Purification and characterization of fructosyltransferase: A low molecular weight enzyme from Aspergillus niger NFCCI2736

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Abstract
A novel transfructosylating enzyme derived from A. niger NFCCI2736 was purified 7.99-fold by ammonium sulphate precipitation (30–80%) followed by DEAE-cellulose ion exchange chromatography. The minimum molecular mass of the purified enzyme was 45 kDa by SDS-PAGE. The Km for fructosyltransferase was 333 mM of sucrose and Vmax was 1.25 x10^3 mM/ mg/ min. The optimum enzyme activity of the purified enzyme was at 5.5 and the maximum stability of the enzyme was at pH 5.00.

The optimum temperature for enzyme activity and enzyme stability coincided at 55 °C. The ammonium ions enhanced the activity of the purified enzyme whereas sodium and manganese ions decreased the activity of purified Ftase.

Keywords: Aspergillus niger, fructooligosaccharides, fructosyltransferase and sucrose.

Introduction
Fructo-oligosaccharides (FOS) are slowly developing immense importance by attracting attention of health-conscious consumers. FOS are vitally recognised in health market due to their low caloric, non-carcinogenic nature and bifidus promoting effect in the intestine6,20,26-28.

FOS are produced by the action of Ftase (fructosyltransferase) enzyme on sucrose by simple transfer reaction. Depending on the number of fructose units, FOS are classified as 1-kestose (GF2), nystose (GF3) and 1F--fructofuranosyl nystose (GF4) having one, two and three fructose molecules respectively6. Microbial sources have been reported to be potent producers of Ftase enzyme. The most exploited group of microbes are fungi and bacteria29. As previously reported, during screening of new potent producers of enzymes showing transfructosylating activities, new strain designated as Aspergillus niger was isolated from soil during previous study2. The extracellular (Ftase) exhibited a substrate specificity towards sucrose. The purification and characterization of the enzyme were studied in detail.

Material and Methods
Chemicals: The food-grade sucrose was used as substrate while other chemicals were of reagent grade of Hi-media. The DEAE-cellulose (D 6418) and the molecular mass markers for sodium do- decyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) were of Sigma. Cellulose dialysis tubing (MW cut off 12 KDa) is of Hi-media.

Enzyme production: A. niger NFCCI2736 was grown aerobically in a batch culture. The medium contained (per litre of distilled water) sucrose 80.0 g, yeast extract 15.0g, MgSO4.7H2O 1.0 g, Carboxyl methyl cellulose 1.0 g and pH was adjusted to 5.50 before sterilization. The spore suspension of A. niger NFCCI2736 was added aseptically to the sterilized medium in 250 ml Erlenmeyer flask and kept in shaking incubator at 30°C for 4 days at 240 rpm.

Crude enzyme preparation: The mycelium was separated after fermentation by filtration and the filtrate was centrifuged at 10000 rpm for 30 min at 4°C. The supernatant was used as source of crude enzyme for salt precipitation by ammonium sulphate. The enzyme activity was measured after each step of purification.

Column Characteristics: The glass column used was of 3 x 18 cm dimension.

Assay of fructosyltransferase enzyme: Quantitative assay of fructosyltransferase was based on the procedure used by Yun et al.30 The filtrate was taken as a crude enzyme with 50% sucrose solution as a substrate at pH 5.50 (0.1M sodium acetate buffer). The mixture was incubated for 1 h at 60°C. The reducing sugars were estimated by Dinitro salicylic acid reagent (Miller). The enzymatic reaction was terminated by keeping the test tube at 100°C in a water bath for 10 min. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of glucose under experimental conditions.

Determination of protein concentration: The protein content was determined following Lowry et al18

Analytical methods: Thin layer chromatography (TLC) is used for qualitative analysis of the reaction products. TLC plates were prepared by coating glass plates with Silica gel and developed with the solvent systems: iso-propyl alcohol: ethyl acetate: water (2:2:1 v/v). The carbohydrates samples loaded in the plates were visualized by heating the plates at 100°C after spraying phenol- sulfuric acid.

Enzyme purification: All operations for enzyme purification were performed at 4°C and centrifugation was
conducted at 6000Xg for 15 min. Crude enzyme solution was concentrated by ammonium sulfate precipitation (30–60% saturation) followed by dialysis at 4 °C overnight against 50 mM sodium acetate buffer (pH 5.50). Concentration of enzyme solution was done using a Hetero- Powdery SN:.50605040.

The concentrated enzyme was loaded on to a DEAE-cellulose pre-equilibrated column with 50 mM sodium acetate buffer (pH 5.50). The non-adsorbed proteins were eluted with 500 ml of linear gradient of 0–0.5 M NaCl at a flow rate of 1 ml/min (5 ml per tube). The fractions showing transfructosylating activity were pooled and concentrated. Proteins were analyzed by SDS-PAGE.

