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Production of Fructoligsaccharides by *Aureobasidium pullulans* Using Immobilization Technique

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Abstract: The high content of fructooligosaccharides production from sucrose using fructosyltransferase from *Aureobasidium pullulans* was investigated in the laboratory by using immobilizing technique. The FOS production was obtained using both extracellular and intracellular enzyme from *Aureobasidium pullulans*. Optimum conditions for enzyme production and enzyme reaction have been determined.

Key words: Fructooligosaccharides, immobilization, fructosyltransferase enzyme, production, laboratory, India

INTRODUCTION

Carbohydrate occur in nature as poly and hetro-functional mono, oligo and polysaccharides and are essential carbon sources for many bacteria, yeast and fungi. Biotechnological conversion of carbohydrates gives rise to broad range of derivatives with interesting and often unique potentials for applications in different types of industries e.g., detergent, cosmetic, food, pharmaceutical and chemical industries. Oligosaccharides are important in sweetener industry because of their wide applications such as low calorie sweetener for diabetics and functional food for enhancing growth of intestinal microorganisms (Oku *et al*., 1984; Hidaka *et al*., 1986; Yun *et al*., 1994). These well-known oligosaccharides are cyclodextrins (Hara *et al*., 1994), isomalto-oligosaccharides (Kohmoto *et al*., 1991), soybean-oligosaccharides (Wada *et al*., 1992) and fructooligosaccharides (Hidaka *et al*., 1988; Jung *et al*., 1993; Yun and Song, 1993). Among these new commercially available products, the Fructo-oligosaccharides (FOS) obtained from sucrose have attracted special attention due to their properties and thus have a great economic potential for the sugar industrial branch. FOS are of low caloric value as they are not hydrolyzed by the gastro-intestinal enzymes, promoting selectively the growth of the bifid bacteria in the colon (Moore *et al*., 2003), helping to eliminate the harmful microbial species to human and animal health and preventing colon cancer (Gibson and Roberfroid, 1995). FOS has also many important physiological properties such as they are non cariogenic, safety for diabetes, reduce cholesterol, phospholipids and triglyceride levels in blood (Tokunaga *et al*. 1989). Fructooligosaccharides (FOS) are oligosaccharides of fructose containing a single glucose moiety. FOS is industrially produced through fructosyl transfer from sucrose using microorganisms especially fungi and these fungal enzymes are too present in many higher plants (asparagus, chicory, onion, Jerusalem artichoke, etc). FOS producing enzymes are assigned as hydrolases (β fructofuranosidases, EC 3.2.1.26) or fructosyltransferases (transfructosidases, EC 2.4.1.9). The maximal FOS production for a particular enzyme depends on the relative rate of transfructosylation and hydrolysis. FOS are mainly composed of 1-kestose (GF_2), nystose (GF3) and 1-β-fructofuranosyl nystose (GF_4) in which fructosyl units (F) are bound at the β (2→1) bonds with terminal α linked D glucose (Sangeetha *et al*., 2005; Yun, 1996; Hidaka *et al*., 1988) (Fig. 1).

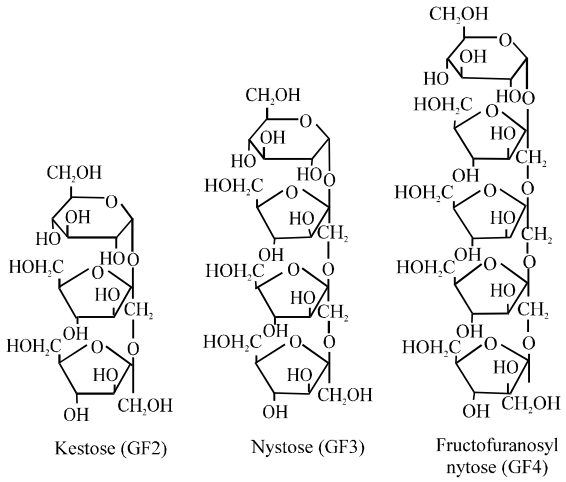

Fig. 1: Composition of FOS

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MATERIALS AND METHODS

Chemicals: All chemicals used were of analytical grade. FOS standards were from Sigma USA and glucose, fructose were from Otto and sucrose of food grade.

Microorganisms and culture conditions: The microbial slant of *Aureobasidium pullulans* was taken from NCL Pune of ATCC No. 20524. The inoculums was developed by transferring of full lope of spores into 50 mL media containing 0.2% yeast extract and 1% sucrose (pH 5.5). The culture were kept in shaker incubator keeping its rpm 220 and temperature 28°C for 24 h.

