Chapter 5

Purification and Characterisation of cyclodextrin glycosyl transferase produced by *Exiguobacterium aurantiacum*

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5.1 Introduction

Purification of the enzyme becomes necessary for understanding properties of the enzyme and recommending its applications. There is a need to concentrate the enzyme before purification by chromatographic methods. The methods used for concentration include ultrafiltration, ammonium sulfate precipitation, solvent precipitation (ethanol, acetone) or reverse osmosis. Many scientists use a combination of various methods to concentrate the CGTase present in the supernatant of the culture fluid. Some researchers have reported the application of ultrafiltration, ammonium sulphate precipitation and starch adsorption purification of CGTase (Tachibana et al., 1999 and Yagi et al., 1986). Purification of CGTase can be achieved by high resolution chromatographic methods to increase the purity of the enzyme. Different methods are used for purification of CGTase. Most of the methods are three step methods, i.e., ammonium sulphate precipitation, dialysis and affinity chromatography / adsorption chromatography / Ion exchange chromatography or Gel permeation chromatography.

Affinity chromatography is a method of separating biochemical mixtures and is based on a highly specific interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand.

Many researchers have used affinity chromatography to purify their CGTases in line with other chromatographic methods. Affinity chromatography is commonly used in the purification of CGTase (Tachibana et al., 1999; Larsen et al., 1998; Volkova et al., 2000; Bovetto et al., 1992; Mori et al., 1994 and Gawande and Patkar, 2001. Gawande & Patkar (2001) utilised an affinity matrix with gelatinized corn starch and purified a CGTase from *Klebsiella pneumoniae* with a 68% yield.

Some other researchers, such as Ferrarotti et al., (1996) have even chosen affinity chromatography as their main chromatographic method to play a vital role in purifying their CGTases. The molecular weight of the enzyme can be estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE).

SDS- PAGE is a technique that separates proteins according to their electrophoretic mobility. SDS is a strong detergent which denatures proteins and renders a net negative charge to them. The electrophoretic mobilities of these
proteins will be a linear function of the logarithms of their molecular weights. Proteins are separated according to their mass and their molecular weight can be calculated using standard molecular weight markers. The bands of the desired protein is then eluted from the gel and then treated and analyzed using Matrix Assisted Laser Desorption Ionization –Time of Flight Mass Spectrophotometry (MALDI – TOF MS).

MALDI is a soft ionization technique used in mass spectrometry which allows the analysis of biomolecules that tend to be fragile and fragment when ionized by more conventional ionization methods. The MALDI is a two step process in which first, desorption is triggered by a UV laser beam. The matrix material consists of crystallized molecules of 3, 5-dimethoxy-4-hydroxycinnamic acid or α-cyano-4-hydroxycinnamic acid which heavily absorbs UV laser such as nitrogen lasers (337 nm). The matrix is desorbed, then ionized which in turn ionizes the peptide. The mass spectrophotometer then measures the mass-to-charge ratio (m/z) of charged peptide which generates a characteristic mass spectrum called peptide mass fingerprint. Computer programs like Mascot (Matrix Science Ltd., London) analyze the peptide mass fingerprint and predict the protein sequence based on sequence homology with existing peptides in database.

In this study, CGTase produced by *E. aurantiacum* was purified using ammonium sulphate precipitation, dialysis, starch adsorption chromatography and ion exchange chromatography. The molecular weight was estimated using SDS-PAGE and the partial sequence was obtained using MALDI – TOF MS and Mascot Peptide mass fingerprinting software.

### 5.2 Materials and Methods

#### 5.2.1 Production of CGTase in 3 L fermentor under optimized conditions

The medium used for production was the medium optimized by Taguchi Design of experiments. One 250 ml flask containing 100 ml nutrient broth pH 10 was inoculated with 16 h old culture of *E. aurantiacum* and incubated at 37°C at 150 rpm for 16 hours. The flask culture was used as seed medium to inoculate 900 ml production medium containing (g/L) soluble starch 10, peptone 5, yeast extract 1
and MgSO$_4$.7H$_2$O 0.02. The pH of the medium was adjusted to 10 using sterile 10% Na$_2$CO$_3$. Fermentation was carried out at 37°C for 24 h with 1 L working volume with air flow rate of 0.5 vvm (volume of air per unit volume of medium per minute) and agitation was 100 rpm. Fermentation was carried out in batch mode till stationary phase approached. After fermentation, the cells were centrifuged at 10,000 g for 20 min at 4°C. Cell free supernatant was used as crude enzyme.

5.2.2 Enzyme assay and protein estimation

The CGTase activity was estimated using the modified method of Goel and Nene (1995) as described in section 2.2.3. The protein content was determined using bovine serum albumin as standard by the method described by Bradford (1976).