**SDS-Polyacrylamide Gel Electrophoresis:** Sodium dodecyl sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli13 using 10% acrylamide gel. The molecular weight markers applied were from the Bangalore Genei SDS PAGE kit (Genei Pvt. Ltd., Bangalore, India).

**Characterization of extracellular fructosyltransferase produced by A. niger NFCCI2736**

**Effect of substrate concentration on the purified fructosyltransferase activity:** The enzyme kinetics was studied by determining the effect of substrate concentration on enzyme activity. 0.01ml of purified enzyme was taken with different concentrations of sucrose ranging from 200 -1600 mM. The K_m and V_max of the enzyme were determined by double reciprocal (Lineweaver Burk plot).

**Effect of pH on activity and stability of the purified fructosyltransferase:** The optimum pH was determined by incubating the enzyme with sucrose at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 followed by standard assay described earlier. To determine the stability, the enzyme was pre-incubated with buffers in the above pH range for 1 h at 55°C before determining Fructosyltransferase activity.

**Effect of temperature on activity and stability of the purified fructosyltransferase:** The enzyme activity was assessed at different incubation temperatures from 30 °C to 80°C with an increment of five degree by standard method. To determine the stability, the enzyme was pre-incubated for 1 h at above temperatures before determining Fructosyltransferase activity.

**Effect of metal ions on the purified fructosyltransferase activity:** The effect of metal ions was determined on the purified enzyme activity by adding 0.1 ml of 10 mM solution of diammonium phosphate, ammonium chloride, ammonium nitrate, ammonium sulfate, urea, diammonium phosphate, citric acid, copper sulphate, sodium chloride, potassium chloride, manganese chloride, calcium chloride and zinc sulphate. The enzyme activity was determined in control (devoid of ions) and experimental sets to determine their inhibitory or stimulatory effect on enzyme activity.

**Results and Discussion**

The protein precipitation using ammonium sulphate using 30 % - 80 % concentrations revealed maximum precipitation at 60% ammonium sulphate with highest activity of specific activity of 204.66 mg protein/ ml. Later, all subsequent purification was carried out at 60% concentration of ammonium sulphate.

The Ftase purification is summarized in table 1. The total activity of the crude enzyme was found to be 15270 U with protein content of 196 mg. Ammonium sulphate precipitation at 60% yielded 76.12% recovery of protein from the crude enzyme with a purification fold of 2.60. DEAE cellulose chromatography yielded 42.28% recovery of the protein with a purification fold of 7.99. The specific activity of fructosyltransferase increased from 77.90 IU/ mg protein (crude enzyme) to 622.5 IU/ mg protein after the DEAE-cellulose chromatography. Fig. 1 depicts the elution pattern of protein at sodium chloride gradient from 0.1 M to 0.5 M. The fructosyltransferase activity was detected only in fractions eluted with 0.3 M gradient. No residual activity was noticed in the remaining fractions.

The finally purified enzyme gave a single protein band as shown in fig. 2(a), the purification yield from the crude extract was significantly high (42.28%). The final enzyme was purified 7.99-fold from the crude enzyme and the minimum molecular mass of the purified enzyme was estimated to be 45 kDa as seen in fig. 2(b) in SDS- PAGE. The overall enzyme purification steps are summarized in table 2.

**Characterization of extracellular fructosyltransferase of A. niger NFCCI2736:** The purified fructosyltransferase was characterized by investigating its enzyme kinetics. The effect of sucrose concentrations, pH and temperature and metal ions was investigated on the enzyme catalyzed reaction. The effect of pH and temperature on activity and stability was also studied. The effect of sucrose concentration was evaluated by determining the K_m and V_max values for fructosyltransferase.

**Effect of substrate concentration on the purified enzyme activity:** The Ftase kinetics was studied by determining the effect of different sucrose concentrations on its activity. The K_m and V_max of the enzyme were determined by double reciprocal (Line weaver-Burk plot) and presented in figure 3. The sucrose concentration used for determining Km was 200 mM - 1600 mM. The enzyme kinetics for substrate utilization revealed that the K_m for fructosyltransferase was found to be 333 mM and V_max was found to be 1.25 x10^3 mM/ mg/ min by LB plot.

**Effect of pH on activity and stability of fructosyltransferase:** The enzyme was incubated with substrate at different pH of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 for determining effect of pH on fructosyltransferase activity. The optimum pH was determined to be 5.50 (Figure
4). For studies on stability, the pre-incubated enzyme with buffers at different pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 was assayed at pH 5.50 for 1 h at 55°C revealing highest stability at pH 5.0. The enzyme stability at different incubated pH is given in figure 4.

