Partial purification of alkaline protease by mutant strain of *Bacillus subtilis*<sup>EMS-6</sup>

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**ABSTRACT**

The present study utilized different methods for purification of alkaline protease by using ammonium sulphate and acetone. Ammonium sulphate was found the best purifying agent and gave 109.09 fold purification of protease with 49.7% yield at 60% saturation. Dialysis was performed by using phosphate and Tris-HCl buffer and phosphate buffer was optimized to good dialyzing agent at pH 8.0.

**Key words:** Alkaline protease, *Bacillus subtilis*<sup>EMS-6</sup>, Partial purification

**INTRODUCTION**

More than 1700 enzymes have been reported in a living cell, each with efficiency unique in nature. Among different industrially important enzymes, proteolytic enzymes are the most important ones representing worldwide sales of about 60% of the total enzymes market. These enzymes are widely used in brewing industry, detergent industry, food industry, laundry industry, leather industry, paper industry, pharmaceutical industry, photography and waste processing industry (Raju, *et al*., 1994; Horikoshi, 1999; Woods, *et al*., 2001; Barredo, 2004; Ahmad, *et al*., 2011).

Proteases are hydrolytic enzymes, because these act upon native proteins and catalyze the breakdown of peptide bonds. These are found in plant tissues, animal tissues and micro-organisms. Microbial source is preferred than the plant and animal source, since they possess almost all the characteristics desirable for their biotechnological applications and can be extracted profitably and economically. Among different microorganisms, especially bacteria of *Bacillus* species are commercially very important producers of proteases (Adinarayana *et al*., 2003; Joo, 2005; Ahmad, *et al*., 2011).

Many procedures have been used for alkaline protease downstream processing especially those which do not cause denaturation of enzyme. A protein may be purified by single step e.g. affinity chromatography or by combination of several steps e.g. fractionation with salts or solvents, ion exchange and gel filtration. It can be carried out by inorganic salts and organic solvents. The most commonly used salt is ammonium sulphate. Various organic solvents such as methanol, ethanol, acetone and diethyl ether can also be used for the precipitation of proteins (Towatana, *et al*., 1999; Reed, 2004; Iftikhar, *et al*., 2011).

Fractionation is one of the easiest and least expensive procedures for the purification of industrial enzymes. This is the oldest and most commonly used
method of fractioning enzymes in which neutral salts such as ammonium sulphate are used at high concentrations. Ammonium sulphate is the most commonly used salt because it is inexpensive, highly soluble, having minimum harmful effects and stability effect on many enzymes and fractionate the enzyme at room temperature. Enzymes having high molecular weight usually precipitate at lower ammonium sulphate saturation (25-35%) and vice versa (Secades & Guijarro, 1999; Lee, et al., 2002).

**Purpose of study:**
The major purpose of the present study was to partially purify alkaline protease with the help of acetone and ammonium sulphate and to assess the efficiency of these used chemicals.

**MATERIALS AND METHODS**

**Bacterial culture collection and maintenance**

The bacterial culture of *Bacillus subtilis* was taken from culture bank of Institute of Industrial Biotechnology, GC University, Lahore. The culture was originally isolated from the soil of a tannery area. The culture was revived by transferring to the slants of peptone-yeast extract agar medium. After taking growth the culture was maintained weekly by transferring to fresh slants and was stored at 4°C in a cool place.

**Fermentation experiment**

Bacterial inoculum was prepared in 250ml Erlenmeyer flask containing 50ml of nutrient broth medium. After sterilization a loop full of bacteria from a fresh slant was transferred to the flask under aseptic conditions. The flasks were then placed in the shaking incubator at 37°C rotating at a speed of 200 rpm for 24 hours. One milliliter of that broth containing rich growth of the bacterium was used as an inoculum.

About 50ml of fermentation medium (g/l: soybean meal, 20; Glucose, 10; polypeptone, 10; KH$_2$PO$_4$, 1.0; (NH$_4$)$_2$SO$_4$, 1.0 and Na$_2$CO$_3$, 5.0) was taken in 250ml Erlenmeyer flasks. The flasks were sterilized and inoculated with 1ml of inoculum under aseptic conditions. Then the flasks were placed in shaking incubator at 37°C at 200 rpm for 48 hrs. After fermentation the broth was centrifuged at 6,000 rpm for 10 minutes and the supernatant was used for enzyme purification and other analytical procedures (Mukhtar & Haq, 2008).

**Assay of proteolytic enzyme**

The method of McDonald & Chen (1965) was used for the assay of proteolytic enzyme. Casein was used as substrate, blue colour was developed by the addition of Folin & Ciocalteau reagent and O.D was obtained at 700 nm.