5.2.3 Ammonium sulphate precipitation and Dialysis of CGTase

Ammonium sulphate was added to five flasks containing 100 ml of the crude enzyme to obtain 40, 50, 60, 70& 80% saturation and incubated overnight at 4°C. After 24 h, the aliquots were centrifuged at 10,000 g for 15 min at 4°C and the weight of each precipitate was recorded. A graph of weight of the precipitate against percentage concentration of ammonium sulphate was plotted and the optimum ammonium sulphate concentration for CGTase was estimated from the graph. The precipitate was dissolved in 50 mM Tris buffer pH 8.5. It was added in the pre treated dialysis bag and dialysed against Tris buffer. The buffer was changed every 2-3 hrs. Dialysis was performed for a minimum period of 6 hours or overnight at 25°C. The enzyme activity and specific activity of the dialysate was estimated. The dialysate was stored at 4°C until further use. The dialysis bag was pre treated by boiling in a solution of 2% sodium bicarbonate containing 1mM EDTA.

5.2.4 Starch adsorption chromatography

Starch adsorption column was prepared by gently heating 10 % (w/v) soluble starch in 50 mM Tris buffer. The gel was poured in an alcohol rinsed glass tube and allowed to set overnight. The column was washed and equilibrated with wash buffer containing Tris Cl buffer pH 7.6 with 1mM CaCl$_2$. The absorbance of the wash was measured at 280 nm (A280) till a constant baseline or readings less than
0.002 was obtained. Two ml of dialysate was then loaded in the equilibrated starch adsorption column. The wash buffer was added and 1 ml fractions were collected. After obtaining the baseline, the elution buffer containing 50 mM Tris Cl pH 7.6 and 1 mm β-cyclodextrin was added. 1 ml fractions were collected and the absorbance at 280 nm and enzyme activity of the fractions was estimated. The fractions showing enzyme activity were pooled and stored as adsorption purified enzyme at 4°C until further use.

5.2.5 Ion Exchange chromatography

For purification of CGTase using ion exchange chromatography, 500 µl of adsorption purified enzyme was loaded in a previously equilibrated DEAE-Sepharose (Sigma) column. The column was washed with 50 mM Tris Cl buffer pH 7.6 with 0.1 M NaCl. The enzyme was eluted with 50 mM Tris Cl buffer pH 7.6 with 0.2 M NaCl. One ml fractions were collected, their absorbance was measured at 280 nm and their enzyme activity was estimated. The fractions showing enzyme activity were pooled and stored as ion exchange purified enzyme at 4°C until further use.

The enzyme activity, protein content and specific activity of the crude enzyme, dialysate, starch adsorption purified enzyme and Ion exchange purified enzyme was calculated.

The adsorption chromatography and ion exchange chromatography purified enzyme was used for characterisation of CGTase.

5.2.6 Effect of pH on purified CGTase activity

The optimum pH for the purified enzyme was determined by replacing 50 mM Tris Cl buffer pH 8.0 in the CGTase assay with the following buffers: 50 mM Phosphate buffer (pH 5 – 7) and 50 mM Tris buffer (pH 8 – 12). The reaction was carried out by incubating 1 % starch and 0.1 ml enzyme in respective buffer for 20 min at 60°C. The enzyme activity was estimated as described by Goel and Nene (1995). A graph of enzyme activity versus pH profile was plotted.
5.2.7 Effect of pH on the stability of purified CGTase

The pH stability of the enzyme was measured by incubating 0.1 ml of purified CGTase enzyme with 1 ml of 50 mM Phosphate buffer (pH 5 – 7) and 50mM Tris buffer (pH 8 – 12) at 60°C, without substrate for 30 minutes. The residual activity of the enzyme was assayed as described in section 2.2.3 by adding 0.1 ml of the enzyme mixture to 1 ml of 1 % soluble starch incubated at 60°C for 20 minutes.

5.2.8 Effect of temperature on purified CGTase activity

The optimum temperature for the purified recombinant CGTase enzyme was determined by incubating the reaction mixture of CGTase assay in 50 mM Tris buffer pH 8.0, at different temperatures, ranging from 10°C – 100°C for 20 minutes. The CGTase activity was assayed as mentioned in section 2.2.3.

5.2.9 Effect of temperature on the stability of purified CGTase

The temperature stability of the enzyme was measured by incubating 0.1 ml of purified CGTase enzyme in 50 mM Tris buffer pH 8.0 without substrate at different temperatures (10°C – 100°C) for 30 minutes. Standard CGTase assay was performed to determine the residual activity. The graph of residual CGTase activity versus temperature was plotted.

5.2.10 Effects of metal ions and inhibitors on purified CGTase activity

To study the effect of metal ions and inhibitors on CGTase activity, 0.1 ml of purified CGTase was added in 50 mM Tris buffer pH 8.0, containing different metals at 5 mM (final concentration), detergents at 10 % concentration and inhibitors at 1 mM (final concentration) and incubated for 10 minutes at 25°C. The CGTase assay was performed to determine the residual activity of the enzyme.

