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Immobilization of fructosyltransferase by chitosan and alginate for efficient production of fructooligosaccharides

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ABSTRACT

The effective system of reusing mycelial fructosyltransferase (FTase) immobilized with two polymers, chitosan and alginate were evaluated for continuous production of fructooligosaccharides (FOS). The alginate beads were successfully developed by maintaining spherical conformation of using 0.3% (w/v) sodium alginate with 0.1% (w/v) of $CaCl_2$ solution for highest transfructosylating activity. The characteristics of free and immobilized FTase were investigated and results showed that optimum pH and temperature of FTase activity were altered by immobilized materials. A successive production of FOS by FTase entrapped alginate beads was observed at an average of 62.96% (w/w) up to 7 days without much losing its activity. The data revealed by HPLC analysis culminate 67.75% (w/w) of FOS formation by FTase entrapped alginate beads and 42.79% (w/w) by chitosan beads in 36 h of enzyme substrate reaction.

1. Introduction

Increasing demand of prebiotic oligosaccharides has led to replace conventional enzymatic processes with immobilized formulations. Recent developments in this technique impart greater stability, re-usability, continuous production, catalytic control and thus are important for economical biotechnological applications of enzymes [1]. Immobilization process in enzyme technology has reached its highest level and one of its important applications in biotransformation process, sucrose to fructooligosaccharides has led to huge development on industrial level [2]. FOS has been greatly explored from last two decades in food and pharmaceutical fields, and is confronted with many functional foods in all criteria of prebiotics. FOS is being prosperously used as an artificial sweetener and is regarded as dietary fibres with low caloric value that promotes growth of bifidobacteria in human colon [3]. In addition, it has broad applications such as non-cariogenicity, non-digestibility, stimulation of calcium and magnesium absorption, also lowering of total cholesterol, phospholipids and triglycerides in serum. The term fructooligosaccharides is preferably being used for fructose oligomers which contain one D-glucose unit and 2-4 D-fructose units bound together by β (2 → 1) glycosidic linkages [4]. FOS can be produced by the action of enzymes possessing transfructosylating activity either fructosyltransferase (EC 2.4.1.9) or β-fructofuranosidase (EC 3.2.1.26) derived from many plants and microorganisms. The concentration of FOS on these plants is quite low and mass production of required enzyme is quite limited by seasonal conditions, therefore industrial production chiefly depends on microbial enzymes. Most of these enzymes have been found in fungi such as *Aspergillus* sp., *Aureobasidium* sp., *Penicillium* sp. and *Fusarium* sp., respectively [5–8]. The main limiting factor in the commercial FOS contains large amount of reaction by-products like glucose, fructose and unreacted sucrose which constrained FOS yield up to 50–60% (w/w). Thus it is pertinent to investigate new potential strains which have high capability and also to develop cost effective biotechnological processes for immense production of FOS. A feasible alternative that has been investigated for FOS production is use of immobilized cell system which avoids the cumbersome process of enzyme extraction and purification procedures. To accomplish cell immobilization system, entrapment of cells within porous matrices is the most appropriate method to confer desirable features into the biocatalyst [9–11].

In our previous screening investigation *Aspergillus flavus* (NFCCI 2364) was reported as the most potent producer containing high titres of FTase for transformation of sucrose to FOS [4]. However, the present study elucidates its performance through immobilization process by sodium alginate and chitosan forming gel beads. The

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main objective of this examination was to study the operational stability and reusability of FTase containing beads for continuous production of FOS.

2. Materials and methods

FOS standards kestose, nystose, 1-fructofuranosyl nystose and sugars standards sucrose, glucose, fructose, were obtained from Wako Pure Chemicals (Japan) and Sigma Aldrich (USA). Other chemicals were purchased from Otto, Hi-media, Merck and Finar India Ltd.

2.1. Microorganism and culture conditions

A. flavus NFCCI 2364 was obtained from NFCCI, Agharkar Research Institute (ARI) Pune, India and was maintained on potato dextrose agar (PDA) medium. The inoculum was prepared by transferring a full loop of spores or mycelia from 5 day old culture to 100 ml medium containing 1% sucrose and 0.2% yeast extract (pH 5.5). The culture was incubated on rotary shaker at 28 °C and 200 rpm for 24 h. For inoculation, 10 ml spore suspension (1.7×10^7 spores/ml) was transferred in 250 ml Erlenmeyer flasks containing 100 ml of sterilized medium sucrose 20%, yeast extract 0.5%, $NaNO_3$ 1%, $MgSO_4{\cdot}7H_2O$ 0.05%, KH_2PO_4 0.25%, NH_4Cl 0.5%, and NaCl 0.25% with an initial pH of 5.5. After 72 h of incubation, culture broth was centrifuged at 6000 rpm at 4 °C using refrigerated centrifuge (Eltek RC 8100 SF, India). The supernatant was used as source of extracellular free enzyme and remaining pellet as source of mycelial FTase.

