CHAPTER – VIII

PARTIAL PURIFICATION AND CHARACTERIZATION OF
GI PRODUCED BY STREPTOMYCES SP. SB - P1

8.1 INTRODUCTION

The purification for any fermentative product is designed by keeping in mind the ultimate usage of the product. The type of function to be performed by the product has direct influence on the required purity of the product. The nature and stability of the product is also of prime importance in designing the purification protocol. Glucose isomerase is used at commercial level in most of the cases in immobilised forms for High Fructose Corn Syrup production. As HFCS shall be used for human consumption therefore the GI preparations should be devoid of any harmful components [Raykovska et al., 2001].

Enzymes are usually sensitive to the environmental changes like temperature and pH. The non covalent interactions which maintain the native structure of protein are disrupted due to elevated temperature and the protein gets denatured. Glucose isomerase being a thermostable enzyme can be handled easily at room temperature.

The production of enzyme is done in submerged fermentation; therefore it is present in diluted form along with other biomolecules. Thus a specific protein concentration step is required. The enzyme is extracellular so we do not need to perform any cell disruption step. Glucose isomerase can be precipitated by ammonium sulphate or acetone. Desalting is usually done either by gel exclusion chromatography on sephadex G-50 or dialysis. This partially purified protein can be subjected to ion exchange chromatography for further resolution of proteins [Raykovska et al., 2001; Ladisch et al. 1977].

The characterization of purified enzyme helps in analyzing the suitable environmental conditions for the maximum activity. For industrial applications isomerisation reaction temperatures greater than 60°C have the advantage of faster
reaction rates, higher equilibrium concentrations of products and decreased viscosity of the substrate and product stream. Therefore, thermostable glucose isomerases with neutral or slightly acidic pH optima have potential industrial application. Owing to the industrial significance of the enzyme, glucose isomerases from various microorganisms have been studied, and their catalytic and physicochemical properties have been reviewed. All glucose isomerases reported so far have pH optima of 7.0 to 9.5. It would be useful to lower the pH optima of glucose isomerase to prevent sugar browning in the production of high-fructose syrup at elevated temperatures. The majority of these enzymes lost most of their catalytic activities under acidic conditions. For example, at pH 6.5, the enzymes from *Streptomyces olivochromogenes* and *Bacillus stearothermophilus* had approximately 50% of their activities, while the enzymes from *Actinoplanes missouriensis* and *Thermotoga maritima* lost about 40% of their activities. Only one xylose isomerase, from *Thermotoga neapolitana* (pH optima at 7.1), showed 80% of its maximum activity between pH 6.1 and 7.6. However, none of these enzymes has maximum activity at acidic pH [Liu et al., 1996].

Glucose isomerase forms one of the major proteins (approx. 10% of total protein) in the cell extracts of high GI producing microbes which makes the purification of the enzyme relatively simple. The growth rate of these bacteria is faster on glucose than on xylose. At present, the molecular mechanism of thermophilicity (i.e. high temperature activity and stability) in these enzymes is not clear and remains to be solved. Cysteine disulphide bonds apparently do not contribute to protein thermostability, as evidenced by full activity in the presence of a reducing agent [Lee and Zeikus 1991].

The amino acid sequence of GI from various sources has been determined. The glucose isomerase gene (*xylA*) from the *Streptomyces* sp. encodes a 386-amino-acid protein (42.7 kDa) showing extensive identities with many other bacterial glucose isomerases [Borgi et al., 2004].

This chapter deals with the partial purification of glucose isomerase produced by *Streptomyces sp. SB - P1* in submerged fermentation process. We have selected an extracellular enzyme producing isolate in order to reduce the cost of downstream processing. The kinetic and molecular characterization of partially purified GI is also aimed.
8.2 MATERIALS AND METHODS

Streptomyces sp. SB - P1 was grown in Medium No. 1 [Appendix - I] at 30°C and 100 RPM for a fermentation period of 96 h. The partial purification was started using 1000 mL of fermented broth. Crude enzyme extract was prepared as described in section 4.2.2.

