Chapter 3

SCREENING AND SELECTION OF STRAIN FOR ALKALINE PROTEASE PRODUCTION BY SUBMERGED FERMENTATION
3.1 MATERIAL AND METHODS

3.1.1 Isolation of bacterial strains for alkaline protease production

Proteolytic bacteria were isolated from alkaline soil samples collected from black cotton soil, groundnut field, milk processing unit, Kotappakonda hill area in Guntur Dist, and Tirumala in Chittur District, Andhrapradesh, India.

These soil samples were suspended in water by vigorous vortexing, serial dilutions were made up to $10^{-9}$ in sterile water. 0.1 ml of appropriate dilution was added to petri plate on skim milk agar.

**Composition of the medium:**

- Peptone : 0.1% w/v
- NaCl : 0.5% w/v
- Agar : 2.0% w/v
- Skim milk : 10% v/v
- pH : 9.5

The plates were incubated at 40°C for 24h. A clear zone of skim milk hydrolysis around the colonies indicated alkaline protease production by the organism. These colonies were picked and purified by streaking on skim milk agar. The purified proteolytic isolates were stored and maintained in nutrient agar slants (pH 9.5) by subculturing at monthly intervals. More than 20 isolates were thus collected and stored.

3.1.2 Screening of the isolates for the alkaline Protease production

The proteolytic bacterial strains were screened for the yield of alkaline protease by submerged fermentation.
Medium for submerged fermentation (Gyp Medium) (Kumar and Bhatla, 2004).

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (G/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5</td>
</tr>
<tr>
<td>Peptone</td>
<td>5</td>
</tr>
<tr>
<td>MgSO₄ 7.H2O</td>
<td>0.2</td>
</tr>
<tr>
<td>K₂H PO₄</td>
<td>1</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>9.5</td>
</tr>
</tbody>
</table>

pH of the medium was adjusted with 1N NaOH or 1N HCl.

3.1.3 Inoculum preparation

Five ml of sterile water was added to 24h old nutrient agar slants (pH 9.5). The cells were scrapped from the slant into sterile water and resultant cell suspension was transferred at 10% level aseptically into 250 ml Erlenmeyer flasks containing 45 ml of sterile inoculum medium. The composition of inoculum medium is peptone 5g, beef extract 3g, NaCl 3g and distilled water 1000ml. These flasks were kept on a rotary shaker (70 rpm) at 40°C for 24h. The contents of the flasks were centrifuged at 4000 rpm for 10 min and the supernatant was decanted. The cell pellets were washed thoroughly with sterile saline followed by sterile distilled water. Finally the cell mass was suspended in sterile saline and used as inoculum for subsequent experiments.

3.1.4 Shake flask fermentation

Five ml of cell suspension (equivalent to 0.03 g dry cell weight) was inoculated in 45 ml basal production medium, i.e Gyp medium. contained in 250ml Erlenmeyer flasks and incubated at 40°C on incubator shaker for 48 h (70 rpm). After incubation the culture broth was centrifuged at 10,000 rpm for 20min. The supernatant was used as the crude enzyme for the assay of alkaline protease activity. The activity was expressed in Uml⁻¹.
3.1.5 Enzyme assay

According to Udandi Boominadhan et al., (2009), the enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M Carbonate – Bicarbonate buffer pH 9.5 and 1ml enzyme solution in a total volume of 3.0ml. Reaction mixture was incubated for 5 min at 40ºC. The reaction was terminated by adding 3ml of 10% ice-cold trichlorocetic acid.

The tubes were incubated for one hour at room temperature. Precipitate was filtered thorough whatman no.1 filter paper and the filtrate was collected. For the color development for the assay of tyrosine in the filtrate, 5ml of 0.4 M Sodium carbonate and 0.5 ml of Folin phenol reagent were added to 1ml of filtrate, vortexed immediately and incubated for 20 min at room temperature O.D was taken at 660 nm. Concentration of tyrosine in the filtrate was read from a standard curve for tyrosine already prepared.

One unit enzyme activity was taken as the amount of enzyme producing 1µg of tyrosine under standard assay conditions and expressed as units ml⁻¹ enzyme.

3.1.6 Identification of the high yielding strain

Various Cultural, Morphological and Biochemical properties of the high yielding strain were studied in the Institute of Microbial Technology, Chandigarh, India.

