolving gel was run at 100V (3mA per well was applied from a DC power supply till the dye front reached just above the lower end).

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<th>Resolving gel (for 10% gel)</th>
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<tr>
<td>1.5M Tris-HCl, pH 8.8</td>
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</tr>
<tr>
<td>Double distilled water</td>
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<td>TEMED</td>
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<td>APS</td>
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<table>
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<th>Volume per gel mold (ml)</th>
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<tr>
<td>Double distilled water</td>
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<tr>
<td>TEMED</td>
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<tr>
<td>APS</td>
<td>0.05</td>
</tr>
</tbody>
</table>
3.2.18.1. Preparation of gel

Two thin glass plates were used for preparing the gels. The plates were held vertically in a stand separated by 0.75 mm spaces on both the sides. The plates were sealed from the bottom with 1 ml of plug solution. The resolving gel solution was added carefully from the open end. The gel was layered with double distilled water from the top to prevent the oxidation of the gel. Plates were left as such for 30 min at room temperature for the polymerization of the gel. After solidification of the resolving gel, the layer of double distilled water was removed from the top. The solution for stacking gel was poured and a comb was inserted for the formation of wells to load the samples. Similarly, on the top, the gel was layered with double distilled water. Gel was allowed to solidify at room temperature, and then the comb was removed. The layer of double distilled water was removed just before loading the samples to the well. The gel was transferred to electrophoresis system after cleaning of the wells with distilled water. The gel plates were immersed in a tank containing running buffer (section 3.1.14).

3.2.18.2. Sample preparation

To 60 μl of enzyme sample, 20 μl of solubilizing buffer (section 3.1.14.) was added. This was used to load the gel.

3.2.18.3. Electrophoresis

The samples from both the cultural conditions were applied separately, in the wells and a constant current of 3 mA per well for the gel was applied from a D.C. power supply till the dye front reached just above the lower end.

3.2.18.4. Staining the gel

After the gel was completely run it was carefully removed from the glass plates and transferred to a flat glass dish for silver staining method devised by Damerval et al. (1987), as follows:

- The proteins in the gel were fixed by incubating the gel for 4-12h at room temperature with gentle shaking in at least 5 gel volumes of fixing solution (ethanol: glacial acetic acid: distilled water, 30:10:60)
• Discarded the fixing solution, and added at least 5 gel volumes of 30% ethanol (15 ml ethanol, 35 ml distilled water). Incubated the gel for 1h at room temperature with gentle shaking.

• Discarded the ethanol and added 10 gel volumes of distilled water. Incubated the gel for 10 min at room temperature.

• Added 50 ml of freshly prepared solution of silver nitrate (0.1%w/v). Incubated the gel for 30 min at room temperature with gentle shaking.

• Discarded the silver nitrate solution and washed both sides of gel (20 seconds each) under a stream of distilled water.

• Added 100 ml of fresh developing solution (section 3.1.15.). Incubated at room temperature with gentle shaking. Watched the gel carefully, as the stained bands of protein appeared within a few min. Continued incubation until the desired contrast was obtained.

• Quenched the reaction by washing the gel in 1% acetic acid solution for few minutes. Then washed the gel several times with distilled water.

Along with this a strip from the gel was cut used for zymogram analysis.

3.2.18.5. Zymogram analysis

Zymography was done to know the exact position of the band of the protein of interest (α-amylase) out of the various bands that appeared on the gel. A strip was cut from the gel in which sample was run and this was put into 50 ml of 0.5% starch solution and incubated in the water bath at 60°C for 10 min. After this the gel strip was put in distilled water and then into the iodine solution. A clearing in a small region of gel was seen against the dark blue background, indicating the position where the protein of our interest i.e. α-amylase has reacted with the starch substrate (so no blue color seen in that region).

3.2.18.6. Comparison of the stained gel with their zymograms

Once the gel was stained and the banding pattern was visible and clear, it was compared with the clearing region of zymogram so as to pin point the protein band of our interest. The clearing zone obtained in the zymogram corresponded to two bands in that region of the stained gel. To locate the exact band of the protein the interest out of the
two bands, the gel was overrun for 1h so that the distance between the two bands is increased and the clearing may appear against a single band only, which would confirm the position of the band of the protein of interest.

After the location of the exact position of the α-amylase, the crude culture filtrates (after 12, 24, 36, 48, 60, 72 and 84h of incubation) from both the cultural conditions were loaded separately, in seven wells of the casted gel to electophoretically study the production pattern of α- amylase.

3.2.19. Concentration and purification of a raw starch liquefying α- amylase from *Bacillus subtilis* subsp. *spizizenii*

For purification of a raw starch liquefying α-amylase, the enzyme was produced in bulk amount by solid state fermentation. The cell free supernatant was pooled and was concentrated by raising to 30% ammonium sulfate precipitation. The resultant precipitate was removed by centrifugation at 4°C. The supernatant obtained was further raised to 60% saturation and left overnight at 4°C. The precipitates formed were collected by centrifugation and dissolved in small volume of 10mM Phosphate buffer, pH 6.5 and was dialyzed against the same buffer. The dialyzed protein sample was further purified by gel filtration chromatography (Sephadex G-200) and ion exchange chromatography (DEAE Sepharose). The protein was purified completely by preparative gel electrophoresis. At all the purification steps, the α-amylase activity (section 3.2.2.2.) and the protein content (section 3.2.20.) was estimated and native PAGE (section 3.2.18.) was run to check the extent of purification of the desired protein. Various steps employed in purifying the α-amylase are discussed as follows:

3.2.19.1. Concentration and dialysis of crude enzyme

Crude preparation of enzyme was concentrated by ammonium sulfate precipitation technique. To 100ml crude sample of enzyme, 17.6g of ammonium sulfate was added slowly at 4°C to get a saturation of 30% and kept for 24h. The resultant precipitate was removed by centrifugation at 4°C. The supernatant obtained was further raised to 60% saturation and left overnight at 4°C. Precipitates formed were separated by centrifugation (10,000 rpm for 20 min) and dissolved in minimum amount of 0.1M Phosphate buffer, pH 6.5. This mixture contained concentrated enzyme and was dialyzed
to remove ammonium sulfate and other low molecular weight impurities from the sample. For dialysis, the tubing of convenient length was cut and was pretreated by boiling the membrane for ½ h in 1mM EDTA. It was then rinsed with deionized water and again boiled for 10 min with 1mM EDTA. The membrane was allowed to cool and was stored at 4°C. Before its use, the membrane was washed with deionized water. Dialysis was done with intermittent change of buffer after every 8h at 4°C and the process continued for 24h. The resulting sample was checked for α- amylase activity, protein content (section 3.2.20.) and banding pattern was seen by native PAGE.

### 3.2.19.2. Gel filtration chromatography

The concentrated and dialyzed sample was further purified by gel filtration chromatography, which was carried out by using Sephadex G-200 column (4cm X 60cm). Sephadex G-200 was swollen by keeping in boiling water bath for 3-4h. The slurry was prepared by decanting the deionized water and replacing it with Phosphate buffer (pH 6.5, 50mM). The equilibrated slurry was packed in the column and the above partially purified α-amylase preparation was loaded on the column previously equilibrated with the same buffer. The elution of the protein was done using buffer at a flow rate of 10ml/h. Fractions of 5ml each were collected. The U.V. absorbance of individual fraction was taken at 280nm. The fractions of a single peak were pooled and concentrated separately. These were then assayed for α-amylase activity, protein content (section 3.2.20.) and banding pattern was seen by native PAGE followed by silver staining.

### 3.2.19.3. Ion exchange chromatography

Ion exchange chromatography was carried out by using DEAE-Sephadex column (2.5cm X 20cm). DEAE-Sephadex was swollen by keeping in boiling water bath for 3-4h. The slurry was prepared by decanting the deionised water and was activated initially with 0.5N NaOH and then with 0.5M glacial acetic acid. This was further replaced by 50mM Phosphate buffer, pH 6.5. The pooled fraction of a single peak of highest specific activity obtained after gel filtration chromatography was loaded on DEAE-Sephadex column. The adsorbed α-amylase was eluted by a linear concentration gradient of sodium chloride from 0.1M to 0.9M in the same buffer. Fractions of 5ml each were collected at a
flow rate of 20ml/h. The fractions of a single peak were pooled separately and assayed for α-amylase activity, protein content (section 3.2.20.) and banding pattern was seen by native PAGE followed by silver staining.

3.2.19.4. Preparative gel electrophoresis

Further purification of the partially purified protein was done by preparative gel electrophoresis. The partially purified protein fraction was poured to cast the gel in native PAGE (section 3.2.18). A part of the runned gel was cut vertically and was used for zymographic studies to locate the position of the protein of interest. Zymography was done similarly (section 3.2.18.5). Both the runned gel and the zymogram were set side by side and the thin area on the runned gel coinciding with the clearing zone of the zymogram was excised from the gel carefully. Gel sections thus obtained were chopped into fine pieces and were crushed carefully. Protein of interest was eluted from this crushed mass of gel by incubating it in minimum amount of elution buffer (0.05 M of Tris HCl pH 7.9, EDTA 0.1mM, NaCl 0.2M and distilled water 100ml; section 3.1.17.) for overnight at 4°C for the elution of the proteins from the gel into the buffer. The eluent containing the purified protein was then extracted carefully from the chopped gel to avoid any contamination. The recovery of the purified protein was checked by determining the α-amylase activity, protein content (section 3.2.20.) and by native PAGE followed by silver staining.

A representative sample after each purification step was taken and these were then loaded in the single gel separately (so that the comparison of each step of purification could be done) along with a zymogram (of purified protein to confirm the presence of α-amylase protein in the sample) and control (without any sample).

3.2.20. Protein estimation

Protein contents of α-amylase samples after every step of purification was estimated by the method of Lowry et al. (1951).

To 2.5ml of working solution (98ml of Lowry A + 1ml of Lowry B +1ml of Lowry C; section 3.1.16.) was added 50μl of sample and 450μl of distilled water. It was mixed thoroughly and allowed to stand at 37°C for 10 min. Added 0.25 ml of 1 N Folin’s reagent (2N Folin Ciocalteau reagent diluted with distilled water to 1N; as
discussed in section 3.1.16.), mixed it rapidly, allowed it to stand at 37°C for 30 min and read absorbance at 660 nm. Protein content was calculated from the standard curve of bovine serum albumin (BSA) as discussed in section 3.1.19. ranging from 0-1 mg, made by using the stock solution of BSA (1mg/ml) as shown in Appendix 5.

