Isolation, Purification and characterization of extracellular β-glucosidase from Bacillus sp.

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ABSTRACT

The present study reports the isolation, purification and characterization of β-glucosidase enzyme from Bacillus species using gel filtration and ion exchange and chromatography. Cellulolytic bacterium producing β-glucosidase activity was isolated from naturally decaying cellulosic material by enrichment culture and serial dilution methods. The organism produced higher level of β-glucosidase activity in the presence of 0.5% sucrose as a carbon source and ammonium sulphate as a nitrogen source in the medium of pH 7.0 at 60°C after 10 hours. The maximum β-glucosidase activity after optimizing the culture condition was 1.7U/mL. Crude enzyme preparation was obtained from culture medium after growing the Bacillus species under optimal condition after 10 hours. The culture supernatant containing the cellulolytic enzymes was lyophilized. The concentrated enzyme sample was purified by a combination of gel filtration using Sephadex G-75 and ion-exchange chromatography through Q-Sepharose, respectively. The purified enzyme showed a single band on SDS-PAGE. The molecular weight of the purified β-glucosidase determined by SDS-PAGE was formed to be 46 kDa. The optimal assay pH and temperature was 7.0 and 60°C. The enzyme was stable between pH 5.5-8.0. Moreover we come to conclusion that enzyme was stable up to 70°C.

Key words: Isolation; purification; characterization; β-glucosidase

Introduction

The imminent storage of fossil fuel has intensified the research for bioconversion of lignocellulosic material to fuels. The availability of solar energy needs to be linked up with cellulosic and hemicelluloses productivity. Thus it is increasingly important to look to the vast annually renewed cellulose and hemicellulose as a substrate for single cell proteins and as a raw material in fermentation for the production of alcohol and other chemicals [12]. The sunlight used in photosynthetic production of cellulose is free, but fossil source of energy are going scarcer and dearer. Because of this, new ways are to be sought to utilize the sunlight conserved in the cellulose skeleton of plants. The use of cellulose as a novel energy source has been reviewed [1].

The market for industrial enzymes has more than doubled since 1983. Estimates for the whole world market are in the range between $ 1.7 and $ 2.0 billion in 2005. Approximately 60 companies produce substantial amount of smaller range, and there are around 400 companies producing industrial quantities

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of a very limited range of enzyme types. About 12 categories of enzymes i.e. amylases, cellulases, hemicellulases, pectinases, proteinases and lipases etc. are used in the industry. Most of them are hydrolytic enzymes used for the depolymerization of natural substances [7].

Cellulases are mixture of several enzymes that act in concert to hydrolyze crystalline cellulose to its monomeric component i.e. glucose. Cellulases can be divided into non aggregating and aggregating enzymes. Cellulases produced by some bacterial such as those from *Clostridium thermocellum*, tend to be cell associated and from tight multi enzyme complexes called cellulosome, on the surface of the cell. Cellulosome exhibits a cellulose binding function that allows the bacterial cell to bind closely to its substrate. Cellulosome is microcellular machine, whose component interacts in a synergistic manner to catalyze the efficient degradation of cellulose [2].

β-glucosidase is one of the component enzymes of the cellualse complex and is widely distributed in nature. It catalyzes the hydrolysis of aryl and alkyl glycosides as well as of cellobiose to glucose which is inhibitory to the endo and exo cellulases during cellulose hydrolysis [4].

The enzyme has great economic significance. An efficient cellobiose hydrolysis requires a large amount of β-glucosidase for the utilization of cellulose residues on an industrial scale β-glucosidase. [10]. β-glucosidase are a heterogeneous. These enzymes have aroused considerable interest primarily because of their involvement in the biological saccharification of cellulolic material [5].

The present study was undertaken to isolate, purify and characterize β-glucosidase enzyme from locally isolated Bacillus species. The purpose of the study was to purify enzyme to be available for the industrial utilization.