8.2.1 Ammonium Sulphate Precipitation

The crude enzyme extract was chilled by keeping it in refrigerator for 2 hours. The extract was saturated to 85% with solid ammonium sulphate. To a total volume of 1000 mL of crude enzyme extract 610 g of ammonium sulphate powder was added. Protein was precipitated from the crude enzyme extract by slow addition and stirring while keeping it on ice bath. After dissolving the whole of ammonium sulphate, the saturated extract was kept at 4°C for 18 hours facilitating precipitation [Raykovska et al., 2001; Liu et al., 1996; Chen and Anderson, 1979].

The precipitates formed next day were collected by centrifuging the saturated extract at 10,000 RPM for 15 mins at 4°C. The pellet was dissolved in 0.05 Molar phosphate buffer. The enzyme assay (4.2.2.1) was performed for crude enzyme extract as well as the pellet dissolved in the buffer.

8.2.2 Gel Filtration for removal of salt on Sephadex G 50

Desalting of the dissolved pellet was done by performing gel exclusion chromatography on Sephadex G – 50. Sephadex G- 50 was soaked overnight by adding 10 g of gel to 100 mL of distilled water. After swelling of the beads it was washed twice and soaked in 0.05 Molar phosphate buffer [Appendix - II]. This was then used to prepare the desalting column.

Ammonium sulphate precipitation yielded 21 mL of the dissolved enzyme, 2 mL was kept as sample and 19 mL was loaded on a 100 mL column of Sephadex G – 50. The column was equilibrated with three times the bed volume of 0.05 M phosphate buffer of pH 7. After loading of the sample one third of the bed volume of elute was collected separately considering that elute is containing buffer without the enzyme. Next fraction of 35 mL was collected as the elute containing protein. Further three
fractions of 5 mL each were collected. All the fractions were used to determine the enzyme activity.

8.2.3 DEAE Cellulose Chromatography

DEAE Cellulose was used for separation of different protein components in the desalted enzyme extract. DEAE Cellulose, the ion exchange resin was weighed 8 g and soaked in 50 mL of distilled water overnight. After 18 h the excess of water was removed and the ion exchange resin was regenerated. The resin was soaked in 1Normal sodium hydroxide solution for 40 minutes. The excess of sodium hydroxide solution was removed and the resin was washed till the pH reaches 7. The alkali treated resin was then soaked in 1Normal hydrochloric acid for 20 minutes. The excess of acid was removed and washed repeatedly with distilled water till the pH reaches 7. After this again 1 Normal sodium hydroxide was added for 30 minutes. The excess of alkali was removed and the resin was washed repeatedly till pH reaches 7. The regenerated resin was used to pack the column. The column was equilibrated with three bed volumes of 0.05 Molar phosphate buffer, pH 7 [Appendix - II]. The fraction number G2, G3 and G4 were pooled as this had high GI activity as compared to others. This sample was loaded on ion exchange resin. For developing a gradient of sodium chloride 1 Molar solution was used. The gradient was developed by siphoning system, using 50 mL of sodium chloride in one beaker and distilled water in another beaker. The sample was loaded on the column and fraction collection started immediately after loading the column. Total 36 fractions were collected of 5 mL each. Protein content in these fractions was detected by measuring the optical density at 280 nanometers in UV-Visible spectrophotometer. All the fractions were used to perform GI assay by the method described in section 4.2.2.1.

8.2.4 Dialysis for Concentration of enzyme

The fractions exhibiting high GI activity were pooled and concentrated by dialysis against solid sucrose. Dialysis was done by pouring the sample in semi-permeable membrane bag and keeping it in a beaker containing solid sucrose for 18 h at refrigerated temperature. The dialysed sample was transferred to sterile eppendorf tubes and stored in the refrigerator.
8.2.5 Molecular Weight Determination by Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE).