3.2.21. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

10% SDS-PAGE was run to check the purity of the purified enzyme as described by Laemmli (1970). Resolving and stacking gels were run at 100V. To make the 10% gel, working solutions were made by mixing the different solutions in the following manner for both the gels:

<table>
<thead>
<tr>
<th>Resolving gel (for 10% gel)</th>
<th>Volume per gel mold (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide solution (30%)</td>
<td>1.7</td>
</tr>
<tr>
<td>1.5M Tris-HCl, pH 8.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>1.95</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002</td>
</tr>
<tr>
<td>APS</td>
<td>0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stacking gel (for 5% gel)</th>
<th>Volume per gel mold (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide solution (30%)</td>
<td>0.83</td>
</tr>
<tr>
<td>1.5M Tris-HCl, pH 6.8</td>
<td>0.63</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>3.4</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002</td>
</tr>
<tr>
<td>APS</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Preparation of gel and electrophoresis (section 3.2.18.) is same except that running gel electrode buffer has 10% SDS in addition (section 3.1.14).

3.2.21.1. Sample preparation

To 60 μl of enzyme sample, 20 μl of sample buffer (solubilizing buffer 1ml, mercaptoethanol 50 μl, SDS 0.001%; section 3.1.14.) was added. The samples were heated in boiling water bath for 3-5 min. After heating, it was centrifuged at 10,000 rpm for 1 min. This was used to load the gel.

3.2.21.2. Staining the gel

The gel was stained by silver staining method (section 3.2.18.4.).

3.2.22. Determination of relative molecular weight of the purified enzyme preparation from Bacillus sp. by SDS polyacrylamide gel electrophoresis

10% SDS-PAGE was run of the purified protein sample as described earlier with the standard molecular weight markers of 205, 97.4, 66, 43, 29, 20.1, 14.3 and 6.5 KDa for the estimation of relative molecular weight.

3.2.22.1. Staining of the gel

The protein separated by SDS-PAGE was stained with a Coomassie blue staining (Sambrook et al., 2001). The runned gel was immersed in the staining solution (methanol 40 ml, acetic acid 10ml, coomassie blue 0.05g and distilled water 50 ml; section 3.1.18.) for 12h at room temperature.

3.2.22.2. Destaining of the gel:

The stained gel was then destained by keeping in destaining solution (methanol 400ml, glacial acetic acid 100ml and distilled water 500ml; section 3.1.18.) for 4h (with intermittent changing of the destaining solution) at room temperature.

The gel was then soaked in distilled water for 1h for the clear visibility of the bands on the gel. The molecular weight of the purified protein on the gel was determined by comparing the Rf values (Relative Front (Rf ) = distance moved by the compound/distance moved by the solvant) of the purified protein with that of the known molecular weight of the standard protein markers.
3.2.23. Characterization of the purified and crude α-amylase preparation

The purified α-amylase preparation were characterized by studying the temperature and pH activity and stability profiles, effect of various metal ions, effector molecules and substrate concentration, its nature, affinity of it towards different raw starches and determining its catalytic domain. Also, the crude raw starch liquefying α-amylase both from solid state and submerged cultures of *Bacillus subtilis* subsp. *spizizenii* was also characterized alongwith, by studying the temperature and pH activity and stability profiles.

3.2.23.1. Temperature activity profile of purified and crude α-amylase

Optimum temperature for enzyme activity of the purified and crude enzyme was determined by assaying the α-amylase activity at different temperatures (40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 and 100°C) at pH 6.5 in 0.1M Phosphate buffer.

3.2.23.2. pH activity profile of purified and crude α-amylase

Optimum pH for α-amylase activity of the purified and crude enzyme was determined by assaying at 60°C using starch substrate solutions made in different buffer systems (pH ranging from 3.0-10.0).

<table>
<thead>
<tr>
<th>Buffers</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M Citrate phosphate buffer</td>
<td>3.0, 3.5, 4.0, 4.5, 5.0, 5.5</td>
</tr>
<tr>
<td>0.1 M Phosphate buffer</td>
<td>6.0, 6.5, 7.0, 7.5, 8.0</td>
</tr>
<tr>
<td>0.1 M glycine-NaoH buffer</td>
<td>8.5, 9.0, 9.5, 10.0</td>
</tr>
</tbody>
</table>

3.2.23.3. Thermostability profile of purified and crude α-amylase

This was studied by incubating the α-amylase preparation, both crude and purified, separately in 0.1M Phosphate buffer, pH 6.5 at temperatures ranging from 50-100°C with and without the addition of thermostabilizers including calcium chloride, sodium chloride, potassium chloride, trehalose and urea at final concentration of 10mM in the reaction mixture. The samples were withdrawn at an interval of 30 min for up to 6h.
and the residual activity was determined by the standard assay under normal conditions (60°C in 0.1M Phosphate buffer, pH 6.5) and expressed in terms of % of control.

3.2.23.4. pH stability profile of purified and crude α-amylase

The enzyme samples both crude and purified were incubated at 37°C in different buffer systems including 0.1M Citrate phosphate, 0.1M Phosphate and 0.1M Glycine-NaOH buffer with variable pH values including 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, 9.0, 9.5, 10.0. The samples were withdrawn after a regular interval of half hour till 6h of incubation and the residual activity was determined by assaying under normal conditions and expressed in terms of % of control.

3.2.23.5. Effect of metal ions on purified α-amylase

This was studied by supplementing various metal salts including aluminium sulfate, barium chloride, calcium chloride, cobalt chloride, copper sulphate, EDTA (ethylenediaminetetraacetic acid), iron chloride, iron nitrate, iron oxide, lead acetate, lithium sulphate, magnesium sulphate, mercuric chloride, potassium chloride, silver nitrate, sodium chloride, sodium molybdate, sodium tungstate and zinc sulphate (at final concentration of 1mM and 10mM each), separately in the reaction mixture of purified enzyme and determining the relative activities under normal assay conditions and expressed in terms of % of control.