Production of fructosyltransferase enzyme: For enzyme production the media was prepared in 250 mL flask and added amount of culture media was 100 mL. The composition of the culture medium was sucrose 20 g, $NaNO_3$ 1 g, K_2HPO_4 0.5 g, yeast extract 0.5 g, $MgSO_4.7H_2O$ 0.5 g and NaCl 0.25 g autoclaving 125°C for 20 min. The 10 mL of 24 h old inoculums was transferred into 100 mL culture media with 1 mL spore suspension containing around 1.89×10^7 spores mL^{-1} and incubated at 30°C with shaking at 240 rpm for 24-120 h with regular sampling at 24 h intervals to measure the enzyme activity. After incubation the culture media is centrifuged at 6000 rpm for 20 min at 4°C. After centrifugation the supernatant was used as a source of extracellular enzyme and the remaining pellet as intracellular enzyme.

Enzyme assay: For determining of enzyme activity 2.5 mL of enzyme is mixed with sucrose solution 60% w/v as substrate, 0.2 M citrate buffer (pH 5.5) and incubated at 55°C for 1 h using shaker incubator from Lark. The reaction was terminated by keeping reaction mixture in water bath of 100°C for 15 min. Glucose, fructose and trisaccharides like GF_2, GF_3 and GF_4, etc. were then determined by HPLC. One unit of activity of transfructosylation is defined as amount of enzyme activity which catalyses the formation of 1 μmol glucose per min and one unit of hydrolytic activity was defined as the amount of enzyme which catalyses the formation of 1 μmol fructose per min under these conditions. Qualitative analysis was done by TLC and quantitave analysis was done by HPLC.

Production of FOS by immobilization technique: After completion of cultivation, the microorganisms were collected and immobilized in sodium alginate to produce fructooligosaccharides. The microorganisms at a certain concentration of 20% w/v were mixed with the sodium alginate and the 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. The immobilized cells (beads) were kept in 2% of calcium chloride solution for enzymatic reaction and the microorganisms at a concentration of 20 g were mixed with the sodium alginate at a concentration of 1% w/v.

Fructooligosaccharide production: FOS production was carried out using 55% concentrations of sucrose w/v). The extracellular enzymes of 2.5 mL were mixed with 7.5 mL sucrose w/v and incubated at 55°C in shaker incubator at 220 rpm. The reaction was carried out as a function of time from 1-36 h. After incubaton the reaction was stopping by putting the reaction mixture in a water bath for 20 min at 100°C. The products in the reaction mixture were analyzed both qualitatively by TLC in Fig. 2 and quantitavely by HPLC (Fig. 3).

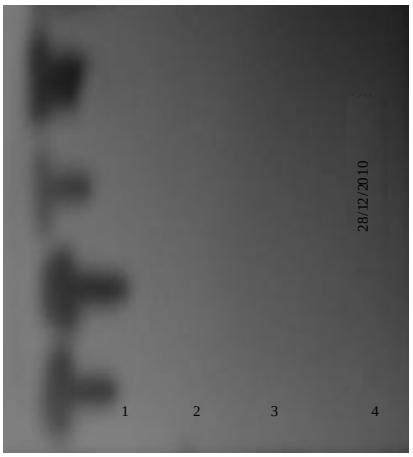

Fig. 2: TLC analysis shows 1: Sucrose; 2: Glucose, 3: Fructose; 4: FOS

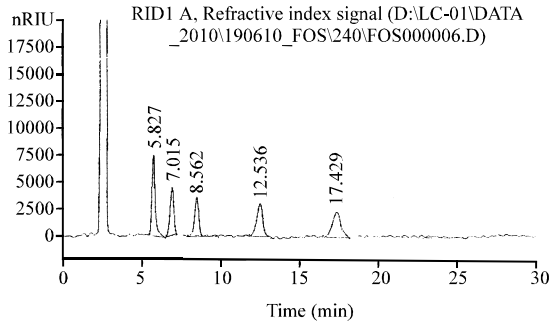

Fig. 3: Typical HPLC chromatogram of standard solution FOS (retention time of fructose-5.827, glucose-7.015, sucrose-8.562, kestose-12.536, nystose-17.429)

The thin layer chromatography coated on glass plates consists of solvent system 1-propanol: ethyl acetate: water 6:2:2 ratio. The plates were developed using spray solution consists of 5 g of urea, 20 mL of M H_3PO_4 and 80 mL of 90% ethanol. The plates were dried in an oven at 80°C overnight.

The quantitative analysis was done by HPLC with a refractive index detector using the shodex column (4.6 mm×25 cm, 5 μm). The analysis was done at room temperature with acetonitrile: water (75:25) as mobile phase at a flow rate of 1.0 mL min^{-1}. The final FOS yield was expressed as the percentage conversion yield based on the initial sucrose concentration.

RESULTS AND DISCUSSION

The main objective of this research was to produce FOS through immobilization technique and the fructooligosaccharides production was made at controlled pH 5.5. Using both extracellular and intracellular enzyme reacting with 55% concentration of sucrose.