5.2.11 Study of Kinetic Parameters of enzyme

The Michales constant ($K_m$) and reaction rate ($V_{max}$) values for the pure enzyme were determined by incubating 0.1 ml of purified CGTase enzyme in 1 ml of 50 mM Tris buffer at various concentrations of soluble starch solution, ranging from 2 mg/ml to 20 mg/ml at 60°C for 10 minutes. The values of $K_m$ and $V_{max}$ were then
determined using Sigma plot enzyme kinetics software. The turnover number ($K_{cat}$) was calculated using $V_{max}$ value.

5.2.12 Stability of CGTase at 4°C (shelf life studies)

The purified enzyme was stored at 4°C and the residual activity was estimated every month.

5.2.13 Molecular weight determination of CGTase using SDS-PAGE

The enzyme for Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was concentrated using acetone precipitation method. This method irreversibly denatures and concentrates proteins for SDS-PAGE (Smith et al, 1985). For acetone precipitation of the enzyme, 1.2 ml of ice cold chilled acetone was added to 0.2 ml of purified CGTase, vortexed for 5 min and incubated overnight in ice at 4°C. After incubation, the sample was centrifuged at 10,000 g for 15 min at 4°C to obtain the enzyme precipitate. The sample for SDS PAGE was prepared by adding 30 µ SDS-PAGE sample buffer to the acetone precipitate and boiling in boiling water bath for 10 min.

The SDS-PAGE was performed as using the vertical electrophoresis unit described by Laemmli (1970). The plates were wiped with alcohol and the cassette was assembled and sealed with 1 % agarose. The resolving gel mix (Table 5.1) was added in cassette and overlayed with water saturated isobutanol. After 40 minutes of polymerisation, the stacking gel mix (Table 5.1) was overlayed over the resolving gel and the comb was inserted. After 20 minutes, the comb was removed and the wells were washed with distilled water. For electrophoresis, 20 µl of sample was loaded in the well and electrophoresed in Tris Glycine electrophoresis buffer at 100 volts for 1 h. After electrophoresis, the gel was removed from the cassette and stained with Commassie brilliant blue R-250 for 1-2 hr and destained using destaining solution till completely decolorised.

The gel was observed using Syngene gel documentation system UK and molecular weight was estimated using molecular weight standards of 6500 – 97400 Da (Bangalore Genei) and G-BOX Syngene software. The bands corresponding to CGTase were excised from the gel and used for MALDI-TOF MS analysis.
Table 5.1: Composition of Stacking and Resolving gel used for SDS – PAGE

<table>
<thead>
<tr>
<th>S. No</th>
<th>Component</th>
<th>Resolving gel mix Volume (ml) / 10 ml gel mould (12 %)</th>
<th>Stacking gel Mix Volume/ 4 ml gel mould</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Water</td>
<td>3.3</td>
<td>2.7</td>
</tr>
<tr>
<td>2</td>
<td>Acrylamide mix (30 % T, 2.6 % C)</td>
<td>4.0</td>
<td>0.67</td>
</tr>
<tr>
<td>3</td>
<td>1.5 M Tris Cl Resolving buffer pH 8.8</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>1.0 M Stacking gel buffer pH 6.8</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>10 % SDS</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>10 % Ammonium persulphate</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>7</td>
<td>TEMED</td>
<td>0.004</td>
<td>0.004</td>
</tr>
</tbody>
</table>

5.2.14 Activity staining of CGTase

For activity staining, native PAGE was performed with 10% polyacrylamide according to the method described by Davis (1964). After electrophoresis, the gel was washed in 0.02 M acetate buffer pH 5.5 containing 3 % soluble starch and then incubated at 40°C for 2 hr. After enzymatic reaction the excess starch was washed off from the gel surface using acetate buffer and the gel was stained with a solution containing 3 % potassium iodide and 1.3 % iodine (Jeang et al., 1999).

5.2.15 Detection of CGTase in phenolphthalein indicator gel

For detection of CGTase by the phenolphthalein indicator method, the enzyme was separated using 10 % native PAGE as described above. After electrophoresis, the gel was removed and indicator gel was uniformly poured over the polyacrylamide gel and was allowed to solidify. The indicator gel was prepared by mixing 0.24 g soluble starch, 0.14 g agar (prepared in 0.2 M phosphate buffer pH 8) and 0.5 ml of 0.4% phenolphthalein at 50°C. After incubation at 37°C for ten minutes, the indicator gel was flooded with a 0.1% (w/v) sodium carbonate solution and observed for CGTase activity (Pakzad et al., 2005).
5.2.16 Analysis of CGTase using MALDI –MS

The band corresponding to CGTase was excised from the gel and transferred to a 0.5 µl microfuge tube. It was washed with 50 mM NH₄HCO₃/acetonitrile 1+1 (v/v) for 15 min till the gel particles shrink. The gel pieces are retreated with 50 mM NH₄HCO₃ and equal volume of acetonitrile (HPLC grade) and incubated for 15 min. After appropriate incubation, the acetonitrile is removed and the gel particles are dried. The dried gel particles are reduced for 45 min at 56°C by addition of freshly prepared 10 mM dithiotreitol (in 50 mM NH₄HCO₃). After incubation, the excess liquid is removed and replaced with freshly prepared 55 mM iodoacetamide (in 50 mM NH₄HCO₃). After incubation for 30 min at room temperature in the dark, the gel particles are washed with 50 mM NH₄HCO₃ and acetonitrile (1+1; v/v).