3.2.23.6. Effect of various surfactants, oxidizing, reducing and chelating agents on purified α-amylase

The effect of various surfactants including Tween 20, Tween 40, Tween 60, Tween 80, Triton X-100 and SDS (sodium dodecyl sulphate) at 0.01% concentration, oxidizing agents including hydrogen peroxide and sodium azide, reducing agents including cysteine HCl and β-mercaptoethanol, and chelating agents including EDTA and sodium citrate all at final 1mM concentration, on the purified enzyme was studied by incorporating these, separately in the reaction mixture and determining the relative activities under normal assay conditions and expressed in terms of % of control.
3.2.23.7. Determination of $K_m$ and $V_{\text{max}}$ for purified $\alpha$-amylase

The effect of substrate concentration on $\alpha$-amylase activity was determined at different concentrations of soluble starch and amylopectin (from 0.1 to 1.0%) as substrates. The $K_m$ and $V_{\text{max}}$ values were determined from Lineweaver-Burk’s plot.

3.2.23.8. Determination of the nature of purified $\alpha$-amylase

This was studied by incorporation of Shiff’s reagent (0.5ml) with purified $\alpha$-amylase preparation (0.5ml) to study whether the enzyme was glycosylated or not.

The Schiff test named after Hugo Schiff is a chemical test for the detection of aldehydes. An enzyme sample was added to the Schiff reagent and when aldehyde is present a characteristic magenta or purple color develops. The Schiff reagent is the reaction product of fuchsine and sodium hydrogen sulfite.

From left to right: Fuchsine, Schiff reagent and fuchsine-aldehyde compound

3.2.23.9. Affinity of enzyme preparation towards different raw starches

To 0.2 g raw starch granules (potato, maize, wheat and rice), 1ml of the enzyme preparation from *Bacillus subtilis* subsp. *spizizenii* was added separately. This was incubated at 37°C for 15 min. After centrifugation the $\alpha$-amylase activity of the supernatant were assayed and compared with that of the original activity and the adsorption rate was calculated as follows:

**Calculations:**

Let the original $\alpha$-amylase activity $= A$ U/ml
Let the α-amylase activity in the supernatant after adsorption on raw starch = B U/ml

\[
\% \text{ Adsorption} = \frac{A-B}{A} \times 100
\]

3.2.23.10. Determination of the catalytic domain of the purified α-amylase

This was studied by the chemical modification of the enzyme by incorporating the purified enzyme with group specific reagents namely, namely 4-bromophenacyl bromide, NBM (n- bromosuccinimide), CMB (p-chloromercuribenzoate), EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide), DEPC (diethyl pyrocarbonate), DFP (diisopropylfluorophosphate), dithiothril, NEM (n-ethylmaleimide), iodoacetamide, β-mercaptoethanol, PCMS (p-chloromercuriphenylsulphonic acid), PMSF (phenyl methyl sulphonyl floriide), PP (pyridoxal phosphate), succinic anhydride and TPCK (tosylphenylalanylchloromethane), seperately at a final concentration of 1mM and 10mM, and determining the residual enzyme activity under standard assay conditions and expressed in terms of % of control.

3.2.24. Evaluation of the direct raw starch digesting α-amylase preparation from the selected strain in the potential biotechnological applications

3.2.24.1. Application of the crude α-amylase preparation from Bacillus subtilis subsp. spizizenii in the direct liquefaction of different raw starches and studies on the pattern of their degradation

The potential application of the direct raw starch liquefying α-amylase preparation was evaluated by studying the extent of hydrolysis of different raw starches including raw insoluble maize, potato, rice and wheat starch granules. The process of direct liquefaction was standardized by altering various parameters including enzyme dose, total solid concentration, incubation time and temperature.

3.2.24.1.1. Optimization of enzyme dose of α-amylase preparation for the direct liquefaction of different raw starches

Effect of the enzyme dose on the direct liquefaction of raw starches including maize, rice, potato and wheat was studied at different concentrations including 2.5, 5.0, 7.5 and 10U/mg raw starch (3125, 6250, 9375 and 12500 U of α-amylase respectively) in the reaction mixture containing 25ml of 5% (1250 mg) of raw starch granules, separately.
at 60°C. 10mM NaCl was added as thermostabilizer. The extent of direct liquefaction of raw starch granules without their gelatinisation was estimated by measuring the decrease in starch content with iodine method (section 3.2.3.3.) and the extent of saccharification was estimated by measuring an increase in glucose levels with DNSA method (section 3.2.3.3.) after 6h of incubation.

A minimum dose of α-amylase required (3125U) was chosen which could effectively degrade raw starch granules and was used in further studies.

3.2.24.1.2. Simultaneous optimization of total solid concentration, incubation time and temperature of the action of α-amylase preparation from Bacillus subtilis subsp. spizizenii for the efficient and direct liquefaction of different raw starches

These were studied simultaneously by varying the concentration of different raw starches including maize, rice, potato and wheat from 5-20% separately, at each temperature ranging from 50-90°C. Different sets of 100ml flasks, each containing 25 ml of 5, 10, 15 and 20% different raw starches separately, was incubated with 3125U enzyme preparation at each temperature ranging from 50-90°C. 10mM NaCl was added as thermostabilizer. This was initially incubated for 6h at each temperature. The extent of direct liquefaction of raw starch granules without their gelatinisation was estimated after an interval of 1h till 6h of incubation by measuring the decrease in starch content with iodine method and the extent of saccharification was estimated by measuring an increase in glucose levels with DNSA method. The crude α-amylase preparation could liquefy the raw starches almost completely at moderate temperatures but could not saccharify it to glucose completely. So, the liquefied starches were thereafter treated (after 6h of incubation at respective temperatures) with glucoamylase preparation (obtained from a laboratory isolate of Aspergillus sp.) for the complete process of hydrolysis to glucose and the extent of saccharification was estimated by measuring an increase in glucose levels with DNSA method after 24h of total incubation. Also, the extent of liquefaction was measured after 24h to observe whether there was any further increase in liquefaction of raw starch.