The enzyme concentrated by dialysis was used for performing SDS PAGE to determine the molecular weight. The gel (13%) used for electrophoresis was prepared. Acrylamide, bisacrylamide, Tris HCl buffer, Sodium Dodecyl Sulphate, ammonium persulphate and N,N,N’,N’-tetra methyl ethylene diamine were mixed according to the details given in appendix II for preparing separating gel. The separating gel was poured in the casting unit leaving 1.5 cm from the top edge of the plate. The stacking gel was prepared by mixing acrylamide, bisacrylamide, Tris HCl and SDS in the ratio given in the appendix II. The comb was placed between the glass plates. The gel was left for polymerization for 45 minutes at room temperature. The gel of size 17 by 17.4 cm was prepared. The running buffer was poured in upper and lower chamber of the unit. The dialysed sample and protein marker used for silver staining was loaded in different wells. The marker composed of Phosphorylase B of molecular weight 97,400 Daltons, Bovine Serum Albumin 66,000 Daltons, Ovalbumin 43,000 Daltons, Carbonic anhydrase 29,000 Daltons, Lactoglobulin 18,400 Daltons and Aprotonin 6,500 Daltons. A constant voltage of 150 volts was applied and the current was run for 4 h.

Gel Staining

The gel was stained by silver staining method. After the run was over, the casting unit was disassembled and the gel was transferred into a staining tray. The gel was fixed with a solution containing 50% methanol + 10% acetic acid + 10% water for 30 minutes. After removing the excess of fixing solution the gel was transferred in 5% methanol for 15 minutes. Thereafter the gel was washed twice with distilled water for 5 minutes. It was then transferred to 0.02% sodium thiosulphate for 2 minutes. Again the gel was washed twice with distilled water for 45 seconds. The gel was then immersed in 0.2% silver nitrate solution for 25 minutes. After silver nitrate treatment, the gel was washed twice with distilled water for 1 minute. The gel was then immersed in developing solution containing 3% sodium carbonate, 37% formaldehyde solution and 0.02% sodium thiosulphate solution for developing the bands. The development was stopped by a stop solution containing 1.4% sodium ethylene diamine tetra acetic acid.
8.2.6 Kinetic characterization of partially purified GI

The kinetic characterization of GI was performed in order to determine optimum condition required for isomerisation of glucose to fructose.

8.2.6.1 Effect of Substrate Concentration on GI activity

The effect of substrate concentration on GI activity was determined by varying the glucose concentration in a series of 20 test tubes from 0.1 Moles to 2 Moles. All the other components of the assay were kept constant according to the details given in section 4.2.2.1.

8.2.6.2 Effect of Temperature on GI activity

The influence of varying temperature in GI activity was determined. A series of test tubes were prepared according to the details described in section 4.2.2.1 and incubated at a wide temperature range. The selected temperatures were 10, 20, 30, 40, 50, 60, 65, 70, 75, 80 and 90°C.

8.2.6.3 Effect of pH on GI activity

The effect of pH on GI activity was checked to find out the optimum pH required for GI activity. The control and test tubes were prepared according to the details given in section 4.2.2.1. The pH was varied in all the tubes using different buffers. Acetate buffer was used for pH 4 and 5 and phosphate buffer was used for pH 6 and 7 [Appendix - II]. Tris buffer was used for pH 8 and 9 and glycine buffer for pH 10 and 11[Appendix - II] [Raykovska et al., 2001].

8.2.6.4 Effect of Metal ions on GI activity

The effect of metal ions on the GI activity was determined. The selected metal salts used were magnesium sulphate, cobalt chloride, manganese sulphate, nickel chloride, calcium sulphate, zinc sulphate, barium chloride, copper sulphate, mercuric chloride, silver nitrate. The metal salts were tested by replacing magnesium sulphate from the enzyme assay mixture. All of them were added as 100 µL of 0.1 M concentration [Appendix - II]. The enzyme assay was performed according to the details given in section 4.2.2.1.
8.3 RESULTS AND DISCUSSION

8.3.1 Ammonium Sulphate Precipitation

The protein precipitated by ammonium sulphate was used to perform GI assay after dissolving it in 0.05 Molar phosphate buffer. A considerable increase in the specific activity was observed. The specific activity of concentrated protein was 2.65 U/mg of protein as compared to 0.32 U/mg of protein for the crude enzyme extract. This step yielded 47.25% recovery and 8.1 times fold purification.