3.1.7 Monitoring the stability of the highest yielding strain

The ability of the highest yielding strain was assessed by sub culturing and testing the yield at monthly intervals. The yield was determined using GYP medium as mentioned earlier and the strain was tested after 5th, 6th, 7th and 8th subcultures.
3.2 RESULTS AND DISCUSSION

3.2.1 Isolation of bacterial cultures

21 bacterial cultures, capable of producing alkaline protease were isolated from, the previously described areas following the method detailed under Material and Methods. The list of isolates is presented in Table-I.

3.2.2 Screening of the isolates for alkaline protease production

The 21 bacterial isolates were screened for the production of alkaline proteases following the method detailed under Materials and Methods. The results are presented in Table-II.

The 21 bacterial cultures produced alkaline proteases at varying levels from 44U ml\(^{-1}\) to 110 U ml\(^{-1}\). Among the cultures tested, the culture number MS6 obtained from alkaline soil of the milk processing unit gave the maximum yield of 110 U ml\(^{-1}\). The MS6 culture was selected for further studies.

3.2.3 Identification of the highest yielding strain

The bacterial strain selected among 21 isolates for high protease activity was found to be a Gram positive motile, aerobic, rod shaped bacterium. It was identified as *Bacillus licheniformis* by Microbial Type Culture Collection center and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India and deposited under Accession No MTCC 10,008. Results are shown in Table-III.
Table I  
List of the Isolates

<table>
<thead>
<tr>
<th>S.No</th>
<th>Source</th>
<th>Isolate number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Black Cotton Soil</td>
<td>BS1</td>
</tr>
<tr>
<td>2</td>
<td>Black Cotton Soil</td>
<td>BS2</td>
</tr>
<tr>
<td>3</td>
<td>Black Cotton Soil</td>
<td>BS3</td>
</tr>
<tr>
<td>4</td>
<td>Black Cotton Soil</td>
<td>BS4</td>
</tr>
<tr>
<td>5</td>
<td>Groundnut field soil</td>
<td>GS1</td>
</tr>
<tr>
<td>6</td>
<td>Groundnut field soil</td>
<td>GS2</td>
</tr>
<tr>
<td>7</td>
<td>Groundnut field soil</td>
<td>GS3</td>
</tr>
<tr>
<td>8</td>
<td>Kotappa Konda Soil</td>
<td>KS1</td>
</tr>
<tr>
<td>9</td>
<td>Kotappa Konda Soil</td>
<td>KS2</td>
</tr>
<tr>
<td>10</td>
<td>Kotappa Konda Soil</td>
<td>KS3</td>
</tr>
<tr>
<td>11</td>
<td>Kotappa Konda Soil</td>
<td>KS4</td>
</tr>
<tr>
<td>12</td>
<td>Milk Processing unit</td>
<td>MS1</td>
</tr>
<tr>
<td>13</td>
<td>Milk Processing unit</td>
<td>MS2</td>
</tr>
<tr>
<td>14</td>
<td>Milk Processing unit</td>
<td>MS3</td>
</tr>
<tr>
<td>15</td>
<td>Milk Processing unit</td>
<td>MS4</td>
</tr>
<tr>
<td>16</td>
<td>Milk Processing unit</td>
<td>MS5</td>
</tr>
<tr>
<td>17</td>
<td>Milk Processing unit</td>
<td>MS6</td>
</tr>
<tr>
<td>18</td>
<td>Tirumala soil</td>
<td>TS1</td>
</tr>
<tr>
<td>19</td>
<td>Tirumala soil</td>
<td>TS2</td>
</tr>
<tr>
<td>20</td>
<td>Tirumala soil</td>
<td>TS3</td>
</tr>
<tr>
<td>21</td>
<td>Tirumala soil</td>
<td>TS4</td>
</tr>
</tbody>
</table>
### Table II

Screening of the isolates for the alkaline proteases production

<table>
<thead>
<tr>
<th>S.No</th>
<th>Isolate number</th>
<th>Enzyme activity Uml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BS1</td>
<td>82</td>
</tr>
<tr>
<td>2</td>
<td>BS2</td>
<td>85</td>
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<tr>
<td>3</td>
<td>BS3</td>
<td>82</td>
</tr>
<tr>
<td>4</td>
<td>BS4</td>
<td>90</td>
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<tr>
<td>5</td>
<td>GS1</td>
<td>94</td>
</tr>
<tr>
<td>6</td>
<td>GS2</td>
<td>78</td>
</tr>
<tr>
<td>7</td>
<td>GS3</td>
<td>92</td>
</tr>
<tr>
<td>8</td>
<td>KS1</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>KS2</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>KS3</td>
<td>104</td>
</tr>
<tr>
<td>11</td>
<td>KS4</td>
<td>54</td>
</tr>
<tr>
<td>12</td>
<td>MS1</td>
<td>44</td>
</tr>
<tr>
<td>13</td>
<td>MS2</td>
<td>92</td>
</tr>
<tr>
<td>14</td>
<td>MS3</td>
<td>100</td>
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<tr>
<td>15</td>
<td>MS4</td>
<td>104</td>
</tr>
<tr>
<td>16</td>
<td>MS5</td>
<td>100</td>
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<td>17</td>
<td>MS6</td>
<td>110</td>
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<td>18</td>
<td>TS1</td>
<td>42</td>
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<tr>
<td>19</td>
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<td>54</td>
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<tr>
<td>20</td>
<td>TS3</td>
<td>72</td>
</tr>
<tr>
<td>21</td>
<td>TS4</td>
<td>78</td>
</tr>
</tbody>
</table>
Table III
Morphological, Physiological and Biochemical Tests for Strain Number MS: 6