3.2.24.1.3. Scanning electron microscopy

To study the pattern of degradation of raw starches including maize, potato, rice and wheat by the action of α-amylase from Bacillus subtilis subsp. spizizenii, scanning
electron microscopy (SEM) of the untreated starch granules and α-amylase treated samples of raw starch granules was performed (Sarikaya et al., 2000). For the SEM of the treated granules, the hydrolyzed sample of the raw maize, potato, rice and wheat were withdrawn separately after regular intervals of time till 6h of incubation from the flasks incubated at their respective optimized temperature of hydrolysis. For the SEM of the untreated granules, the raw starch granules including maize, potato, rice and wheat were dispensed in distilled water. All the samples were treated as follows:

- The samples were centrifuged at 10,000 rpm for 5 min. The pellet obtained was washed twice with pure ethanol and again centrifuged at 10,000 rpm for 5 min.
- The samples were then washed twice with t-butyl alcohol (2-methyl-2-propanol) and again centrifuged (10,000 rpm for 5 min).
- Samples were then dried and were attached to a SEM stub with silver plate.
- The mounted samples were then spatter-coated with gold using fine coat, JEOL ion sputter, model JFC-100. The gold coated stubs were examined at different magnifications under scanning electron microscope; model JSM 6100 SM JEOL at 10 KV.

### 3.2.24.1.4. Detection of the end products of raw starch hydrolysis by paper chromatography

Samples of the reaction mixture drawn after 6 h (when treated only with α-amylase preparation from *Bacillus subtilis* subsp. *spizizenii*) and 24 h (when treated both with α-amylase preparation from *Bacillus subtilis* subsp. *spizizenii* and glucoamylase preparation from *Aspergillus* sp.) of incubation, from the hydrolysis of 20% of different raw starches at their respective optimized temperatures of hydrolysis were analyzed by paper chromatography to identify the end product of hydrolysis so as to confirm the extent of liquefaction and saccharification of different raw starches (Hayashida et al., 1988). Samples from hydrolysis mixture were taken and the paper chromatogram was developed in developing reagent (butanol:pyridine:water :: 6:4:3 by volume; as discussed in section 3.1.20.) using Whatman no.1 paper. Glucose and maltose were used as reference to analyze the products. The products were detected by spraying with a detection reagent (acetone 100 ml, phthalic acid 0.66g and aniline 93ml; section 3.1.20.).
3.2.24.2. Evaluation of α-amylase preparation in the direct liquefaction of different crude starchy biomass, followed by simultaneous saccharification and fermentation, for ethanol production

Various starchy substrates are currently being explored as potential alternatives of molasses for commercial ethanol production in India. In the present study different starchy substrates including maize, sorghum, rice and wheat flour, procured from local grain market of Chandigarh, for the ethanol production has been used and were evaluated for hydrolysis.

3.2.24.2.1. Determination of starch content of various starchy substrates

To estimate the starch content of the above mentioned grains, these were finely grinded. To 10g of each flour was added in distilled water and the final volume was made to 100ml in 500ml flasks. To these was added Termamyl™ (bacterial α-amylase; 0.015 ml; 2.5 lac units), it was then kept in water bath at 90°C for 30-45 min and later steam cooked at 5 lb/inch² in the autoclave for 45 min for the complete gelatinization of starches. Later the flasks were cooled to 90-95°C and again Termamyl™ (0. 015 ml; 2.5 lac units) was added and were incubated at same temperature for 45 min -1h for the complete liquefaction of starchy substrates. The flasks were further cooled to 55°C followed by the addition of AMG™ (amyloglucosidase, fungal glucoamylase; 0.06ml; 7000U) for the saccharification of liquefied mash and were kept at 55°C for 4h in water bath. Finally appropriate dilutions were made and the reducing sugars were assayed in terms of glucose by DNSA method (Miller, 1959). The starch was then calculated from the glucose by stoichiometrical calculation wherein, 1.11g glucose is obtainable from 1g of starch.

Calculations:
From the O.D. of test compared to Standard curve, let the glucose content

= A µg (in 0.1 ml of sample)
= A × 1000 µg (in 100 ml of sample)

From the O.D. of substrate control compared to Standard curve, let the glucose content

= B µg (in 0.1 ml of sample)
= B × 1000 µg (in 100 ml of sample)
From the O.D. of enzyme control compared to Standard curve, let the glucose content

\[ C \, \mu g \, (in \, 0.1 \, ml \, of \, sample) \]

\[ = C \times 1000 \, \mu g \, (in \, 100 \, ml \, sample) \]

Total amount of glucose formed from the starchy substrate (D)

\[ = A - (B+C) \, \mu g \]

Maximum amount of glucose that can be formed from 10g (10000mg) of starch (E)

\[ = 0000 \times 1.11 = 1111 \, mg \]

\[ = 1111 \times 1000 \, \mu g \]

Total g of starch content in 10g of sample (F)

\[ = D \times 10 \]

\[ E \]

% Starch content

\[ = F \times 100 \]

\[ 10 \]

3.2.24.2.2. Ethanol production from cereal grains by employing conventional method followed by fermentation to ethanol

The complete conventional process of producing alcohol from starchy biomass includes the enzymatic hydrolysis of starch to glucose followed by fermentation of liberated sugars to ethanol.