8.3.2 Gel Filtration for removal of salt Sephadex G 50

The desalting of concentrated protein containing enzyme was performed on sephadex G – 50. The fraction number G2, G3 and G4 contained maximum amount of enzyme and they were pooled. The specific activity determined for this step of purification was 5.1 U/mg of protein. The fold purification calculated was 15.59 times with 38.2% recovery.

8.3.3 Ion Exchange for separation of proteins

The desalted enzyme was subjected to ion exchange chromatography for separation of other proteins from our enzyme. The representation of optical density for all the fractions measured at 280 nm in UV-Vis spectrophotometer is given in Fig. 8.1. The fractions containing high amount of protein did not exactly coincide with the high enzyme containing fractions. The high activity was found in the preceding few fractions. The fractions with high activity were pooled and enzyme kinetics studies were performed with them. The specific activity after this step of purification was 39.86 U/mg of protein with 121.8 times fold purification and 26.4% recovery.

8.3.4 Dialysis for Concentration of enzyme

The enzyme preparation was dialysed to concentrate the purified enzyme. This step yielded 18% recovery with 216 fold purification having a specific activity of 70.67 U/mg of protein. The detailed results for all the purification steps are presented in Table 8.1.
Partial purification and characterization of GI produced by Streptomyces sp. SB - P1

Fig. 8.1: Optical density plot for the fractions obtained after ion exchange chromatography

Table 8.1: Summary of Glucose Isomerase purification steps

<table>
<thead>
<tr>
<th>Steps</th>
<th>Fraction</th>
<th>Activity [U]</th>
<th>Total Enzyme Activity</th>
<th>TP [mg]</th>
<th>Total Protein Content [mg]</th>
<th>Specific Activity</th>
<th>Fold Purification</th>
<th>Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude Enzyme Extract</td>
<td>3.81</td>
<td>3810</td>
<td>11.64</td>
<td>11644.83</td>
<td>0.33</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>85% Amm Sulp Precipitation</td>
<td>51.44</td>
<td>1800.23</td>
<td>19.41</td>
<td>679.28</td>
<td>2.65</td>
<td>8.1</td>
<td>47.25</td>
</tr>
<tr>
<td>3</td>
<td>Gel Exclusion Chromatography Sephadex G-50</td>
<td>20.79</td>
<td>1455.42</td>
<td>4.06</td>
<td>285.30</td>
<td>5.10</td>
<td>15.59</td>
<td>38.2</td>
</tr>
<tr>
<td>4</td>
<td>Ion Exchage Chromatography DEAE Cellulose</td>
<td>67.06</td>
<td>1005.84</td>
<td>1.68</td>
<td>25.23</td>
<td>39.87</td>
<td>121.85</td>
<td>26.4</td>
</tr>
<tr>
<td>5</td>
<td>Dialysis</td>
<td>137.16</td>
<td>685.8</td>
<td>1.94</td>
<td>9.70</td>
<td>70.67</td>
<td>216</td>
<td>18</td>
</tr>
</tbody>
</table>
8.3.5 Molecular Weight Determination by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.

The glucose isomerase enzyme is a tetramer of four polypeptide chains of 43,000 D. We observed the band of partially purified GI on SDS PAGE gel near the marker band of 43,000 D. The results of SDS PAGE are shown in Plate. 8.1. Lane I and II contains partially purified glucose isomerase and Lane III is showing protein markers.

Gong et al., (1980) reported the presence of one protein band of glucose isomerase purified from *Actinoplanes missouriensis* on SDS-polyacrylamide gel. The molecular weight of this polypeptide band was measured to be 42,000 daltons. The molecular weight of glucose isomerase from *Bacillus coagulans* (58,000) Daltons is considerably larger than glucose isomerase from *Streptomyces* sp [Kwon et al., 1987].