**Material and methods**

*Sample Collection*

Different samples of naturally decaying cellulotic materials like wheat straw, rice straw, and bagasse and, soil were collected from various sites of Lahore and Muridkay (a city near Lahore), Punjab Province. The samples were tested for the presence of cellulolytic microorganisms.

*Isolation of the Organism*

A number of cellulolytic bacterial strains were isolated from the different samples collected. Isolation was done by culture technique and serial dilution methods using modified Han, s medium (MH). Avicel (Sigma Chemical Co., USA), 5g/L as a carbon source was used for the isolation of active cellulolytic bacterial strains. The bacterial colonies which produced haloes around the colonies were picked and selected for further study.

*Culture*

Inoculum was prepared in 25 mL of Modified Han's (MH) medium containing 0.2 mL of 10 % sucrose (Sigma Chemical Co., USA), taken in 250 mL Erlenmeyer flask and inoculated with cells from single colony of freshly grown slants. The supernatant was used as extracellular enzyme source.

**Optimization of the culture conditions**

*Effect of Time Course*

The effect of the time course on cell growth and β-glucosidase production was studied by withdrawing 1.0 mL of culture samples from the culture medium aseptically after different time intervals. The cell growth at each time interval was monitored by measuring absorbance at 600 nm on spectrophotometer.

*Effect of Temperature*

The effect of temperature on cell growth and β-glucosidase production was studied by incubating 50 mL of the culture medium at various temperatures. A temperature range of 45 to 65°C was used.

*Effect of pH*

The effect of initial pH of the medium on β-glucosidase production was studied by varying the pH of the culture medium. The pH of the medium was adjusted to 5.0 to 7.5 with NaOH.

*Effect of Nitrogen Sources*

The effect of various nitrogen sources on β-glucosidase production was studied by using a number of nitrogen sources such as (NH₄)₂S0₄, (NH₄)HPO₄, NaNO₃, KNO₃, urea etc. Nitrogen sources were used at a concentration of 0.1 %.

*Effect of Carbon Sources*

The effect of cellobiose, sucrose, lactose, glucose, and Avicel (0.5%) on the production of β-glucosidase was studied after specific time intervals. For cellobiose, sucrose and lactose, 10 % stock solution was prepared and separately autoclaved. 2.5 mL of autoclaved stock solution was added to 50 mL medium as a carbon source.

*Enzyme assay*
**β-Glucosidase activity**

Assay of β-Glucosidase activity was performed by measuring the release of reducing sugars by the dinitrosalicylic acid (DNS) method [6]. 0.5 mL of the appropriately diluted enzyme was mixed with 0.5 mL of salcin dissolved in 0.05 M phosphate buffer (pH 7.0) and incubated at 60°C for 10 minutes. After incubation, 3 mL of DNS reagent was added to the enzyme mixture and heated in boiling water for 15 minutes. For the estimation of the reducing sugars in the test solution, a reference blank was prepared by taking 0.5 mL of salcin, 3.0 mL DNS and 0.5 mL of the enzyme of the same dilution as used for the test. The mixture was placed in boiling water bath for 15 minutes and cooled to room temperature. The absorbance was then measured at 600nm. The absorbance of the sample corrected by subtraction of enzyme blank was used to calculate glucose concentration from the glucose standard curve. Enzyme activity (U/ml) was determined by the following formula:

\[
\text{Activity (U/mL)} = \frac{\text{Glucose Cone.} \times \text{dilution factor x 2}}{\text{Time of incubation (min)}}
\]

One unit of enzyme activity is defined as the amount of enzyme, which released 1 mmol of reducing sugars equivalent, to glucose per minute under the assay conditions described.

**Estimation of Protein**

Soluble proteins in the culture supernatant and in pooled fractions of purification experiments were estimated by dye binding method of Bradford using bovine scum albumin (Sigma Chemical Co., USA) as a standard.

**Purification**

The bacterial culture was centrifuged at 12,000 rpm for 10 minutes. To concentrate the enzyme solution it was lyophilized. Enzyme activity measured after lyophilization.