3.2.24.2.3. Conventional enzymatic hydrolysis of starchy substrates to reducing sugars

The conventional procedure employed for the hydrolysis of starchy biomass uses standard commercial amylases including Termamyl™ (bacterial α-amylases) and AMG™ (amylglucosidases, fungal glucoamylase), all marketed by Novo Industria/s, Denmark. All the four starchy substrates were hydrolysed to glucose using the standard enzymes with the following procedure:

Gelatinisation

The starchy grains including maize, rice, sorghum and wheat were finely grinded. To 100g of each flour, taken separately in 1L round bottom flasks with flat base, was
added tap water (temperature of water was 90°C) with constant stirring and 10mM CaCl₂·2H₂O (acted as thermostabilizer for standard enzyme used in the present study) was incorporated making the final volume to 500ml. This was followed by the addition of 0.015ml of Termamyl™ (2.5 lac units) and the temperature was maintained at 90 - 95°C in a water bath for 30 min. The mash was then cooked under pressure in autoclave at 105°C for 30 min.

**Liquefaction**

After gelatinisation, the contents of each flask were allowed to cool and 0.015ml (2.5 lac units) of Termamyl™ was added in each flask. The flasks were kept in water bath and the temperature inside the flasks was maintained between 90-95°C for 1h.

**Saccharification**

The liquefied mash obtained was cooled down to a temperature of 50-55°C and 0.06ml (7000U) of AMG™ was added in all the flasks for the saccharification process. The flasks were kept at the same temperature for 4h and the reducing sugars thus liberated were determined by DNSA method (Miller, 1959) as discussed in section 3.1.9. after regular interval of time.

**Fermentation**

The reducing sugars thus liberated were fermented to ethanol by the inoculation of 1g of distiller’s yeast *Saccharomyces cerevisae* having a viable yeast cell count of 1x10¹⁰ cells/g in all the flasks, separately. Fermentation of the mashes was done at 32°C in BOD incubator for total time period of 72h. The progress in fermentation was checked at regular intervals of 24h by observing the fall in specific gravity, change in pH and determining the alcohol content. A representative of the each sample was also kept at the same conditions of incubation without the inoculation of yeast strain from which reducing sugars liberated were determined by DNSA method (Miller, 1959) after a regular interval of 24h till 72h of incubation.

**3.2.24.2.4. Determination of alcohol content**

The alcohol content in the fermented mash was determined by using Sike’s hydrometer. The final volume of the mash was noted and 250ml of the sample was distilled to atleast half of the total volume, in a laboratory distillation assembly. The volume of the distillate was then made to 250ml with distilled water and the alcohol
content (°P) was calculated by putting a Sike’s hydrometer in the distillate taken in a 250ml glass cylinder and relating the reading corresponding to the hydrometer scale with the standard alcohol tables. The °P was converted to %v/v by dividing it with a factor of 1.75 (1%v/v of alcohol corresponds to 1.75 °P).

Calculations:
Conversion Efficiency (C.E.) = \frac{\text{reducing sugars produced in grain mash}}{\text{reducing sugars obtainable from starch in grain}} \times 100

Fermentation Efficiency (F.E.) = \frac{\text{alcohol produced in fermented mash}}{\text{alcohol obtainable from fermented sugars in mash}} \times 100

Alcohol obtainable from sugars, according to Gay – Lussac’s equation is 51.1%, which comes out to be 0.64/g glucose after employing the specific gravity factor.

Overall Efficiency (O.E.) = \frac{\text{Conversion efficiency} \times \text{Fermentation efficiency} \times 100}{100 \times 100}

3.2.24.3. Application of a-amylase preparation in the direct liquefaction of crude starchy biomass, followed by simultaneous saccharification and fermentation, for ethanol production

The potential application of the direct raw starch liquefying α-amylase preparation was evaluated by using it in the direct liquefaction of raw starchy substrates at moderate temperatures without involving the process of gelatinisation. The crude α-amylase preparation could liquefy the raw starches almost completely at moderate temperatures but could not saccharify it to glucose completely. So, the liquefied starches were thereafter treated with glucoamylase preparation from a laboratory isolate of Aspergillus sp. for the complete process of hydrolysis to glucose and thereafter it was inoculated with yeast Saccharomyces cerevisae for the process of fermentation to occur. The detailed procedure used for the production of ethanol from starchy biomass is discussed below.

Also, the process of direct liquefaction of various raw starchy biomass including maize, rice, sorghum and wheat was standardized by altering various parameters including incubation temperature, enzyme dose, incubation time, enzyme dose, metal
ions and its concentration and total solid concentration. A similar procedure was adopted throughout the standardization as discussed below.

**Incubation temperature**

This was studied by incubating the flasks containing different flours including maize, rice, sorghum and wheat flour at different temperatures in water baths including 50, 55, 60, 65, 70, 75, 80, 85 and 90°C, respectively.