Morphological Tests:

<table>
<thead>
<tr>
<th>Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony Morphology</td>
<td>MS-6</td>
</tr>
<tr>
<td>Configuration</td>
<td>Round</td>
</tr>
<tr>
<td>Margin</td>
<td>Wavy</td>
</tr>
<tr>
<td>Elevations</td>
<td>Convex</td>
</tr>
<tr>
<td>Surface</td>
<td>Smooth</td>
</tr>
<tr>
<td>Density</td>
<td>Opaque</td>
</tr>
<tr>
<td>Pigments</td>
<td>Cream</td>
</tr>
<tr>
<td>Gram’s Reaction</td>
<td>+</td>
</tr>
<tr>
<td>Shape</td>
<td>Rods</td>
</tr>
<tr>
<td>Size</td>
<td>Moderate</td>
</tr>
<tr>
<td>Arrangement</td>
<td>Chains</td>
</tr>
<tr>
<td>Spore</td>
<td></td>
</tr>
<tr>
<td>Endospore/granules</td>
<td>+</td>
</tr>
<tr>
<td>Position</td>
<td>Sub terminal</td>
</tr>
<tr>
<td>Shape</td>
<td>Oval</td>
</tr>
<tr>
<td>Sporangia Bulging</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
</tr>
<tr>
<td>Florescence(UV)</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table III (Contd)

**Physiological Tests**

<table>
<thead>
<tr>
<th>Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth at Temp.</strong></td>
<td>MS-6</td>
</tr>
<tr>
<td>$4^\circ$C</td>
<td>-</td>
</tr>
<tr>
<td>$10^\circ$C</td>
<td>+</td>
</tr>
<tr>
<td>$15^\circ$C</td>
<td>+</td>
</tr>
<tr>
<td>$25^\circ$C</td>
<td>+</td>
</tr>
<tr>
<td>$30^\circ$C</td>
<td>+</td>
</tr>
<tr>
<td>$37^\circ$C</td>
<td>+</td>
</tr>
<tr>
<td>$42^\circ$C</td>
<td>+</td>
</tr>
<tr>
<td>$55^\circ$C</td>
<td>+</td>
</tr>
<tr>
<td>$65^\circ$C</td>
<td>-</td>
</tr>
<tr>
<td><strong>Growth at pH</strong></td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>5.7</td>
<td>+</td>
</tr>
<tr>
<td>6.8</td>
<td>+</td>
</tr>
<tr>
<td>8.0</td>
<td>+</td>
</tr>
<tr>
<td>9.0</td>
<td>+</td>
</tr>
<tr>
<td>11.0</td>
<td>+</td>
</tr>
<tr>
<td><strong>Growth on NaCl (%)</strong></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>+</td>
</tr>
<tr>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>7.0</td>
<td>+</td>
</tr>
<tr>
<td>8.5</td>
<td>+</td>
</tr>
<tr>
<td>10.0</td>
<td>+</td>
</tr>
<tr>
<td><strong>Growth Under Anaerobic Condition</strong></td>
<td>+/-</td>
</tr>
</tbody>
</table>
### Table III (Contd)

**Biochemical Tests:**

<table>
<thead>
<tr>
<th>Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MS-6</strong></td>
<td></td>
</tr>
<tr>
<td>Growth on MacConkey Agar</td>
<td>-</td>
</tr>
<tr>
<td>Indole Test</td>
<td>-</td>
</tr>
<tr>
<td>Methyl Red Test</td>
<td>-</td>
</tr>
<tr>
<td>Voges Proskauer Test</td>
<td>-</td>
</tr>
<tr>
<td>Citrate Utilization</td>
<td>+</td>
</tr>
<tr>
<td>Gas Production from Glucose</td>
<td>-</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Starch Hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Urea Hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate Reduction</td>
<td>+</td>
</tr>
<tr>
<td>H$_2$S Production</td>
<td>-</td>
</tr>
<tr>
<td>Cytochrome Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Catalase Test</td>
<td>+</td>
</tr>
<tr>
<td>Oxidation/ Fermentation (O/F)</td>
<td>F</td>
</tr>
<tr>
<td>Gelatin Hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>+</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>-</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>-</td>
</tr>
</tbody>
</table>
Table III (Contd)