Freshly prepared trypsin solution (Sigma, Proteomics grade) (in 25 mM NH₄HCO₃) was added to the reduced and alkylated gel and incubated at 37°C for 30 minutes. The excess enzyme solution was removed and 25 mM NH₄HCO₃ (approx. 2-3µl) was added to keep the gel moist. The peptides from the gel were extracted by addition of extraction buffer (50/50 trifluoroacetic acid and 0.1% acetonitrile) in the microfuge tube containing the treated gel.

After extraction, the sample was mixed with crystallized 3, 5- dimethoxy-4- hydroxy cinammic acid and spotted on a MALDI plate. The solvents vaporize leaving recrystallised matrix. The matrix and analyte (CGTase from the gel) were co-crystallised on the spot. The plate was analyzed on a MALDI- TOF MS analyser (Bruker Daltonics GmBh) according to manufacturers instructions. The peaks and the peptide mass generated by the mass spectrophotometer was analyzed using Mascot search engine (Matrix Science) that uses mass spectrometry data to identify proteins from primary sequence databases. The primary sequence was also analysed using BLASTp (Basic Local Assignment Search Tool for proteins, NCBI).
5.3 Results

5.3.1 Enzyme production

The CGTase production increased with incubation period (Fig. 5.1). It was optimum at 18 h and activity decreased after stationary phase. The optical density increased with time and was maximum at 6h.

\[ \text{Fig. 5.1 : } \text{Time course of CGTase production by E. aurantiacum using soluble starch as a substrate} \]

After appropriate incubation, the broth was centrifuged and the cell free supernatant was used for ammonium sulphate precipitation.

5.3.2 Ammonium sulphate precipitation and dialysis

The optimum concentration of ammonium sulphate required for maximum yield of CGTase was found to be 70 % based on the weight of the precipitate (Fig. 5.2). Some researchers recommend addition of 80 % concentration of ammonium sulphate for precipitation of CGTase (Tachibana et al., 1999).
When the concentration of ammonium sulphate increases, the solubility of CGTase decreases and the enzyme precipitates due to a process called salting out. The salts and other contaminants were eliminated from the enzyme precipitate using a dialysis membrane. The dialysate was loaded in a starch adsorption column.

5.3.3 Starch adsorption chromatography

The enzyme was purified by starch adsorption chromatography. It was observed that the enzyme started to elute at the fourth fraction with enzyme activity of 8 U/ml and reached its peak at the tenth fraction with 24 U/ml. The concentration then decreased subsequently to 0.5 U/ml at the fourteenth fraction. All the ten fractions (No. 4 to 14) which demonstrated CGTase activity were pooled and stored at 4°C as adsorption purified CGTase. The absorbance at 280 nm increased reaching a peak at fraction no. 9 and 10 corresponding to the eluants containing maximum enzyme activity (Fig. 5.3).
CGTase is generally purified by two steps purification procedures which were also used by Volkova et al. (2000) to obtain pure CGTase from Bacillus sp. 1070 by using β-CD Sepharose 4B chromatography and immobilized metal ion affinity chromatography. CGTase binds to cyclodextrin present in column. The presence of C-terminal raw starch binding motif on the CGTase plays an important role for binding the CGTase to CD affinity that is used in this column (Wind et al., 1995). Other researchers also have suggested that raw starch binding domain in domain E were capable of binding strongly to cyclodextrins (Penninga et al., 1996). Gawande and Patkar (1995) reported purification of a novel raw starch degrading cyclomaltodextrin glucanotransferase (CGTase; E.C. 2.4.1.19) produced by Bacillus firmus by ultrafiltration, affinity and gel filtration chromatography.

5.3.4 Ion Exchange chromatography

The enzyme purified by starch adsorption column was applied to a DEAE-Sepharose anion exchange column in a 5 ml syringe with 7 cm X 1.5 cm dimension filled with 4 ml of resin at a flow rate of 1ml/min. There was a complete loss of CGTase activity when the enzyme was purified via DEAE-sepharose. Two distinct peaks corresponding to protein fractions were observed at fraction 25 and 37. But both the fractions demonstrated absence of CGTase activity (Fig.5.4).

Tachibana et al. (1999) have purified CGTase using ion exchange chromatography, hydrophobic interaction chromatography and affinity chromatography. Wind et al. (1998) purified amylase with CD producing activity from *Thermoanaerobacterium thermosulfurigenes* in a single step using affinity chromatography. Savergave et al. (2008) have also reported DEAE-sepharose as an efficient exchange column for CGTase. The enzyme activity and yield of the enzyme obtained by the two step purification method is presented in Table 5.2.