Effect of temperature on activity and stability of the purified fructosyltransferase: The enzyme activity was determined at different incubation temperatures of 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C and 80°C (Figure 5). The highest activity of Ftase was noticed at 55°C. The effect of temperature on stability of the enzyme was determined by pre-incubating the purified enzyme at different incubation temperatures of 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C and 80°C for 1 h. The enzyme assay was performed at 55 °C revealing highest stability at 55°C (Figure 5).

Effect of additives on the purified fructosyltransferase activity: The effect of additives was determined on the purified enzyme activity by adding 0.1 ml of 10 mM solution of di-ammonium phosphate, ammonium chloride, ammonium nitrate, ammonium sulphate, urea, di-ammonium phosphate, citric acid, copper sulphate, sodium chloride, potassium chloride, manganese chloride, calcium chloride and zinc sulphate. The enzyme activity was determined in control (devoid of ions) and experimental situations to determine their inhibitory or stimulatory effect on enzyme activity. All the ammonium salts and potassium chloride were found to have stimulatory effect on the enzyme catalyzed reactions. Copper sulphate totally inhibited the activity of the purified enzyme. Other salts which had inhibitory effect upon the enzyme catalyzed reactions included sodium chloride, manganese chloride and calcium chloride (Table 2).

The Ftase purified from A. niger NFCCI2736 exhibited a single band of 45KDa. The purified Ftase from Aspergillus niger AS0023 exhibited two bands of 600 KDa and 309 KDa and in SDS PAGE a single band of 168 KDa which indicated a polymeric form of the enzyme. The monomeric form of purified fructosyltransferase from Bacillus macerans exhibited molecular weight of 66 KDa. Fructosyltransferase purified from many microbes revealed a monomeric form ranging between 45 KDa to 220 KDa. Sucrase from Termotmyces clypeatus of extremely low molecular weight of 13.5 KDa exhibited activity at high sucrose concentration.11

In the present study, the enzyme kinetics for substrate utilization by Ftase in A. niger NFCCI2736 revealed that the K_m for fructosyltransferase was found to be 333 mM and V_max was found to be 1.25 x10^3 mM/ mg/ min. The purified fructosyltransferase originating from different microbial sources revealed K_m values 4.0 to 770.4 mM.11 Jung et al reported K_m to be 330 g l^{-1} and V_max 130g l^{-1}h^{-1}.14 The K_m value of the enzyme from Aspergillus aculeatus was 0.53 M^{-1}.

The effect of pH on stability and activity of the purified Ftase in present study was found to be 5.0 and 5.5 respectively. Many workers have reported the optimum pH values to be from 5.0-6.0.1,10 However, very high pH 7.5 and very low pH 4.5 for purified fructosyltransferase from Actinomyces viscous and Rhodotorula sp have been reported.11,21

The effect of temperature on stability and activity of the purified Ftase investigated was found to be 55°C. Most of the purified fructosyltransferase exhibited the optimum temperature range between 50°C-60°C. On the contrary, Ghazi et al reported purified fructosyltransferase from Aspergillus aculeatus exhibiting optimum temperature of 65°C. According to reports of Homman et al, a fructosyltransferase purified from Bacillus megaterium exhibited optimum temperature of 45°C.

Table 3 gives a comparative account of purification work conducted on Ftase from different microbial sources. The comparisons of the results on purification of enzyme from Aspergillus sp reveal that the present study has isolated an enzyme of 45KDa which is monomeric and dissimilar to earlier reports.

Figure 1: Elution profile of DEAE-cellulose chromatography. The elution was carried out with a linear gradient from 0.1 M to 0.5 M NaCl in pH 5.5. The absorbance at Y-axis is relative values to blank.

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Figure 2(a): SDS-PAGE profile, Lane A-Markers; Lane B- Ammonium sulphate precipitate, Lane C-11th fraction, Lane D-12th fraction of chromatographic eluent
Figure 2(b): Plot of mobility of markers against their molecular mass

Table 1
Purification of fructosyltransferase from *Aspergillus niger*

<table>
<thead>
<tr>
<th>Purification stages</th>
<th>Volume (ml)</th>
<th>Total activity (IU)</th>
<th>Protein (mg)</th>
<th>Specific activity (U/ mg protein)</th>
<th>Purification fold</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>200</td>
<td>15270</td>
<td>196</td>
<td>77.90</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>20</td>
<td>11625</td>
<td>56.80</td>
<td>204.66</td>
<td>2.60</td>
<td>76.12</td>
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<tr>
<td>DEAE cellulose</td>
<td>5</td>
<td>7221</td>
<td>11.60</td>
<td>622.50</td>
<td>7.99</td>
<td>42.28</td>
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</table>

