Purification and immobilization of fructosyl transferase for production of fructo-oligosaccharide(s) from sucrose

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A protocol for commercial production of a non-digestible sweetener, fructo-oligosaccharide(s) from sucrose has been developed. The extracellular enzyme fructosyl transferase was isolated and purified from Aureobasidium pullulans. The enzyme was covalently immobilized on CNBr activated agarose for its economical viability and for continuous use.

Fructo-oligosaccharide(s), a recent market entries are a class of non-digestible sweeteners, consisting of a mixture of linear and branched oligosaccharide(s) of GFn series, (G-glucose and F-fructose'). These short chained fructo-oligosaccharide(s) are a low energy bulk ingredients that have a taste profile similar to that of sucrose, with physical and chemical properties that match precisely with those of sucrose in wide range of food applications. It is also suggested that fructo-oligosaccharide(s) selectively stimulate Bifidobacteria growth. Since Bifidobacteria are considered beneficial genera, they contribute to the establishment of a more healthy colonic environment. These fructo-oligosaccharide(s) are present in low quantities and are naturally occurring sugars in many plants, such as onion, asparagus, wheat, rye, triticcales and chicory roots. As these fructo-oligosaccharide(s) are widely spread in nature, they are considered as ingredients rather than additives.

Fructo-oligosaccharide(s) can be obtained by enzymatic action of a fungal enzyme system, fructosyl transferase on sucrose. Sucrose plays the dual role of fructose donor and fructose acceptor. The reaction leads to a mixture of short chain fructo-oligosaccharide(s) including kestose (GF2), nystose (GF3) and fructo-furanosyl nystose (GF4) in which one to four fructose units are bound to β(2→1) position of sucrose.

Supply of fructo-oligosaccharide(s) is rather limited due to limited content of it present in plants. Alternatively these can be produced using fructosyl transferase enzyme system, from sucrose which is a major agricultural product.

The continuous use or reuse of the enzyme as biocatalyst is made possible using the technology called enzyme immobilization, where by the movement of enzyme in space is completely or severely restricted, resulting into a water soluble forms of enzyme. For efficient use of enzyme, immobilization techniques has created many opportunities. Applications of immobilization techniques for enzymic conversions of sugar are always attractive. Uncharged structure and small size of sugar molecule minimizes unwanted diffusional restrictions, and simultaneously the use of high substrate concentrations prevent the contamination problems in industrial operation.

Material and Methods

Enzyme production—The extracellular enzyme fructosyl transferase (EC. 2.4.1.9) was detected in cultural filtrate of Aureobasidium pullulans NCLM 1049. The culture obtained from National Chemical Laboratory, Pune, India was maintained and subcultured after a month on potato dextrose agar slants at 4°C. The culture medium containing sucrose, 10%; yeast extract, 1%; K2HPO4, 0.5%; MgSO4 H2O, 0.05% and NaNO3, 0.15% was observed suitable for the optimum growth of the fungus at which the maximum enzyme secretion occurred.

The conventional procedures for purifications were used. All purification procedures were performed at 4°C unless otherwise indicated. The cultural filtrate was dialysed against 25 mM potassium phosphate buffer, (pH 7) and then applied to DEAE-Sepharose...
CL 6-B anion exchanger column (1.6×70cm, flow rate 25ml/hr), which was equilibrated in same buffer. The column was washed with 250ml of buffer, and the stepwise elution of bound proteins were done using 0.2 and 0.5 M NaCl in same buffer. The enzyme activity of each fraction was determined using the assay procedure described as below. The protein content of each fraction was measured using Lowry’s method10 and carbohydrate content was also determined11. The pooled fractions containing maximum activity were dialysed and then lyophilized to concentrate the same. Then it was rechromatographed on Bio Gel P-100 column (1.5 x 72cm, flow rate 10ml/hr). The eluent buffer used was 25mM, potassium phosphate buffer, pH 7.

Enzyme assay—Fructosyl transferase activity was determined by measuring glucose released from sucrose during transfer reaction. The reaction mixture consisted of 2.4ml of sucrose solution (100mg/ml) prepared in 100 mM citrate buffer, (pH 5.5) and 0.1ml properly diluted enzyme solution, which was incubated at 50°C for 15min and then the reaction was terminated by immersing the assay tubes in boiling water bath for 2 min. Glucose released was measured using specific glucose oxidase-peroxidase coupled enzymatic assay method12. Parallel conditions were maintained for the assay of immobilized enzyme, where 0.1 ml of enzyme bound diluted gel beads were used. Agarose beads (6%) were activated using CNBr treatment. The beads were washed repeatedly at 4°C and used for coupling with fructosyl transferase enzyme. The enzyme was maintained at 4°C and pH 7.0.

One enzyme unit was defined as the amount of enzyme responsible for the release of 1.0 mg of glucose per min under standard assay conditions (at pH 5.5; 100 mg/ml of sucrose and 50°C). The effect of pH on fructosyl transferase activity was studied using an assay mixture in which properly diluted enzyme solution (0.1ml) was added to sucrose (100mg/ml) suspended in 100mM citrate phosphate buffer (pH 2 to 8) and 100 mM carbonate-bicarbonate buffer (pH 9 to 11) at 50°C. Effect of temperature on fructosyl transferase activity was also studied between 5°C and 100°C in an assay mixture containing enzyme (0.1 ml) and sucrose (100 mg/ml) suspended in 100 mM of citrate buffer, (pH 5.5). Effect of substrate concentration on enzyme activity was examined using an assay mixture in which required diluted enzyme solution (0.1ml) was added to sucrose solution at different concentration ranging from 10 to 900 mg/ml in 100mM citrate buffer, (pH 5.5).