Production of FOS: Using immobilization technique of cells their often occurs causes some changes in kinetic patterns of the resulting product. When extracellular enzyme was used the FOS concentration was observed after 42 h which corresponded to 46% but when intracellular enzyme was used the FOS concentration was little higher 54% w/w. The samples were taken at regular intervals after 2 h of reaction FOS formation was 15% and goes on increasing and reaches upto 46% by using extracellular enzyme (Fig. 4) but in intracellular enzyme it was 20% after 2 h of reaction and reaches 54% during end of reaction time 42 h (Fig. 5). Concentration of sucrose was much in the beginning but it soon becomes less as the fructosyl enzymes transfers fructose molecules from one sucrose molecules which acts as donor and transfers it to next sucrose molecules which acts as recipient forms large oligomers like kestose (GF_2) and nystose (GF_3). There was no formation of fructofuranosyl nystose (GF_4) upto 22 h but after 30 h of reaction its formation starts and goes on increasing and reaches upto 4% in 48 h. Fructose in the reaction was less in beginning but goes on increasing because fructosyltransferase enzyme transferase fructose from one sucrose molecules to another. Also in case of glucose it was less in the beginning but as the reaction proceeds it was liberated free and it inhabits for fructooligosaccharides production.

These conditions are identical to those reported by Jung *et al.* (1993) and Sangeetha *et al.* (2004) with FTF isolated from AP. However, the yield of conversion, the sugar composition and the kinetic for the enzyme reaction

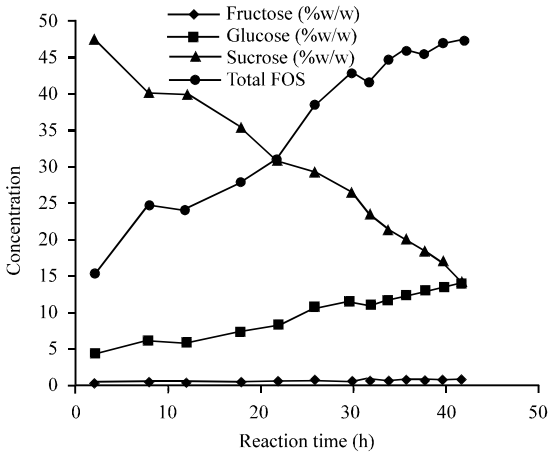

Fig. 4: Concentration of FOS in 42 h using extracellular enzyme

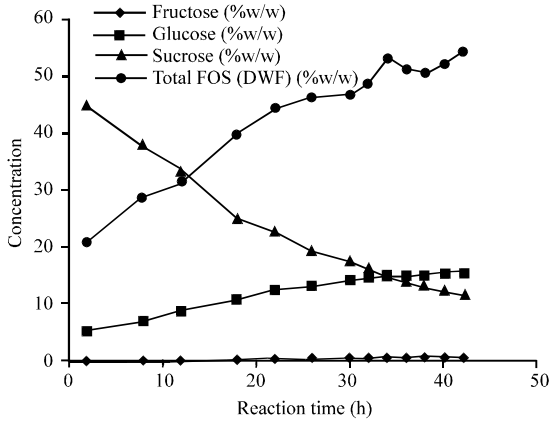

Fig. 5: Concentration of FOS during 42 h using intracellular enzyme

were different. Their yield of conversion was markedly higher than the results. Yun *et al.* (1994) demonstrated that the conversion yield obtained using FTF alone is limited to only 55-60% (w/w) because the glucose liberated during the enzymatic reaction acts as a competitive inhibitor. To enhance FOS conversion, they suggested using a mixed system constituted of FTF combined with enzymes able to eliminate glucose in the reaction mixture. They obtained very good results using a system containing FTF and glucose oxidase (Yun *et al.*, 1994). Under optimal conditions, sucrose was completely consumed in 25 h, glucose was converted into gluconic acid and conversion yields up to 90% were obtained. This system is nevertheless not ideal because of the glucose oxidase being inactive at high temperature and the reaction has to be carried out at 55°C with limited sucrose concentration of 55% w/v. Sangeetha *et al.* (2004) used

both culture fluid and intracellular enzyme of Aureobasidium Pollulans CFR77 and their result was 55% of culture fluid and 56% of intracellular enzyme in 24 h of reaction mixture. But their sucrose concentration was 80%.

CONCLUSION

Using fructosyltransferase enzyme from *Aureobasidium pullulans* both the extracellular and intracellular cell mass were immobilized in sodium alginate to form small gel beads. About 5.2 g of beads was reacted with 55% concentration of sucrose and resultant product was 54% of FOS which is much >46% from extracellular enzyme.

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