Acid Production from carbohydrates:

<table>
<thead>
<tr>
<th>Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-6</td>
<td></td>
</tr>
<tr>
<td>Adonitol</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>-</td>
</tr>
<tr>
<td>Dextrose</td>
<td>+</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
</tr>
<tr>
<td>Inositol</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
</tr>
<tr>
<td>Melibiose</td>
<td>-</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
</tr>
<tr>
<td>Salicin</td>
<td>-</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>-</td>
</tr>
</tbody>
</table>

On the basis of above tests, the organism has been identified as follows:

<table>
<thead>
<tr>
<th>S.N</th>
<th>Strain Designation</th>
<th>Identity</th>
<th>MTCC No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MS-6</td>
<td><em>Bacillus licheniformis</em></td>
<td>10,008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Gardner and Proy)</td>
<td></td>
</tr>
</tbody>
</table>
3.2.4 Stability of the highest yielding strain

Repeated sub culturing was not affecting the yield by *Bacillus licheniformis*. The original yield shown by this strain which was determined after the second subculture was $110 \text{U/ml}^{-1}$. More or less similar yields were obtained in the tests performed after 5\textsuperscript{th}, 6\textsuperscript{th}, 7\textsuperscript{th} and 8\textsuperscript{th} subcultures also. Since no signs of instability were shown by the strain the results were suggestive of the stable high yielding nature of the strain.

The naturally occurring alkaline environments comprise alkaline soils, soda lakes, alkaline springs etc. Isolation and screening of bacteria from these natural environments can be supposed to be useful for obtaining bacterial strains with the potential of yielding alkaline protease. So in this study for obtaining the suitable strains for alkaline protease production the proteolytic bacteria from alkaline soils were isolated and screened. The soil samples for this purpose were collected from alkaline soils at five locations, viz., Black cotton soil, Groundnut field soil, Kotappakonda soil, Milk processing unit and Tirumala soil. 21 bacterial strains were isolated from these five soils and screened for alkaline protease production.

The diverse group of bacteria that thrive well in alkaline environment can be categorized into two broad groups alkalotolerants and alkalophiles. Alkalotolerants show optimal growth between pH 7.0 – 9.0 but cannot grow above pH 9.5. The alkalophiles can be further divided into two groups facultative alkalophiles and obligate alkalophiles. Facultative alkalophiles can grow at neutral pH while obligate alkalophiles cannot. Both these groups will grow at pH 10.0 (Krulwich and Guffanti, 1989). So in a medium with pH 10.0 or above only the alkalophiles are supposed to grow.

Since the casein agar used in this study was having pH 9.5, the isolates obtained could be alkalophiles, either facultative or obligate. The use of alkaline casein or milk agar for the isolation of alkaline protease producing bacteria has been reported by some workers. (Durham *et al.*, 1987); Nihalani and Satyanarayana, 1992; Gessesse and Geshe, 1997).
Since the incubation of plates was performed at 40°C, the isolates obtained may either be thermo tolerant or thermophilic also. So the method used in this study can be considered to be an easy and simple one for the isolation of proteolytic strains which could possibly be the good source of thermo stable alkaline proteases.

The proteolytic strains isolated from alkaline soils were tested for the yield of alkaline protease by submerged fermentation. The yield of alkaline protease by the isolates was found to be ranging from 44 Uml⁻¹ to 110 Uml⁻¹. There were only four isolates producing 42 Uml⁻¹, 44 Uml⁻¹, 54 Uml⁻¹, and 54 Uml⁻¹. There were six isolates producing alkaline protease at a level ranging from 100 Uml⁻¹ to 98 Uml⁻¹. Among all these best enzyme activity of 110 Uml⁻¹ was shown by MS6. The highest yielding strain viz., MS6 was identified as *Bacillus licheniformis*. That *Bacillus licheniformis* produces good amount of protease in submerged culture has been reported by Aunstrup (1974).

Before selecting the MS6 strain for further studies its ability to maintain the high yielding nature was studied by sub culturing and testing the yields at monthly intervals. Repeated sub culturing has not affected the yield of the strain.