**Gel filtration**

The concentrated enzyme sample was chromatographed through Sephadex G-75. The swollen Sephadex G-75 suspension was packed in a column (1.6x 66 cm) and equilibrated with 0.05M phosphate buffer (pH 7.0). 2 mL of concentrated enzyme sample was subjected to gel filtration. The enzyme sample was loaded on the column and bound proteins were eluted with 0.05M phosphate buffer (pH 7.0). A total of 40 fractions (2.5 mL each) were collected. The b-glucosidase activity and protein concentration were estimated in each fraction. b-glucosidase active fractions were pooled and subjected to further purification processes.

**Ion Exchange Chromatography**

Preswollen Q-Sepharose gel (Sigma Chemical Co., USA) was equilibrated with buffer of different pH by washing 10 times with 10 mL of 0.5 M Tris-HCl buffer (pH 8.5). The gel was then equilibrated with 0.01 M Tris-HCl buffer by washing 5 times with 10 mL buffer of the same pH. The gel was allowed to settle and supernatant was assayed for β-Glucosidase activity. The pH at which maximum activity bound to the gel was selected for ion-exchange chromatography,

For selection of salt-gradient for Q-Sepharose chromatography, the gel was equilibrated with 0.05M Tris-HCl buffer at the selected pH. The gel was then allowed to settle and supernatant was assayed for β-Glucosidase activity. The salt concentration at which least activity bound to the gel was selected.

2 mL of concentrated and partially purified enzyme sample was fractionated on Q-Sepharose column equilibrated with 0.05M Tris-HCl buffer (pH 8.5). The enzyme sample was loaded on the column and the bound proteins were eluted by a linear gradient of 0.5M NaCl in Tris-HCl buffer (pH 8.5). A total of 30 fractions (2 mL) were collected. Enzyme activity and protein concentrations were estimated in each fraction. Active fractions were concentrated by lyophilization for electrophoresis.

**SDS - PAGE**

Homogeneity and purity of the protein sample was checked by SDS-PAGE after various steps of purification. 12% polyacrylamide gel was prepared for this purpose. 5µL of enzyme was incubated with 2 mL of the loading dye containing dithiothreitol, SDS and bromophenol blue at 7.7 %, 10.0 % and 0.1 % w/v and 50 % glycerol (v/v) in 0.4M Tris buffer (pH 6.8) in boiling water bath for one minute. The samples were loaded on a 12% polyacrylamide gel and electrophoresed at a constant voltage of 150 V for 3 hours. Gel was further subjected to staining and destaining solutions.

**Characterization of the enzyme**

**Molecular weight determination**

The molecular weight of the purified enzyme was determined by electrophoresis on 12% SDS-PAGE gel. Enzyme samples were loaded on the gel along with standard markers (The reference proteins
used were: phosphorylase B (MW29,000), bovine serum albumin (MW68,000), ovalbumin (MW43,000), carbonic anhydrase (MW 29,000), β-lactoglobulin (MW18,400) and lysozyme (MW 14,300).

**pH Stability**

To determine the pH stability of the enzyme, the enzyme was incubated for 30 minutes at room temperature with buffer solutions of varying pH range. The pH range of the phosphate buffers used was of pH 5.5-9.5. The residual activity was then determined at 60°C under standard assay conditions.

**Assay pH**

In order to determine the optimum assay pH, for β-glucosidase activity, the assay was carried out using 0.05M phosphate buffer ranging from pH 6-9. Assay temperature

In order to determine the optimum assay temperature for β-glucosidase activity, the assay was carried out at temperature range of 50-70°C.

**Results and discussion**

**Screening for Cellulolytic Microorganisms**

A number of cellulolytic bacterial strains were isolated by enrichment culture and serial dilution methods as described in materials and methods. The cellulolytic bacterial strains which produced strong haloes was selected for further study (Figure-1).