Effect of pH, temperature and substrate concentration were also studied for immobilized fructosyl transferase using parallel assay procedures where 0.1 ml of enzyme bound gel slurry was used.

Immobilization of fructosyl transferase—Agarose gel beads (25 ml) were thoroughly washed and soaked in ice cold carbonate-bicarbonate buffer, (200 mM; pH 11.5) the supernatant was discarded and the gel slurry was kept in ice bath. Cyanogen bromide (CNBr; 50 mg) crystals were added, well stirred and kept overnight at 4°C. Unreacted cyanogen bromide was removed using ice cold distilled water till the gel beads were free from it as, tested with by AgNO₃ solution. These activated beads were soaked in iced cold potassium phosphate buffer (50 mM, pH 7.0) and 1.1ml (100 units) of fructosyl transferase was added, gently stirred and kept overnight at 4°C with occasional stirring13. Excess was removed with repeated washing of the gel beads with the same buffer. The bound activity was determined using standard assay procedure as mentioned earlier. The activity loss of bound enzyme which was stored at 4°C, was periodically studied for five months.

Reactor operation—The reactor containing 100 ml of sucrose (500 mg/ml) prepared in citrate buffer, (100 mM; pH 5.5) along with 25 units of either free fructosyl transferase or immobilized fructosyl transferase was operated at 50°C for 10 hr with continuous agitation. After completion of reaction the immobilized enzyme was separated using centrifugation, washed with potassium phosphate buffer, (100 mM; pH 7.0) and stored at 4°C. It was repeatedly used for several batches without any activity loss.

Analysis of fructo-oligosaccharide(s)—The qualitative analysis of the product was carried out using ascending type of paper chromatography using butanol:ethanol:water (3:2:1) as a solvent system and separated sugars were visualized using benzidine TCA colour spray reagent15. For quantitative analysis Bio Gel P-2 gel filtration chromatography was used. (1.5x70 cm, flow rate 10ml/hr). Elution was done in distilled water. Fractions containing fructo-oligosaccharides (Fraction No.62 to 82) were hydrolysed in 0.05 N HCl at 98°C for 1 hr. The hydrolyzate was neutralized using few drops of 0.1 N NaOH solution. Ascending type of paper chromatography of this hydrolyzate was done using
butanol:acetone:water (4:5:1) as solvent system. This hydrolyzate was analysed for specific glucose by glucose oxidase peroxidase method and for total carbohydrate using phenol- H$_2$SO$_4$ method. Ratio of glucose to fructose was determined (difference between total carbohydrates and glucose was fructose).

**Results and Discussion**

Preliminary studies showed that *Aureobasidium pullulans* was able to produce extracellular fructosyl transferase when grown in sucrose containing medium. Maximum enzyme concentration was obtained from 72 hr old culture. The best results were obtained when growth medium was constantly stirred for 250 rpm to provide aeration. Chromatography of the cultural filtrate on DEAE Sepharose CL 6-B (Fig. 1) eliminated almost all protein contaminants present in it. Subsequent chromatography on Bio Gel P-100 (Fig. 2) displayed the homogeneity of the enzyme preparation. Carbohydrate content of each fraction showing enzyme activity (Figs 1, 2) was more than the protein content. It was 75% (w/w) of the protein content, which indicates that fructosyl transferase is a heavily glycosylated protein. The enzyme was stable, as 100% activity was retained for both free and immobilized form of enzyme, when stored for 5 months at 4°C. Further investigations are needed to study whether the stability at elevated temperature and the storage stability of enzyme is contributed by the heavily glycosylated nature.

This purified enzyme solution was used for immobilization. The parameters for both free and immobilized fructosyl transferase were determined and compared. The immobilization process does not result into alteration in optimum temperature, optimum pH and Km (Michealis constant; Fig. 3A, B, C). The free enzyme showed progressive increase in activity upto 30% (w/v) sucrose solution and stabilized at 70% (w/v) sucrose which declined at still lower temperatures.
Fig. 3—Effect of (A) temperature; (B) pH; and (C) substrate concentration.

Fig. 4—Paper chromatographic analysis of (A) reaction product-fructo-oligosaccharide(s); and (B) acid hydrolyzed fructo-oligosaccharide(s).

Table 1—Stability study of immobilized fructosyl Transferase

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<tr>
<th>Days</th>
<th>Bound enzyme activity (Units)</th>
<th>Activity loss (%)</th>
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<tr>
<td>0</td>
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<tr>
<td>150</td>
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higher concentration of sucrose. For immobilized enzyme, activity started to decline over 50% (w/v) concentration of sucrose. Maximum activity of the enzyme was observed at 50% (w/v) sucrose concentration, pH 5.5 and 50°C.

Covalent binding between enzyme and support (agarose gel) provided most stable immobilized fructosyl transferase preparation. The bound enzyme was stable for several months, when stored at 4°C. This was repeatedly used for several batches without
The enzyme cost is a critical factor for industrial production of fructo-oligosaccharide(s). The technology described is best for continuous use or reuse of fructosyl transferase. The overall system developed is economically more viable and quality intensive and can be exploited for the industrial applications.

References