Different microbial glucose isomerases characterized previously vary in molecular weight from 80000 to 200000 and are composed of two or four identical subunits. Molecular weight of glucose isomerase produced by *Clostridium thermosulfurogenes* and *Thermoanaerobacter strain B6A* is 200000 Daltons with tetrameric subunit composition [Lee and Zeikus, 1991] are similar to the 195000 Daltons enzyme present in *Lactobacillus brevis* [Yamanaka, 1968]. Glucose isomerases characterized from *Streptomyces* sp. and Arthrobacter was observed to be 1,85,000 Daltons [Kasumi et al., 1981; Smith et al., 1991]. GI from *Bacillus coagulans* and *Flavobacterium arborescens* also ranged between 157000 and 185 000 with tetrameric subunit compositions. The size and shape of glucose isomerase crystals vary from different sources. Glucose isomerases from *E. coli*, alkalophillic Bacillus, *Actinoplanes missouriensis* and *Streptomyces olivochromogenes* are dimers, with molecular weight of 80000-120000 Daltons [Lee and Zeikus, 1991].
Lane I and Lane II- Partially purified GI from *Streptomyces sp. SB - P1*

Lane III – Marker details:-

1. Phosphorylase B – 97,000 D
2. Bovine Serum Albumin – 66,000 D
3. Ovalbumin – 43,000 D
4. Carbonic anhydrase – 29,000 D
5. Lactoglobulin – 18,400 D
6. Aprotonin – 6,500 D

Plate 8.1: SDS PAGE of partially purified GI from *Streptomyces sp. SB - P1*, Lane I and II showing a band near 43,000D protein marker and Lane III showing protein marker bands.

8.3.6 Kinetic characterization of purified GI

8.3.6.1 Effect of substrate concentration on GI activity

The effect of substrate concentration on GI activity was studied. The activity increased progressively till 1.2 Moles and there was no further increase observed. The $K_m$ calculated for glucose by Lineweaver Burk plot is 0.99 Moles and $V_{max}$ was 9.78 U/mg. The results are depicted in Fig. 8.2 and 8.3. Glucose isomerase produced from *Actinoplanes missouriensis* exhibited $K_m$ value of 1.33 Moles for glucose [Gong et al., 1980]. Dhungel et al., (2007) reported 0.45 M as $K_m$ and 0.18 U/mg as $V_{max}$ for glucose by using psychrotolerant *Streptomyces* sp. Raykovska et al., (2001) observed the $K_m$ and $V_{max}$ as 55.5 mM and 31.3 U/mg for *Streptomyces thermovulgaris*. Lee and Zeikus, (1991) reported $K_m$ of 140 and 120 mM for glucose by GI from *C. thermosulfurogenes* and *Thermoanaerobacter* respectively. Liu et al., (1996) worked out $K_m$ for *Thermoanaerobacterium* strain JW/SL-YS 489 to be 130 mM and $V_{max}$ 6 U/mg. Fan et al., (2011) observed $K_m$ and $V_{max}$ values to be 11 mM and 25 U/mg respectively for xylose. Brown et al., (1993) *Thermotoga maritima* exhibited $K_m$ value of 118 mM for glucose. The enzyme possesses higher $K_m$ value for glucose than
fructose. *Lama et al., (2001)* studied the glucose isomerase catalytic properties of *Bacillus thermoantarcticus* and observed $K_m$ and $V_{max}$ values to be 167 mM and 6.3 U/mg respectively. *Ogbo and Odibo, (2007)* determined $K_m$ and $V_{max}$ for glucose isomerase produced by *Saccharococcus caldoxylosilyticus* to be 334 mM and 0.92 U/mg of protein.

![Fig. 8.2: Effect of substrate concentration on GI activity](image1)

![Fig.8.3: Lineweaver Burk plot for GI activity](image2)
8.3.6.2 Effect of Temperature on GI activity

Glucose isomerase is known to be a thermostable enzyme which has optimum activity at high temperature. The maximum activity of enzyme produced by our isolate *Streptomyces sp. SB - P1* was observed at 75°C. The activity was good enough on and above 50°C with a broad optimum range from 60 to 80°C. This kind of broad range for optimum activity is beneficial for industrial purposes because maintenance of very critical and narrow temperature range is difficult at commercial level. The results are depicted in Fig. 8.4.