**Optimisation of the culture conditions**

**Effect of time course**

The effect of time course on cell growth and β-glucosidase production were determined by measuring the absorbance of cell growth and determining the cellulase activity at different time intervals. The results indicated that maximum β-glucosidase activity was observed after 10 hours of fermentation with sucrose as a carbon source (Table-1). After 10 hours a decline in growth and β-glucosidase activity was noticed.

**Effect of temperature**

The effect of temperature on the cell growth and β-glucosidase production was studied by growing the organism at different temperature. As the fermentation temperature was increased from 45°C to 70°C. Cell growth and β-glucosidase activity in the culture supernatant gradually increased from 0.55 U/mL to 1.72 U/mL (Table-2). After 60°C, the cell growth and β-glucosidase activity gradually decrease. Since the maximum β-glucosidase activity was observed at 60°C, this temperature was selected in the subsequent experiments to determine the effect of other parameters for growth and β-glucosidase production.

**Effect of pH**

The effect of initial pH of the culture medium on the cell growth and β-glucosidase production was studied by growing the organism at different pH values ranging from 5.0 to 8.5 at 55°C. As the pH of the culture medium increased from 5.5 to 7.0, β-glucosidase activity increased from 0.8 to 1.72 U/mL. The maximum cell growth was measured 1.72 at pH 7.0. Increasing the pH to 8.5, the β-glucosidase activity was reduced to 0.425 U/mL. Since maximum β-glucosidase activity was observed at pH 7.0, so the pH of the medium was selected as 7.0 (Table-3).

**Effect of Nitrogen Sources**

The effect of various nitrogen sources on cell growth and β-glucosidase production was studied by using a number of nitrogen sources at a concentration of 0.1%. The results showed that the highest level of β-glucosidase activity observed was 1.7U/mL with (NH₄)₂SO₄ as the nitrogen source (Table-4). Ammonium hydrogen phosphate, sodium nitrate, potassium nitrate and urea produced lower levels of β-glucosidase activity.

**Effect of Carbon Sources**

To study the effect of different carbon sources on cell growth and β-glucosidase production, a number of carbon sources at a concentration of 0.5% were used. The maximum yield of β-glucosidase activity was obtained after 10 hours of fermentation when sucrose was used as a carbon source. Sucrose was the best amongst the carbon sources i.e. glucose, lactose, cellobiose and avicel. The maximum β-glucosidase activity was 1.72 U/mL, when sucrose was used as the carbon source (Table-5). The minimum cell growth and β-glucosidase activity was observed when avicel was used as carbon source. Therefore, sucrose was selected as the carbon source for the production of β-glucosidase.
As a result of screening 6 cellulytic bacterial strains were isolated which produced strong haloes and the one which give strong haloes was selected for further study.

Table 1: Effect of time course on cell growth and \( \beta \)-glucosidase activity.

<table>
<thead>
<tr>
<th>Fermentation period (hours)</th>
<th>Cell growth O.D. (_{600})</th>
<th>( \beta )-glucosidase activity U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.76</td>
<td>0.45</td>
</tr>
<tr>
<td>8</td>
<td>1.414</td>
<td>1.23</td>
</tr>
<tr>
<td>10</td>
<td>1.636</td>
<td>1.7</td>
</tr>
<tr>
<td>12</td>
<td>1.402</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Table 2: Effect of temperature on cell growth and \( \beta \)-glucosidase production after 10 hours of formation.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Cell growth O.D. (_{600})</th>
<th>( \beta )-glucosidase activity U/mL</th>
<th>Soluble protein mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>0.825</td>
<td>0.55</td>
<td>0.043</td>
</tr>
<tr>
<td>50</td>
<td>1.221</td>
<td>1.52</td>
<td>0.067</td>
</tr>
<tr>
<td>55</td>
<td>1.321</td>
<td>1.7</td>
<td>0.070</td>
</tr>
<tr>
<td>60</td>
<td>0.846</td>
<td>1.72</td>
<td>0.062</td>
</tr>
<tr>
<td>65</td>
<td>0.625</td>
<td>0.68</td>
<td>0.033</td>
</tr>
</tbody>
</table>

Table 3: Effect of initial pH of medium on cell growth and \( \beta \)-glucosidase production.