The starchy grains including maize, rice, sorghum and wheat were finely grinded. Four sets, each set containing nine flasks were taken. To all the four sets was added 100g of grounded maize, rice, sorghum and wheat flour respectively in 1L round bottom flasks with flat base and were dispersed with constant stirring in tap water making the final volume to 500ml. The initial pH of the flasks was set to 6.5. This was followed by the addition of 2 lac units of crude α-amylase preparation from *Bacillus* sp. in each flask. Taking one flask from each set at a time, all flasks were incubated at different temperatures in water baths including 50, 55, 60, 65, 70, 75, 80, 85 and 90°C, respectively for 2h with intermittent shaking. This led to the direct liquefaction of raw starchy biomass without involving the process of gelatinsation at high temperatures. The liquefied mash obtained was cooled down to a temperature of 50-55°C and the pH was again adjusted to 5.5 (optimim pH for the glucoamylase to act). 7000U of glucoamylase was added in all the flasks for the saccharification process and the flasks were kept at the same temperature for 2h. Thereafter all the flasks were further cooled to 32°C and were inoculated with distiller’s yeast *Saccharomyces cerevisae* and incubated at 32°C for 72h. The progress in fermentation was checked at regular intervals of 24h by observing the fall in specific gravity and change in pH. The alcohol content was determined after 72h of incubation. A representative of the each sample was also kept at the same conditions of incubation without the inoculation of yeast strain from which reducing sugars liberated were determined by DNSA method (Miller, 1959) after 24, 48 and 72h of incubation.

A minimum temperature at which the raw starchy biomass was effectively degraded was chosen for all types of raw starches separately, and was used in subsequent studies for the respective raw starches.
Enzyme dose

The direct liquefaction with α-amylase preparation was evaluated by varying the dose of enzyme preparation including 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.25, 2.50, 2.75 and 3.00 lac units.

A minimum enzyme dose which was sufficient enough to effectively degrade raw starchy biomass was chosen for all types of raw starches separately, and was used in subsequent studies for the respective raw starches.

Incubation time

This was studied by incubating the flasks containing different flours including maize, rice, sorghum and wheat flour for different time periods including \( \frac{1}{2}, 1, 1\frac{1}{2}, 2, 2\frac{1}{2}, 3, 3\frac{1}{2} \) and 4h respectively.

A minimum incubation time which was sufficient enough to effectively degrade raw starchy biomass was chosen for all types of raw starches separately, and was used in subsequent studies for the respective raw starches.

Metal ion as thermostabilizer

This was studied by supplementing various metal salts including calcium chloride, potassium chloride and sodium chloride as thermostabilizers (at final concentration of 10mM each), separately in the reaction mixture of starch hydrolysis.

Since sodium chloride proved to be the best thermostabilizer, the effect of different concentrations (5, 10, 15mM; also 20mM concentration was taken for sorghum flour) of the same was also studied in the similar set of experiments and the optimum concentration giving the best thermostabilizing effect to the α-amylase preparation was chosen for all types of raw starches separately, and was used in subsequent studies for the respective raw starches.

Total solids

The direct liquefaction with α-amylase preparation was evaluated by varying the total solid concentration including 10 (40g), 15 (60g), 20 (80g), 25 (100g), 30 (120g), 35 (140g), 40 (160g), 45 (180g) and 50% (200g) of all the four types of flours including maize, wheat, rice and sorghum flour.
A maximum solid concentration which was effectively degraded by α-amylase preparation by *Bacillus* sp. was chosen for all types of raw starches separately, and was used in subsequent studies for the respective raw starches.

**Hydrolysis of crude starchy biomass by their direct liquefaction using α-amylase preparation from *Bacillus subtilis* subsp. *spizizenii* under optimized conditions of hydrolysis, followed by simultaneous saccharification by standard commercial glucoamylase AMG™ (amylglucosidase) and fermentation, for ethanol production**

Four flasks were taken, each for the hydrolysis of different grounded flours including maize, rice, sorghum and wheat respectively. To the flasks, was added the respective maximum optimized solid concentration of the respective flours in 1L round bottom flasks with flat base and were dispersed with constant stirring in tap water making the final volume to 500ml. The initial pH of the flasks was set to 6.5. This was followed by the addition of respective optimized units of crude α-amylase preparation from *Bacillus* sp. and sodium chloride in each flask for the direct liquefaction of respective flours. The different sets of flasks were incubated at their respective optimized temperature and time of liquefaction for the direct liquefaction of respective raw starches, separately. This led to the direct liquefaction of raw starchy biomass without involving the process of gelatinisation at high temperatures. The liquefied mash obtained was cooled down to a temperature of 50-55°C. 0.06ml of AMG™ was added in all the flasks for the saccharification process and the flasks were kept at the same temperature for 2h. Thereafter all the flasks were further cooled to 32°C and were inoculated with distiller’s yeast *Saccharomyces cerevisae* and incubated at 32°C for 72h. The progress in fermentation was checked at regular intervals of 24h by observing the fall in specific gravity and change in pH. The alcohol content was determined after 72h of incubation. A representative of the each sample was also kept at the same conditions of incubation without the inoculation of yeast strain from which reducing sugars liberated were determined by DNSA method (Miller, 1959) after 24, 48 and 72h of incubation.
3.2.24.4. Scale up of the process of ethanol production from the various starchy grains

Ethanol production from different starchy grains including maize, rice, sorghum and wheat was scaled up from 100g of grains as substrate to ½, 1, 1½, 2, 2½ and 3kg of grains.