**Protein estimation**

The method of Bradford 1976 was used for the estimation of protein content in the fermentation broth.
Mutagenesis
Ethyl methane sulphonate (EMS) was used as a chemical mutagen for inducing mutation in *B. subtilis*. Wild bacteria were grown in peptone-yeast extract medium for 24 hrs in 250ml flasks. After taking growth, the broth was centrifuged at 5000 rpm for 10 min. Supernatant was discarded and the pellet was washed with sterilized saline water and resuspended in 5ml of 0.2 M Tris. 150 µl of EMS was added in the tube and shaken well. The medium was incubated at 37ºC for 2 hrs. After prescribed period, the medium was centrifuged and pellet was washed. The suspension was poured on peptone-yeast extract casein-agar plates and placed in incubator at 37ºC for 24-48 hrs.

Purification of alkaline protease
1. Ammonium sulphate precipitation
Solid ammonium sulphate was added to 100 ml of crude cell free broth of protease at the concentration of 30% (w/v). The suspension was stirred for half an hour at 4ºC in cold room. After sufficient shaking the precipitates were collected by centrifugation at 15,000 rpm for 15 minutes at 4ºC. Similarly, enzyme solution was then treated with 40, 50 and 60% (w/v) ammonium sulphate to achieve the desired saturation from 30% to 80 % at 4ºC. Proteolytic activity was determined in the supernatant after each treatment and precipitates were collected for further purification.

2. Acetone Precipitation
Crude enzyme extract was mixed at 4ºC with cold acetone and kept at 0ºC for 12 hrs. The resultant precipitates were pelleted by centrifugation at 15,000 rpm for 15 minutes at 4ºC. The pelleted precipitates after removing supernatant were re-suspended in potassium phosphate / Tris-HCl buffer to one fifth of the starting volume. Then the proteolytic activity was measured.

Dialysis
The precipitates obtained by ammonium sulphate and acetone were dialyzed in small volume of the dialysis buffer using 12,000 molecular weight cut off dialyzed bag, which was placed in one litre of the buffer (pH 7.0) for 24 hrs at 4ºC. The proteolytic activity of the dialyzed material was determined.

RESULTS AND DISCUSSIONS
Ammonium sulphate and acetone fractionation
The protease was purified by using ammonium sulphate and acetone fractionation. The fractionation was carried out from 30 to 80 % saturation of acetone and ammonium sulphate. In case of acetone precipitation, the fractionation was started from 40% saturation and at 70% saturation it reached at maximum after which it started to decline sharply. There was 54.97 purification fold with 40.5% yield and the specific activity was 2.419µg/ml/mg (Table 1). While on the other hand, in ammonium sulphate precipitation, maximum enzyme purification was achieved at 60% saturation corresponding to 109.09 fold purification with 49.7% yield and specific activity was 4.8µg/ml/mg (Table 2).
Acetone is a good purifying agent for proteins but it causes the denaturation of the enzyme which was evident in the present work, therefore it was not commonly used in purification procedures. On the other hand ammonium sulphate is most commonly used salt in industry as well as in laboratory procedures and does not cause the denaturation of proteins. The purification of alkaline protease using acetone at 80% saturation was also reported by Thangam & Rajkumar (2002) and Omran (2005). While several workers have reported the purification of protease with ammonium sulphate at 60% saturation (Secades & Guijarro, 1999; Cabral, et al., 2004; Gupta, et al., 2005; Hezayen, et al., 2009; Gaur, et al., 2010).

Table 1: Purification of alkaline protease produced by B. subtilis\textsuperscript{EMS-6} using acetone precipitation

<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Total Protein (µg/ml)</th>
<th>Specific Activity (U/µg/ml)</th>
<th>Percent Yield</th>
<th>Purification Folds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude broth</td>
<td>4200</td>
<td>0.044</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>30%</td>
<td>1500</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>40%</td>
<td>535</td>
<td>0.0336</td>
<td>9.7</td>
<td>0.764</td>
</tr>
<tr>
<td>50%</td>
<td>321</td>
<td>0.090</td>
<td>15.7</td>
<td>2.045</td>
</tr>
<tr>
<td>60%</td>
<td>181142</td>
<td>0.373</td>
<td>28.6</td>
<td>8.47</td>
</tr>
<tr>
<td>70%</td>
<td>331</td>
<td>2.419</td>
<td>40.5</td>
<td>54.977</td>
</tr>
<tr>
<td>80%</td>
<td>17</td>
<td>0.059</td>
<td>0.545</td>
<td>1.341</td>
</tr>
</tbody>
</table>

The purification procedure was carried out at 4\textdegree C with constant stirring.

Dialysis

Two buffers named potassium phosphate and Tris HCl were used for the re-suspension of fractioned enzyme and dialysis procedure. It was observed that 44.86% yield and 117.86 folds purification of the protease was obtained when potassium phosphate buffer was used for this purpose. On the other hand, Tris-HCl buffer gave 36.757% yield and 110.38 folds of protease purification. The pH of both buffers was kept at 7.5.