**Table 5.2 :** Purification of CGTase produced by *E. aurantiacum*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein mg/ml</th>
<th>Total Units</th>
<th>Specific Activity U/mg</th>
<th>Yield %</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>30</td>
<td>75</td>
<td>0.05</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Dialysate</td>
<td>19</td>
<td>87</td>
<td>0.45</td>
<td>86.2</td>
<td>9</td>
</tr>
<tr>
<td>Starch Adsorption</td>
<td>17</td>
<td>144</td>
<td>1.263</td>
<td>52.08</td>
<td>25</td>
</tr>
</tbody>
</table>

CGTase was successfully purified using Starch Adsorption chromatography with 25 fold purification. Ion exchange chromatography was also used but there was no increase in enzyme activity. After repeated elutions, the enzyme was recovered from the pooled fractions, but there was loss in enzyme activity. The enzyme activity of affinity purified fraction was 24 U/ml. The affinity purified enzyme was
thus used as purified enzyme for the further studies on characterisation of CGTase. A two step method for CGTase purification is demonstrated in the present studies.

5.3.5 Effect of pH on purified CGTase activity

The effect of pH on activity of purified CGTase is illustrated in Fig. 5.5. The optimum pH for purified CGTase of *E. aurantiacum* was found to be 9 which is suitable for cyclisation and hence production of cyclodextrins.

![Fig. 5.5: Effect of pH on activity of CGTase](image)

Most of the reported purified CGTase exhibited optimum pH ranging from 5.0 to 8.0. Purified CGTase from *Bacillus stearothermophilus* (Ahn *et al*., 1990 and Tachibana *et al*., 1999) exhibited an optimum pH of 5.0 - 5.5 while Bovetto *et al*. (1992) found that the optimum pH for *Bacillus circulans* E 192 CGTase was pH 5.5. CGTase from recombinant *Brevibacillus brevis* CD162 (Myung *et al*., 1998) has an optimum pH of 8.0.

5.3.6 Effect of pH on the stability of purified CGTase

The effect of pH on stability of CGTase was studied (Fig. 5.6).

![Fig. 5.6: Effect of pH on stability of CGTase](image)
E. aurantiacum CGTase was stable from pH 5 – 12, but demonstrated greater stability at alkaline pH scale). As alkaliphiles are preferred sources of CGTase, the enzyme is generally stable at alkaline pH.

5.3.7 Effect of temperature on purified CGTase activity

The results of effect of temperature on the activity of purified CGTase are presented in Fig. 5.7.

**Fig. 5.7:** Effect of temperature of CGTase activity

Purified CGTase from E. aurantiacum had temperature optimum of 50 °C (Fig. 5.7). The optimum temperature of CGTase activity for most mesophiles and alkaliphiles is generally in the range of 30 – 40 °C. Likewise, CGTases from thermophiles show a higher optimum temperature for enzyme activity. E. aurantiacum is a mesophile with optimum growth temperature of 37 °C but the enzyme CGTase it produces is moderately thermostable. CGTase from Thermoanaerobacterium thermosulfurigenes EM1 (Wind *et al*., 1998) and Thermococcus kodakaraensis KODI (Rashid *et al*., 2002) exhibited higher range of optimum temperatures of 90°C to 100°C, 80°C to 85°C and 80°C respectively.

5.3.8 Effect of Temperature on the stability of purified CGTase

The results of effect of temperature on stability of purified CGTase are illustrated in Fig. 5.8. The enzyme was found to be stable in temperature range of 50 to 60°C.
Generally CGTases from mesophiles like *Klebsiella pneumoniae* AS-22 (Gawande and Patkar, 2001) have optimum temperature range of 35-50°C. CGTase from *Brevibacillus brevis* AS-22 (Gawande et al., 2001) has optimum temperature range of 35-50°C. CGTase from *Bacillus clarkii* 7364 (Takada et al., 2003) and *Brevibacillus brevis* CD162 (Myung et al., 1998) were stable up to 40°C, 45°C and 50°C respectively. The stability studied revealed that *E. aurantiacum* was stable at 10°C upto six months.

5.3.9 Effects of metal ions, inhibitors and detergents on CGTase

The effect of metal ions, inhibitors and detergents on purified CGTase activity was studied and the results are presented in Fig. 5.9.
This enzyme was shown to be metal independent since it retains more than 90% of activity with EDTA. This demonstrates that it is not a metallo-enzyme as no metal ions significantly enhances its activity. Additionally, K⁺, Mg²⁺ and Ca²⁺, do not lower the CGTase activity either. But strong inhibition of CGTase activity was observed by Fe³⁺ metal ions with complete loss of activity which suggests the presence of histidine and tyrosine residues in the active center.