![Fig.8.4: Effect of temperature on GI activity](image)

Glucose isomerases are generally classified into two types of enzymes according to their thermal stability. Glucose isomerase from *Lactobacillus brevis* [Yamanaka, 1968] and *E. coli*, which are active at 37-50°C, belong to the thermolabile type of glucose isomerase. Glucose isomerases from certain mesophilic microbial sources [e.g. *S. phaeochromogenes* and *A. missouriensis*], however, can display quite high thermal stability. Glucose isomerases produced from *C. thermosulfurogenes* and *Thermoanaerobacter, Thermotoga maritima, Thermotoga neapolitana* are highly thermostable, and they belong to the latter type of glucose isomerase [Lee and Zeikus, 1991]

The optimum temperature reported for thermophilic organisms is usually above 80°C. Brown et al., (1993) observed 105-110°C as optimum temperature for *Thermotoga maritima. Thermoanaerobacter ethanolicus* was found to isomerise best at

8.3.6.3 Effect of pH on GI activity

The optimum pH range observed for GI produced by Streptomyces sp. SB - P1 was from 6 to 9. The highest activity was observed at pH 7 but the analysis indicates slightly alkalophillic preference for the enzyme activity which is in accordance with most of the earlier reports. The results are presented in Fig. 8.5.

![Fig. 8.5: Effect of pH on GI activity](image)

The pH optima for mesophilic as well as thermophilic microorganisms fall between a range of 5 to 9. This also supports the structure and amino acid sequences

### 8.6.6.4 Effect of Metal Ions on GI activity

The effect of various cations was checked on glucose isomerase activity. Magnesium and cobalt are the known activators of the glucose isomerase and enhanced the activity of GI produced from *Streptomyces* sp. SB - P1 also. Manganese is the next level of choice of some glucose isomerases which also proved to be an activator for our enzyme. Calcium was also found to activate the enzyme to some extent. Nickel, zinc and iron had very little effect on glucose isomerase activity. Barium, Mercury and copper totally inhibited the enzyme activity as also stated by Lee and Zeikus, (1991). The results are depicted in Fig. 8.6.
Glucose isomerase from different microbial sources, respond differently to the presence of divalent cations, Fan et al., (2011) *Thermoanaerobacter ethanolicus* reported a great deal of enhancement in activity in the presence of magnesium and cobalt ions. Ogbo and Odibo, (2007) *Saccharococcus caldoxylosilyticus* requires Mn>Mg>Co. Copper was found to inhibit GI activity but calcium did not show such an inhibition. Lee and Zeikus, (1991) stated that the treatment of enzyme preparation with EDTA renders enzyme totally inactive. The addition of cobalt and magnesium did not show a synergistic effect on glucose isomerase activity. Manganese did not enhance the glucose isomerase activity whereas it decreased the activation effect of cobalt or magnesium. Glucose isomerase from *B. coagulans* also requires these two cations for optimum activity.

### 8.4 CONCLUSIONS

The purification of enzyme is the most important step in industrial production of the enzyme. Various methods have been described by investigators for the purification of glucose isomerase. After screening a few methods we have designed a process for partial purification of glucose isomerase. Fractionation with ammonium sulphate gave a high degree of purification of the enzyme. The enzyme was precipitated by saturation of crude extract up to 85%. There was a progressive increase in the specific activity of enzyme through the purification steps. We observed
216 fold purification of enzyme with specific activity of 70.67 Units/mg of protein and 18% enzyme recovery in the finally dialysed sample. We observed a clear band of our partially purified enzyme near the protein marker of 43,000 Daltons which indicates the molecular weight of our enzyme as the same.

The $K_m$ calculated for glucose by Lineweaver Burk plot is 0.987 Moles and $V_{max}$ was 9.78 U/mg. The characterization of enzyme yielded maximum glucose isomerase activity at pH 7 and 75°C. GI produced by Streptomyces sp. SB - P1 was maximally activated by the presence of both the cations magnesium and cobalt. The individual effects shown by magnesium was highest followed by cobalt and manganese. Calcium also activated the enzyme to a small extent but all other metal ions were not at all useful. The pH and temperature range both were quiet broad which establishes fair enough chances of industrializing the process.