2.2. Preparation of sodium alginate beads by whole cell immobilization

Immobilization of mycelial cells containing intracellular enzyme was done by mixing FTase with 0.3% (w/v) of sodium alginate in warm water (50 °C) to form slurry. For entrapping the cell bound FTase, mycelia-alginate mixture was extruded drop wise through needle (1D 1.0 mm) into 0.1 M $CaCl_2$ solution by peristaltic pump. Droplets were instantly transformed into spherical beads (2–3 mm in diameter) by exchange of Na^+ in droplets and Ca^+ in solution. To maintain the spherical conformation, alginate beads were cured by immersing them into 0.2 M calcium chloride ($CaCl_2$) solution in 0.1 M sodium acetate buffer pH 4.8 for 24 h at 4 °C before use (Supplementary Fig. A1). Thus obtained beads were used as source of cell-bound FTase for generation of FOS from 60% (w/v) sucrose.

2.3. Preparation of chitosan beads by whole cell immobilization

The preparation of chitosan beads was done by mixing 0.1% (w/v) of chitosan into 10 ml of 4% (w/v) glacial acetic acid solution under mild stirring for 1 h. The activated chitosan was mixed with fungal mycelia and was introduced into 20 ml syringe. The slurry was then extruded through 5 mm diameter needle using compressed air. The droplets were pulled off in 20% (w/v) tripolyphosphate (TPP) solution and beads formed were hardened for 2 h. After cross-linking reaction, the beads were rinsed with deonized water for making solution neutral and were stored in a 0.1 M sodium acetate buffer (pH 5.5) at 4 °C. Thus prepared chitosan beads were then added to flask containing 20 ml glutaraldehyde solution (1% w/v) and shaken at 40 rpm for 1 h at room temperature. Before putting beads into sucrose 60% (w/v), they were again washed with distilled water to remove excess of glutaraldehyde (Supplementary Fig. A2).

Table 1
Effect of different sodium alginate concentrations on bead formation for transfructosylating activity of *A. flavus*.

Concentration of sodium alginate (% w/v)	Transfructosylating activity (Ut) (U/ml)	Bead diameter (mm)
1	18.16 ± 1.48	1.80 ± 0.98
2	29.53 ± 1.60	2.20 ± 1.21
3	37.33 ± 1.37	2.90 ± 1.54
4	20.42 ± 1.14	3.30 ± 1.88
5	11.07 ± 0.99	4.10 ± 2.11

2.4. Enzyme assay

The transfructosylating (Ut) activity for free FTase was determined by incubating 250 μl cell-free supernatant with 750 μl of sucrose 60% (w/v) in 0.1 M citrate buffer (pH 5.5) at 55 °C for 1 h in water bath. For estimation of immobilized FTase activity, 1 g of alginate and chitosan beads were added to 3 ml of sucrose (60% w/v) solution. The reaction was stopped by boiling the mixture in water bath at 100 °C for 10 min. Transfructosylating activity was estimated by taking 10 μl of appropriately diluted reaction mixture and mixing it with 1 ml test reagent (Glucose oxidase-peroxidase, GOD-POD kit, Sigma). The glucose released was measured by spectrophotometer (Hitachi Techcom, India) at 505 nm. One unit of fructosyltransferase was defined as the amount of enzyme activity required to produce 1 μmol of glucose per minute under the described conditions.

2.5. Continuous production and analysis of fructooligosaccharides

The cell bound FTase entrapped in chitosan and alginate beads (each 5 g) were reacted with sucrose (60% w/v) at 55 °C for 24 h and percentage of FOS formation was analyzed by HPLC. After 24 h, these beads were decanted from reaction mixture and reused for next batch for FOS formation. This process was repeated for several batches until FOS formation was maximally declined. At the end of each incubation period the enzyme substrate reaction was arrested by boiling in water bath at 100 °C. Analysis was done by HPLC (Waters, USA) having 515 HPLC pump with RI detector 2414 and injection valve of 20 μl. Sugar-pak column was used for identification and quantification of FOS derivatives kestose (GF_2), nystose (GF_3), 1-fructofuranosyl nystose (GF_4) and sugars such as sucrose (GF), glucose (G) and fructose (F), respectively. The solvent system used was water as mobile phase at flow rate of 0.2 ml/min, and calculation of analysis was done by Empower 2 build software 2154.

2.6. Statistical analyses

The data in the tables and figures were expressed in triplicates as mean ± standard deviation. Using one-way analysis of variance (ANOVA), a difference was considered statistically significant if the *P* value was less than 0.05.

3. Results and discussion

3.1. Effect of sodium alginate and $CaCl_2$ concentration on entrapment of FTase for transfructosylating activity

For developing stable beads to impart better entrapment efficiency of FTase, different concentrations of sodium alginate and $CaCl_2$ were evaluated. The highest transfructosylating activity (Ut) was found when mycelia containing FTase was entrapped using 3% (w/v) of sodium alginate (Table 1). The FTase activity decreased below this concentration due to large pore size causing leakage of