<table>
<thead>
<tr>
<th>Medium pH</th>
<th>Cell growth O.D. (_{600})</th>
<th>( \beta )-glucosidase activity U/mL</th>
<th>Soluble protein mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>0.926</td>
<td>0.55</td>
<td>0.043</td>
</tr>
<tr>
<td>5.5</td>
<td>1.125</td>
<td>0.825</td>
<td>0.052</td>
</tr>
<tr>
<td>6.0</td>
<td>1.231</td>
<td>0.910</td>
<td>0.057</td>
</tr>
<tr>
<td>6.5</td>
<td>1.382</td>
<td>1.2</td>
<td>0.063</td>
</tr>
<tr>
<td>7.0</td>
<td>1.583</td>
<td>1.7</td>
<td>0.072</td>
</tr>
<tr>
<td>7.5</td>
<td>1.625</td>
<td>1.67</td>
<td>0.066</td>
</tr>
<tr>
<td>8.0</td>
<td>1.135</td>
<td>0.735</td>
<td>0.051</td>
</tr>
<tr>
<td>8.5</td>
<td>1.025</td>
<td>0.425</td>
<td>0.039</td>
</tr>
</tbody>
</table>

Table 4: Effect of nitrogen source on cell growth and \( \beta \)-glucosidase production.

<table>
<thead>
<tr>
<th>Nitrogen source (0.1%)</th>
<th>Cell growth O.D. (_{600})</th>
<th>( \beta )-glucosidase activity U/mL</th>
<th>Soluble protein mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH(_4))(_2)SO(_4)</td>
<td>1.32</td>
<td>1.70</td>
<td>0.072</td>
</tr>
<tr>
<td>(NH(_4))(_2)HPO(_4)</td>
<td>1.22</td>
<td>1.25</td>
<td>0.066</td>
</tr>
<tr>
<td>NaNO(_3)</td>
<td>1.20</td>
<td>1.15</td>
<td>0.057</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>1.15</td>
<td>0.92</td>
<td>0.043</td>
</tr>
<tr>
<td>Urea</td>
<td>0.95</td>
<td>0.52</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Table 5: Effect of different carbon sources on cell growth and the production of \( \beta \)-glucosidase.

<table>
<thead>
<tr>
<th>Carbon sources</th>
<th>Cell growth O.D. (_{600})</th>
<th>( \beta )-glucosidase activity U/mL</th>
<th>Soluble protein mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.024</td>
<td>0.23</td>
<td>0.054</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.414</td>
<td>1.72</td>
<td>0.073</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.342</td>
<td>0.25</td>
<td>0.058</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.345</td>
<td>0.51</td>
<td>0.062</td>
</tr>
<tr>
<td>Avicel</td>
<td>0.406</td>
<td>0.20</td>
<td>0.043</td>
</tr>
</tbody>
</table>

Purification

The enzyme \( \beta \)-glucosidase was purified from the culture supernatant by gel filtration and ion exchange chromatography.

Gel filtration

The concentrated enzyme sample containing 160U of \( \beta \)-glucosidase activity and 3.0 mg protein was subjected to gel filtration on sephadex G-75.
column. Elution was done with 0.05M phosphate buffer (pH 7.0). The β-glucosidase activity and protein contents were determined in each fraction. Activate fractions (16-38) were pooled and assayed for total β-glucosidase activity and protein. A recovery of 65 units of β-glucosidase activity with a specific activity was obtained (Figure-2).

 Ion exchange chromatography

The partially purified sample was fractioned on a Q-Sepharose chromatographic column. Elution was done with increasing gradient of NaCl (0.05-IM) in 0.05M Tris HCL buffer of pH 8.5. The fractions were analyzed for β-glucosidase activity and protein content. Active fractions (8-32) were pooled and assayed for total β-glucosidase activity and protein content. A recovery of 55 units of β-glucosidase activity with a specific activity of 183 U/mg was obtained (Figure-3).