For the hydrolysis of the different quantities of each starchy biomass including maize, rice, sorghum and wheat to reducing sugars and subsequently their fermentation to ethanol, similar procedure was adopted (section 3.2.24.3.). The different quantities of flours including ½, 1, 1½, 2, 2½ and 3kg were dispersed in big vessels separately, with constant stirring in 2.5, 5, 7.5, 10, 12.5 and 15 L of tap water respectively. The initial pH in all the reaction mixtures was set to 6.5. This was followed by the addition of respective optimized units of crude α-amylase preparation from Bacillus sp. and sodium chloride in each mixture for the direct liquefaction of respective flours. These were incubated at their respective optimized temperature and time of liquefaction for the direct liquefaction of respective raw starches, separately. This led to the direct liquefaction of raw starchy biomass without involving the process of gelatinisation at high temperatures. The liquefied mash obtained was cooled down to a temperature of 50-55°C. 7000U of glucoamylase prepared from Aspergillus sp./100g of flour was added in all the reaction mixtures for the saccharification process and was kept at the same temperature for 2h. Thereafter these were further cooled to 32°C and were inoculated with distiller’s yeast Saccharomyces cerevisae and incubated at 32°C for 72h. The progress in fermentation was checked at regular intervals of 24h by observing the fall in specific gravity and change in pH. The alcohol content was determined after 72h of incubation. A representative of each sample was also kept at the same conditions of incubation without the inoculation of yeast strain from which reducing sugars liberated were determined by DNSA method (Miller, 1959) after 24, 48 and 72h of incubation.

3.2.24.5. Desizing of textile fibers by α-amylase preparation from Bacillus subtilis subsp. spizizenii

The α-amylase preparation was evaluated for its ability to remove the size from the fabrics in the presence of various metal ions, chelating ion and surfactants. The process of desizing of the textile fiber by the α-amylase was compared with the
commercial α-amylase preparation procured from MAPS, India (usually employed in industries) by replacing the latter with the former. The process of desizing with the crude α-amylase preparation was standardized by altering various parameters including temperature, metal ions, surfactants, and enzyme dose.

To check the extent of desizing of the fabric, the cloth pieces were taken out and were treated with 2% iodine solution by spotting at the centre of the cloth and the color of the cloth was noted down which ranged from blue to yellow. A similar procedure was adopted throughout the standardization as discussed in the next section. The results were reported qualitatively based on the color which was noted down.

The symbols used for different colors obtained were:

<table>
<thead>
<tr>
<th>Color</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>-</td>
</tr>
<tr>
<td>Bluish brown</td>
<td>-</td>
</tr>
<tr>
<td>Brownish blue</td>
<td>+ -</td>
</tr>
<tr>
<td>Brown</td>
<td>+</td>
</tr>
<tr>
<td>Brownish yellow</td>
<td>+ +</td>
</tr>
<tr>
<td>Yellowish brown</td>
<td>+ + +</td>
</tr>
<tr>
<td>Yellow</td>
<td>+ + + +</td>
</tr>
</tbody>
</table>

**Effect of varying temperature**

The sized fabric was cut into six 5x5 cm² pieces and each of five pieces were put separately into five 100ml Erlenmeyer flask containing 25ml of distilled water and crude α-amylase preparation (1000U). The remaining one piece of sized fabric was put into 100ml of Erlenmeyer flask containing only 25ml of distilled water, which was taken as
control. The flasks were then incubated at different temperatures including 50, 60, 70, 80 and 90°C respectively, in a New Brunswick water bath shaker at 150 rpm. The flasks were repeatedly withdrawn after a time interval of 10 min till a total time of 60 min of incubation at the respective temperatures. Every time the flasks were withdrawn, the cloth pieces were taken out and were treated with 2% iodine solution by spotting at the centre of the cloth and the color of the cloth was noted down which ranged from blue to yellow. A control (as discussed before in this section) was also run along at every temperature with similar set of conditions except the addition of crude $\alpha$-amylase preparation in it.

The least temperature (60°C) which effectively desized the cloth was chosen and was used in the subsequent studies.

Effect of different metal ions and chelating agents at a final concentration of 10mM

This was studied by supplementing metal ions including calcium chloride, potassium chloride, sodium chloride and EDTA (chelating ion) at a final concentration of 10mM, separately in the reaction mixture.

The metal ion (sodium chloride) which effectively desized the cloth in a minimum time was chosen and was used in the subsequent studies.

Effect of different surfactants

This was studied by supplementing different surfactants (0.01% v/w) including Tween 20, Tween 40, Tween 60, Tween 80, Triton X-100 and commercially used surfactant (X) used in desizing industries, separately in the reaction mixture.

The surfactant (commercially used surfactant, X) which effectively desized the cloth in a minimum time was chosen and was used in the subsequent studies.

Effect of different enzyme dose

This was studied by adding different enzyme dose including 1000, 1500, 2000, 2500, 3000, 3500 and 4000U, separately in the reaction mixture.

The minimum enzyme dose (1500U) which effectively desized the cloth in a minimum time of 10 min was chosen.
Comparison of the desizing of sized fabric by using α-amylase preparation from *Bacillus subtilis* subsp. *spizizenii* under optimized conditions of desizing and by commercial α-amylase preparation (Palkozyme) obtained from MAPS, India

The sized fabric was cut into two 5x5 cm² pieces and each piece was put separately into each of two 100ml Erlenmeyer flask, containing 25ml of distilled water and 0.01%(v/w) of commercially used surfactant. To the first flask was added 1500U of α-amylase preparation from *Bacillus subtilis* subsp. *spizizenii* and 10mM of sodium chloride and to the other was added commercial α-amylase preparation (0.1% w/w of cloth, 1500U) and 10mM calcium chloride. The flasks were then incubated at 60°C (optimum for desizing by α-amylase preparation from *Bacillus subtilis* subsp. *spizizenii*) and 70°C (optimum for desizing by commercially available α-amylase), respectively in a New Brunswick water bath shaker at 150 rpm. The flasks were repeatedly withdrawn after a time interval of 10 min till a total time of 60 min of incubation. Every time the flasks were withdrawn, the cloth pieces were taken out and were treated with 2% iodine solution by spotting at the centre of the cloth and the color of the cloth was noted down which ranged from blue to yellow.