Table 2
Effect of additives on purified enzyme fructosyltransferase

<table>
<thead>
<tr>
<th>Additives (10 mM)</th>
<th>Relative activity (%)</th>
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</thead>
<tbody>
<tr>
<td>Control (without metal ions)</td>
<td>100</td>
</tr>
<tr>
<td>Di-ammonium phosphate</td>
<td>152</td>
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<tr>
<td>Ammonium chloride</td>
<td>165</td>
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<tr>
<td>Ammonium nitrate</td>
<td>178</td>
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<tr>
<td>Ammonium sulphate</td>
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<tr>
<td>Urea</td>
<td>115</td>
</tr>
<tr>
<td>Di-ammonium phosphate</td>
<td>109</td>
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<tr>
<td>Citric acid</td>
<td>86</td>
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<tr>
<td>Copper sulphate</td>
<td>Nil</td>
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<tr>
<td>Sodium chloride</td>
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<tr>
<td>Potassium chloride</td>
<td>108</td>
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<tr>
<td>Manganese chloride</td>
<td>96</td>
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<tr>
<td>Calcium chloride</td>
<td>64</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>126</td>
</tr>
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</table>
### Table 3
Review of characterization of purified microbial FTase

<table>
<thead>
<tr>
<th>Source of FTase and nature of enzyme</th>
<th>Purification steps</th>
<th>Purification fold</th>
<th>Molecular weight (kDa)</th>
<th>Activated by</th>
<th>Inactivated by</th>
</tr>
</thead>
</table>
| **Bacillus macerans** EG-6 Extracellular<sup>23</sup> | ➢ ASP  
➢ CM-sepharose  
➢ CL 6B  
➢ Resource-Q  
➢ Phenyl sepharose SR  
➢ Mono-S column | 63.5 | 66KDa | Not reported | Not reported |
| **Arthrobacter oxydans** J17-21 Extracellular<sup>13</sup> | ➢ Q-Sepharose  
➢ Mono-Q HR 5/5  
➢ gel permeation chromatography | 95.5 | 54K Da | CaCl<sub>2</sub> EDTA | MnCl<sub>2</sub> and CuSO<sub>4</sub> completely by FeSO<sub>4</sub> and Ag<sub>2</sub>SO<sub>4</sub> |
| **Microbacterium laevaniformans** ATCC 15953 Extracellular<sup>22</sup> | ➢ Ammonium sulfate precipitation,  
➢ DEAE-Sepharose Fast Flow  
➢ Sephacryl S-100 HR | 64 | 64kDa | Not reported | CuSO<sub>4</sub> and HgCl<sub>2</sub> and moderately inhibited by ZnSO<sub>4</sub>. |
| **Microbacterium sp. AL-210** Extracellular<sup>4</sup> | ➢ Resource-Q FPLC  
➢ Superdex 200HR FPLC. | 98.8 | 46KDa | CaCl<sub>2</sub>, MgCl, CoCl<sub>2</sub>, MnCl<sub>2</sub> | FeCl<sub>2</sub>, AgNO<sub>3</sub> EDTA, KMnO<sub>4</sub> and 2-Mercaptoethanol |
| **Aspergillus niger** AS0023 Extracellular<sup>17</sup> | ➢ ASP  
➢ DEAE-sephadex A-25  
➢ Sephacryl S-200  
➢ Con A sepharose 4B | 78.5 | Polymeric upto 600KDa | Not reported | Not reported |
| **A. Aculeatus** Commercial enzyme preparation<sup>7</sup> | ➢ FPLC  
➢ DEAE –Sepharose | 107 | Dimer 135 | Dithiotreitol 2-mercaptopoethanol, sodium dodecylsulphate and Tween 80. T | Hg<sup>2+</sup> and Zn<sup>2+</sup>. |
| **A. Pullulans** Intracellular<sup>16</sup> | ➢ ASP  
➢ DEAE-cellulose  
➢ Sephadex G-200 | 79.44 | 147KDa 170KDa | Not reported | Not reported |
| **Aspergillus niger** IMI303386 Intracellular<sup>19</sup> | ➢ APS  
➢ DEAE Sepharose Fast Flow  
➢ Ultrogel AcA44 | 50 | 120 to 130 kDa | Ba<sup>2+</sup>, Mg<sup>2+</sup>, Cu<sup>2+</sup> and sodium-EDTA | Hg<sup>2+</sup>, Ag<sup>+</sup> and Ni<sup>2+</sup> |
| **Aspergillus niger** NFCC12736 Extracellular* | ➢ APS  
➢ DEAE-cellulose | 7.99 | 45KDa | All ammonium salts | Complete inhibition by CuSO<sub>4</sub> |

*Present study
Figure 3: Line weaver Burk plot of Ftase

Figure 4: Effect of pH on activity and stability of purified fructosyltransferase from *Aspergillus niger*

Figure 5: Effect of temperature on stability and activity of purified fructosyltransferase from *Aspergillus niger*
References


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