Molecular weight determination

To determine the molecular weight of the purified enzyme. The purified fractions were subjected to SDS-PAGE that demonstrated one band of purified β-glucosidase. According to the position of the purified β-glucosidase band in the lane 4, its molecular weight was calculated to be 46kDa (Figure-4).

pH stability

pH stability of the β-glucosidase was investigated by pre-incubating the enzyme in buffer solution of various pH for 30 minutes at room temperature and residual activity was then determined at 60°C. β-glucosidase was stable over the pH range of 5.0-8.0. Below pH 5.0 and above pH 8.0 enzyme activities were gradually lost (Figure-5).

Thermal stability

Thermal stability of β-glucosidase was determined by pre-incubating the enzyme in phosphate buffer of pH 7.0 at various temperatures ranging from 50 to 80°C for 2 hours. The residual activity was then determined under standard conditions. The enzyme was stable up to 60°C. After 60°C there was gradual decrease in activity (Figure-6).

Assay pH

In order to obtain optimum assay pH, assay was carried out using 0.05M phosphate buffer ranging form 5-9. β-glucosidase activity increased up to pH 7.0, as the pH was increased further; there was a gradual decrease in activity (Figure-7).

Assay Temperature

The effect of temperature on β-glucosidase activity was determined by assaying at various temperature ranging from 50°C to 80°C for 10 minutes. β-glucosidase activity increased with increasing temperature up to 60°C. After 60°C there was a decrease in β-glucosidase activity (Figure-8).

Discussions

The main objective of the present research was to isolate cellulosic microbes from the local flora, which can effectively utilize cellulosic materials. A large number of decaying samples from different habitats were screened for the presence of bacteria capable of producing β-glucosidase activity. The most potent of the cellulytic bacterial strain was isolated and the production of β-glucosidase activity was optimized by studying different culture parameters, such as time course, temperature, pH, nitrogen sources and carbon sources (Figure-1).

The bacterium produced highest level of β-glucosidase activity when grown at 55°C in the presence of sucrose for 10 hours. The results are in agreement with there reported by Au et al., for cellulytic bacteria Acidothermus cellulolyticus. The bacterium showed high level of cell growth and β-glucosidase production when grown at pH 7.0 in the presence of sucrose for 10 hours. Above this pH the production was decreased (Table-3). Singh and Kumar [17] isolated Bacillus brevis from soil, which secreted extracellular cellulase. Production of cellulase was found to be optimal at pH 5.5. Robson and Chambliss [13] reported a Bacillus strain growing at pH 7.0 and producing β-glucosidases activity in the culture supernatant. An alkalophilic Bacillus strain producing cellulase activity was reported to grow well at higher pH ranging from 8.0 to 11.0.

A study on the effect of nitrogen sources on cellulase production was carried out using various inorganic and organic nitrogen sources (Table-4). The maximum enzyme activity was produced when ammonium sulfate was used as a nitrogenous source. Cellulomonas sp. has been reported to grow well when 0.3-0.6% ammonium sulphate was used in the fermentation medium [14].

The effect of different carbon sources on β-glucosidases production was also studied. When bacterium was grown in the presence of 0.5% sucrose as a carbon source, it showed higher levels of β-glucosidases activities as compared to those produced in the presence of 0.5% sucrose, cellobiose, glucose, and Avicel (Table-5). [16] studied β-glucosidases production of Acidothermus cellulolyticus when grown on a variety of carbon sources. Highest cell growth and cellulase production was obtained in

Fig. 2: Gel filtration through Sephadex G-75 column of culture supernatant after concentration lyophilization.

Fig. 3: Q-Sepharose chromatography of culture supernatant after gel filtration

Fig. 4: SDS-PAGE analysis of β-glucosidase purification. Lane 1: Standard markers; Lane 2: Crude enzyme; Lane 3: After gel filtration; Lane 4: After ion-exchange chromatography.