Cu²⁺ and Zn²⁺ neither decreased nor enhanced CGTase activity of *E. aurantiacum*. The majority of the CGTases are inhibited by Cu²⁺ and Zn²⁺ (Tonkova, 1998) including the recombinant CGTase of Bacillus sp. TS1-1. CGTase from *Brevibacterium* sp. No. 9605 (Mori *et al.*, 1994), *Bacillus halophilus* INMIA-3849 (Abelian *et al.*, 2002), *Bacillus firmus* (Yim *et al.*, 1997), *Bacillus* sp. AL-6 (Fujita *et al.*, 1990) and *Bacillus agaradhaerens* (Martins and Hatti-Kaul, 2002) were strongly inhibited by Zn²⁺.

It is interesting to find out that K⁺ and Ca²⁺ in the form of KCl and CaCl₂ help to promote the CGTase activity. Previous studies show that CGTase from *Brevibacterium* sp. 9605 (Mori *et al.*, 1994), *Bacillus autolyticus* 11149 (Tomita *et al.*, 1993) and *Paenibacillus* sp. F8 (Larsen *et al.*, 1998) exhibited more stable activity in the presence of Ca²⁺.

In the presence of Hg²⁺ ions CGTase retains 100% of its activity, that probably proves the absence of sulfur-containing amino acids in the active center of the enzyme. The detergents tween 80 and SDS caused partial loss of activity. Iodoacetamide binds covalently with the thiol group of cysteine so the protein cannot form disulfide bonds rendering the enzyme inactive. Iodoacetamide does not inhibit CGTase activity of *E. aurantiacum* suggesting absence of cysteine in active site. However DTT caused about 60% decrease in activity suggesting presence of sulphide linkages in the enzyme which may be responsible for thermostability. Phenyl MethylSulfonyl Fluoride (PMSF) inhibits serine proteases specifically. There was decrease in activity but not a complete loss in activity due to PMSF, which suggest that serine may not be present in active site of *E. aurantiacum* CGTase.
5.3.10 Study of kinetic parameters of purified CGTase

The Michaelis-Menten constant (K_m) and the maximum forward velocity of the reaction (V_max) were determined by Sigmaplot® Enzyme Kinetics Module using the Lineweaver Burke plot (Fig. 5.10).

![Lineweaver Burke plot for E. aurantiacum CGTase](image)

**Fig. 5.10**: Lineweaver Burke plot for *E. aurantiacum* CGTase

The K_m and V_max value calculated by Sigmaplot enzyme kinetics module for *E. aurantiacum* CGTase was 0.022 mg/ml and 57 µg of β-cyclodextrin/ml/min which shows greater affinity for substrate. The Kcat value (turnover number) was calculated using V_max as 316.7 s⁻¹. CGTase from *Bacillus circulans* E 192 (Bovetto et al., 1992) had a K_m of 5.7 mg/ml and CGTase from *Bacillus firmus* (Gawande et al., 1998) with its K_m equal to 1.21 mg/ml. Gawande and Patkar (2001) reported that the K_m value of *Klebsiella pneumoniae* AS-22 CGTase was 1.35 mg/ml, while Martins and Hatti-Kaul (2002) have estimated K_m of CGTase from *Bacillus agaradhaerens* to be 21.2 mg/ml.

5.3.11 Determination of molecular weight of CGTase

The CGTase purified by chromatography was precipitated using acetone. The concentrated precipitate was freeze dried and used for sample preparation. CGTase produced by *E. aurantiacum* was purified using a 12 % SDS-PAGE stained by
Coomassie brilliant blue (Fig.5.15). The gel was analyzed and molecular weight determined by using molecular weight standards of 6500 – 97400 Da (Bangalore Genei) and G-BOX Syngene software. A single band of purified CGTase (Fig.5.11 a, well 5) was observed suggesting the homogeneity of the purified enzyme.

Fig: 5.11 a) Well 3: Crude CGTase, Well 4 : Dialysate, Well 5: Affinity purified CGTase (arrow), Well 7: Molecular weight markers, Well 8: Ion exchange purified fraction ; b) Activity staining of CGTase using iodine (arrow) and c) Staining of CGTase using phenolphthalein indicator (arrow).

Two separate bands were observed in the dialysate in well 4 (Fig. 5.11). A single band was obtained in well 5 which contained the starch adsorption purified CGTase demonstrating purity of the enzyme. A similar band is also observed in well 4 containing dialysate which confirms the presence of CGTase in dialysate. This band was eluted from the gel and further processed for MALDI-MS analysis. The band obtained in well 8 depicts the protein purified by ion exchange chromatography. There is complete loss in CGTase activity after the starch adsorption purified enzyme was subjected to ion exchange chromatography. A single band of approximately 90 kDa was purified by ion exchange. This band was also present in the dialysate. Moreover, it is known that most of the CGTase-secreting bacterial strains produce other amylolytic enzymes, such as alpha amylase or glucoamylase (MW = 90-95 k Da) and these starch hydrolyzing enzyme may have been purified by ion exchange chromatography.
A single band in well 5 clearly confirms the presence of purified CGTase from *E. aurantiacum*. The molecular weight was estimated to be 77.84 kDa by graph of Log molecular weight versus distance travelled (Rf) generated by analysis of SDS-PAGE by Syngene software. Most of the purified CGTases from various *Bacillus* species have Molecular Weight (MW) in the range of 68 kDa to 88 kDa. MW of CGTase from *Bacillus* sp. KC201 is 75kDa (Kitamo *et al*., 1992), of CGTase from *Brevibacillus brevis* CD162 is 75kDa (Myung *et al*., 1998) and of CGTase from *Bacillus* sp. HA3-3-2, is 68 kDa (Nomoto *et al*., 1986). The MW of CGTase from *E. aurantiacum* is comparable to the MW of CGTases produced by other bacilli.