It is suggested that it does not contain such elements which hinders the activity of enzyme or it may have some stabilizing effects on the enzyme structure. There are several reports for the use of phosphate buffer for the purification of protease (Adinarayana, et al., 2003; Gupta, et al., 2005). However, some workers have also reported the use of Tris HCl buffer for the purification of alkaline protease (Toni, et al., 2002; Cabral, et al., 2004).

Effect of pH of dialysis buffer on the purification of alkaline protease

The potassium phosphate buffer was used for the re-suspension of the purified enzyme pellet and dialysis during the purification procedure. The effect of pH of potassium phosphate buffer (7.0-9.0) was studied on the purification of alkaline protease. It was observed that there was a gradual increase in the specific activity of precipitated protease when pH of the dialysis buffer was increased from 7.0-8.0 while maximum activity was observed at buffer pH of 8.0, i.e., 134µg/ml of protease. Above pH 8.0, there was a gradual decrease in the
activity of purified enzyme. According to most of the reports, the best pH of the buffer recorded is 7.5 (Toni, et al., 2002) whereas Cabral et al. (2004) reported 8.5 as the best pH of dialysis buffer for the partial purification of alkaline protease while in the present study the buffer having pH 8.0 was found to be the best as also reported by (Thangam, et al., 2002; Hinode, et al., 1991).

Table 2: Purification of alkaline protease produced by B. subtilis<sup>EMS-6</sup> using ammonium sulphate precipitation

<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Total Protein (µg/ml)</th>
<th>Specific Activity (U/µg/ml)</th>
<th>Percent Yield</th>
<th>Purification Folds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude broth</td>
<td>4200</td>
<td>0.044</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>30%</td>
<td>1200</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>40%</td>
<td>190</td>
<td>0.142</td>
<td>14.6</td>
<td>3.227</td>
</tr>
<tr>
<td>50%</td>
<td>121</td>
<td>0.479</td>
<td>31.3</td>
<td>10.886</td>
</tr>
<tr>
<td>60%</td>
<td>18</td>
<td>4.8</td>
<td>49.7</td>
<td>109.09</td>
</tr>
<tr>
<td>70%</td>
<td>3</td>
<td>0.033</td>
<td>0.5</td>
<td>0.75</td>
</tr>
</tbody>
</table>

The purification procedure was carried out at 4°C with constant stirring.

Table 3: Effect of dialysis buffer on purification of alkaline protease produced by B. subtilis<sup>EMS-6</sup>

<table>
<thead>
<tr>
<th>Dialysis Buffer</th>
<th>Total Protein (mg)</th>
<th>Total Activity (µg/ml)</th>
<th>Specific Activity (µg/ml/mg)</th>
<th>Percent Yield</th>
<th>Purification Folds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>4200</td>
<td>185</td>
<td>0.044</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Potassium phosphate buffer</td>
<td>16</td>
<td>83</td>
<td>5.186</td>
<td>44.86</td>
<td>117.86</td>
</tr>
<tr>
<td>Tris-HCl buffer</td>
<td>14</td>
<td>68</td>
<td>4.857</td>
<td>36.757</td>
<td>110.38</td>
</tr>
</tbody>
</table>

The purification procedure was carried out at 4°C with constant stirring. Ammonium sulphate precipitation at 60% saturation; dialysis time=24hrs.

Table 4: Effect of pH of dialysis buffer on purification of alkaline protease produced by B. subtilis<sup>EMS-6</sup>

<table>
<thead>
<tr>
<th>pH of Dialysis Buffer</th>
<th>Total Protein (mg)</th>
<th>Total Activity (µg/ml)</th>
<th>Specific Activity (µg/ml/mg)</th>
<th>Percent Yield</th>
<th>Purification Folds</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>18</td>
<td>80</td>
<td>4.44</td>
<td>43.24</td>
<td>100.91</td>
</tr>
<tr>
<td>7.5</td>
<td>16</td>
<td>83</td>
<td>5.186</td>
<td>44.86</td>
<td>117.86</td>
</tr>
<tr>
<td>8.0</td>
<td>15</td>
<td>89</td>
<td>5.933</td>
<td>18.11</td>
<td>134.84</td>
</tr>
<tr>
<td>8.5</td>
<td>17</td>
<td>86</td>
<td>5.05</td>
<td>46.49</td>
<td>114.77</td>
</tr>
<tr>
<td>9.0</td>
<td>19</td>
<td>78</td>
<td>4.105</td>
<td>42.16</td>
<td>93.29</td>
</tr>
</tbody>
</table>
REFERENCES