Fig. 5: Effect of pH on β-glucosidase activity
Fig. 6: Effect of temperature on \(\beta\)-glucosidase activity

![Graph showing the effect of temperature on \(\beta\)-glucosidase activity.]

Fig. 7: Effect of pH on the stability of \(\beta\)-glucosidase

![Graph showing the effect of pH on the stability of \(\beta\)-glucosidase.]

Fig. 8: Effect of temperature on the stability of \(\beta\)-glucosidase

![Graph showing the effect of temperature on the stability of \(\beta\)-glucosidase.]

the presence of cellobiose as the carbon source. Stoppok et al., [17] reported the production of endoglucanase and \(\beta\)-glucosidases from *Cellulomonas uda* when grown on microcrystalline cellulose, CMC, printed newspapers and some mono and disaccharides. Christakopoulos et al., [3] isolated an alkaline endoglucanase, secreted by *Bacillus pumilus* grown in submerged culture on a combination of oat split xylan and corn starch as carbon sources.

For the purification of the bacterial \(\beta\)-glucosidases, the bacteria were grown for 10 hours using optimal conditions for maximum enzyme yield. The culture supernatant containing the cellulytic enzymes was lyophilized and stored at \(-20^\circ\text{C}\). This concentrated enzyme sample was subjected to gel filtration using Sephadex G-75. A total of 40 fractions were collected which showed one major \(\beta\)-glucosidases peak and two major protein peaks on the chromatogram.

The partially purified enzyme sample was then subjected to ion-exchange chromatography through Q-Sepharose. 0.05 M Tris-HCl buffers of pH 8.5 and a linear gradient of 0.05-1 M NaCl was used for the elution of the enzyme sample. The chromatogram showed 16 fractions with one major \(\beta\)-glucosidases peak and one major protein peak. Osmundsson et al., [11] isolated two extracellular cellulose from the culture supernatant of *Sporotaphaga myxocoides* by using a similar two-step purification process of gel filtration and ion-exchange chromatography. Lau et al., [9] reported the purification of cellulase from; the culture supernatant of *Cellulomonas biazeotea* using a four-step purification process which included ammonium sulphate precipitation, gel filtration, ion-exchange chromatography and reversed-phase high performance
liquid chromatography. The enzyme was purified over 260-fold. Roy et al. [15] purified an extracellular endoglucanase from *Myceliophthora Thermophila* by ammonium sulphate precipitation and two consecutive ion-exchange chromatographic steps on DEAE-Sepharose A-50 columns. The enzyme was purified 13.8 fold.

To check the purity of the β-glucosidases, crude and purified enzyme fractions were subjected to SDS-PAGE on a 12% polyacrylamide gel. Crude enzyme sample showed a number of smeared bands while three bands and one band was obtained after gel filtration and single band after ion-exchange chromatography, respectively (Figure-4).

The molecular weight of the purified β-glucosidases determined by SDS-PAGE was found to be 46 kDa. Lau et al. [9] reported the purification of a novel cellobiose of *Cellulomonas biozotea* with a molecular mass of 109 kDa as determined by SDS-PAGE.

The β-glucosidase showed optimum activity at pH 7.0 and was stable in the pH-range of 6.5-8.0. Kaur et al., [8] reported the purification and characterization of β-glucosidase from a newly isolated thermophilic fungus, *Melanocarpus* sp. The molecular weight of β-glucosidase was determined to be ~92 and 102 kDa with SDS PAGE and gel filtration, respectively. The enzyme optimally active at 60°C and pH 6.0, though was stable at 50°C and pH 5.0 - 6.0.

The optimal temperature for β-glucosidase activity was found to be 60°C and the enzyme was stable, in the temperature range of 50-70°C for two hours. Above 60°C, a decline in enzyme activity was observed. Marques et al., [10] purified and characterized an β-glucosidase from *Trichoderma viride*. The enzyme had a temperature optimum of 50°C. It was stable at 55°C for 46 hours, and it retained approximately 20% of its activity after 30 minutes at 80°C.

### References