### 5.3.12 Activity staining and phenolphthalein staining of gel

The purified CGTase was subjected to non denaturing native gel electrophoresis and stained using iodine and phenolphthalein. The zymogram of CGTase demonstrated its starch hydrolytic activity which was clearly observed in iodine gel (Fig. 5.11, b) whereas a faint colorless band was observed in pink background of phenolphthalein gel (Fig. 5.11, c). The phenolphthalein indicator gel method differentiates CGTase from other starch hydrolytic enzymes.

### 5.3.13 MALDI – TOF MS analysis

The CGTase band was picked up from the 1-D SDS-PAGE gel, extracted, mixed with crystallized 3,5- dimethoxy-4-hydroxy cinnamic acid and spotted on a MALDI plate. The plate was analyzed on a MALDI-TOF MS analyser (Bruker Daltonics GmBh) according to manufacturer’s instructions and the peaks generated are shown in Fig.5.12.
The peaks generated (Fig. 5.13) and the peptide mass was analyzed using Mascot search engine (Matrix Science) that uses mass spectrometry data to identify proteins from primary sequence databases. The primary sequence was also analysed using BLASTp (NCBI).
The MASCOT analysis revealed that the CGTase enzyme did not demonstrate significant homology to any of the known and existing CGTase sequences deposited in Swissprot and Uniprot (Fig. 5.14). A total of 33 peaks were generated out of which three peptide fragments matched peptide fragments of glycosylase OS-Colwellia psychrerythrae (strain 34H / ATCC BAA-681) GN=mutM PE=3 SV=3 FPG_COLP3 with Intensity Coverage of 0.5 % (1710 cnts) and Sequence Coverage MS of 12.9%.
It is observed from the above figure that all the three peptides end in Lysine and trypsin had cleaved CGTase at lysine (K). The presence of acidic amino acids is seen in peptide 1 (Asp 39, Glu 40, Asp 91 and Glu 253). Proline which is responsible to give rigidity to the enzyme is observed (Pro 38, Pro 48) and a single amino acid of tryprophan (Try 35) and histidine (His 90) was also observed. The isoelectric point of CGTase from *E. aurantiacum* is predicted as 9.1.

**5.4 Discussion**

The most important parameter in study of any enzyme is to obtain it in pure form suitable for appropriate applications and to characterize it for better understanding of its properties. There are many approaches for purification of CGTase from bacteria. A simple two step process involving ammonium sulphate precipitation and dialysis, followed by starch adsorption chromatography with 52% yield is reported as also described by Higuti *et al.* (2004) and Ibrahim *et al.* (2012). Many researchers have used three steps procedure to purify their CGTase, such as Gawande *et al.* (1998) on *Bacillus firmus* (Ultrafiltration + starch affinity chromatography + gel filtration) and Sohn *et al.* (1997) on *Bacillus firmus* (ammonium sulfate + DEAE-Sephadex A-50 column + Sephadex G-100 column). Other researchers have attempted to use more steps to purify their CGTase, viz. Tachibana *et al.* (1999) used ammonium sulfate + Resource Q column + phenyl-Superose column + α-CD affinity column for purification of CGTase from *Thermococcus sp.* Mori *et al.* (1994) purified CGTase produced by

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Brevibacterium sp. No. 9605 using ultrafiltration + butyl-Toyopearl 650M column + γ-CD Sepharose column + Toyopearl HW-55S column.

Matioli et al. (1998) purified CGTase from alkaliphilic Bacillus to up to 157-fold by biospecific affinity chromatography. Pongsawasdi and Yagisawa (1988) purified a CGTase from Bacillus circulans with a 30% recovery by adsorption on corn starch followed by size-exclusion chromatography. Martins & Hatti-Kaul (2002) purified a CGTase from Bacillus agaradhaerens with a yield of 50% by adsorption to corn starch in the presence of 1 M ammonium sulphate followed by elution with β-CD solution.

Rosso et al. (2002) isolated and purified a CGTase from B. circulans using α-cyclodextrin-derivatised Sepharose 4B affinity chromatography (Ferrarotti et al., 1996). Many of the researchers use gel permeation chromatography including Sephadex G-150, Sephadex 75 HR, Biogel A-500, Biogel P-100, Biogel P-150, Sephareryl S-100, Sephareryl S-200 in their purification steps (Nakamura and Horikoshi., 1976; Larsen et al. 1998; Pongsawasdi and Yagisawa, 1988; Gawande and Patkar, 2001). Purification of E. aurantiacum CGTase using DEAE-Sepharose resulted in loss in enzyme activity and the single protein band in the SDS –PAGE suggest purification of some other peptide instead of CGTase. The characterization of CGTase revealed that the pH optimum was 9 and the enzyme was stable over a wide range of pH (5-11). This property is useful in application of the enzyme in detergent industry. There are a few CGTase enzymes that showed higher optimum pH such as CGTase from Brevibacterium sp. 9605 (Mori et al., 1994) and Bacillus clarkii 7364 (Takada et al., 2003). Both exhibited an optimum pH of 10. CGTase from E. aurantiacum demonstrates pH optimum of 9 which is suitable for cyclisation and hence production of cyclodextrins. pH plays a significant role in preserving biological activity and stability of CGTase because changes in pH may alter the three dimensional structure of the enzyme, as well as changing the native ionic form of the active site. Thus, it is vital to ensure that the pH of the buffer is always monitored to maintain the optimal stability of the enzyme. CGTase from other organisms such as Bacillus agaradhaerens (Martins and Hatti-Kaul, 2002) and Bacillus firmus (Sohn et al., 1997) exhibited stability over a wide range of pH, from pH 5.0 - 11.4 and pH 5.5 - 9.0, respectively. CGTase from Klebsiella pneumoniae AS-22 was stable between pH 6.0-9.0 (Gawande and Patkar, 2001).
E. aurantiacum CGTase showed significant activity in a wide temperature range, 30°C–70°C, showing maximal enzyme activity at 50°C. Temperature optima in the range of 55–65°C have been previously reported for CGTases from various alkaliphiles (Alves-Prado et al., 2008; Martins and Hatti-Kaul, 2002 and Ibrahim et al., 2012). This shows that CGTase of E. aurantiacum is an Intermediate thermostable enzyme (ITS) and can be explored for its applications in bakery and food industry. The effect of various metal ions and inhibitors revealed that CGTase is not a metallo enzyme as no metal ions significantly enhanced its activity and the enzyme retained 90 % activity in presence of EDTA which are similar to the reports of CGTase of Amphibacillus sp. and Bacillus pseudoalkaliphilus (Ibrahim et al., 2012; Atanasova et al., 2009). The metal ions like Fe$^{3+}$ and Zn$^{2+}$ catalyze the oxidation of the amino acid residues present in active centre including tryptophan, glutamic acid, aspartic acid and histidine, which are essential for cyclization reaction, causing the reaction rate of CGTase to decrease (Martins and Hatti-Kaul, 2002), cause transition state stabilization, as well as substrate binding and guiding linear starch chain into the active site (Uitdehaag et al., 1999; Knegtel et al., 1995; Penninga et al., 1996 and Nakamura et al., 1992). Thus, the cyclization reaction rate of CGTase will be decreased tremendously if these crucial amino acid residues are not well preserved. This suggests presence of glutamic and aspartic acid in active centre. The MALDI-TOF MS analysis of CGTase of E. aurantiacum showed the presence of acidic amino acids in peptide 1 (Asp 39, Glu 40, Asp 91 and Glu 253). Since CGTase produced by E. aurantiacum has optimum activity at alkaline pH, the presence of acidic amino acids in the enzyme protein is apparent.

The $K_m$ and $V_{max}$ values of CGTase produced by E.aurantiacum are 0.022 mg/ml and 57 μg of β-cyclodextrin/ml/min respectively show greater affinity of the CGTase to soluble starch. The $K_{cat}$ value (turnover number) of CGTase of E. aurantiacum was 316.7 s$^{-1}$ which is very high as compared to CGTase of Bacillus macerans ($K_{cat}$= 99.8 s$^{-1}$) (Jeang et al., 1999).

The molecular weight of CGTase produced by E. aurantiacum was estimated as 77.84 k Da which is comparable to most of the CGTases having molecular weight in the range of 70 – 110 kDa (Cao et al., 2005 ; Hirano et al., 2006; Savergave et al., 2008 and Ibrahim et al., 2012). The MALDI–TOF MS analysis revealed that
the enzyme had about 40% homology to sequence of glycosylase of OS-Colwellia psychrerythraea suggesting that the CGTase produced by E. aurantiacum may be a novel enzyme which can be confirmed in future using N-terminal amino acid sequencing. The isoelectric point was predicted as 9.1. CGTase produced by E. aurantiacum isolated from Lonar lake is thus a novel intermediate thermostable and alkaline enzyme produced extracellularly with high affinity for substrate.

5.5 Conclusions

Purification and characterization of alkaline CGTase from E. aurantiacum resulted in 25 fold purification. The enzyme was purified to homogeneity with a molecular weight of 77.84 kDa. The enzyme was completely inhibited by Fe, partially inhibited by DTT and not inhibited by metal ions. Calcium ions enhanced activity of CGTase. The enzyme was detected in starch and phenolphthalein indicator gels successfully. The enzyme was moderately thermostable suggesting its application in food industry.