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Fructooligosaccharides production from sucrose by *Aspergillus sp.* N74 immobilized in calcium alginate

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Batch fructooligosaccharides (FOS) production by fructosyltransferase from *Aspergillus sp.* N74 immobilized in calcium alginate was studied. The used biomass for immobilization was obtained in 250 ml shake flask from the culture of 10^6 *Aspergillus sp.* N74 spores in 100 mL medium during 48 h. After biomass immobilization, the sucrose bioconversion was carried out with a mean dry weight biomass:reaction volume ratio of 0.4:100. pH, temperature and initial sucrose concentration effect on FOS production was evaluated, obtaining the higher transfructosylating activity and hydrolytic activity relation (3.78 to 5.62) at pH 5.0, 55ºC and 55-80% initial sucrose concentration for 5 h; at these conditions were obtained the greatest FOS productions (~ 50 % $^{w}/_{w}$ in sucrose basis). The results suggest that the fructosyltransferase from the native strain *Aspergillus sp.* N74 could be an appropriated enzyme for the commercial production of FOS.

1. Introduction

Fructooligosaccharides (FOS) are oligosaccharides of fructose containing a single glucose moiety, they are produced by the action of fructosyl transferasa (FTase, E.C. 2.4.1.9) from many plants and microorganisms. The FOS formed contains fructosyl units bounded at the β-2,1 position of sucrose, they are mainly composed by 1-kestose, nystose and 1-β-fructofuranosyl nystose (Sangeetha *et al.*, 2005b; Kaplan and Hutkins 2000; Yun, 1996; Hidaka *et al.*, 1988). FOS with low polymeric grade display better therapeutic properties than those with a high polymeric degree. They are about 0.4 and 0.6 times as sweet as sucrose and have been used in the pharmaceutical industry as a functional sweetener (Sangeetha *et al.*, 2005b; Biedrzycka and Bielecka, 2004; Heyer and Wendenduerg, 2001; Yun, 1996; Kühbauch, 1972). FOS present properties such as low caloric values, noncariogenic properties, decrease levels of phospholipids, triglycerides and cholesterol, help gut absorption of calcium and magnesium, are useful for diabetic products and are used as prebiotics to stimulate the bifidobacteria growth in

the human colon (Sangeetha *et al.*, 2005b; Biedrzycka and Bielecka, 2004; Roberfroid and Delzenne, 1998; Yun, 1996; Crittenden and Playne, 1996; Yamashita *et al.,* 1984).

FOS are industrially produced from sucrose by microbial enzymes with transfructosylating activity. Most of these enzymes have been found in fungi such as *Aspergillus*, *Aureobasidum, Arthrobacter* and *Fusarium*. Nevertheless, commercial FOS may contain glucose, fructose and sucrose in more than 500 g per kg of total FOS dry weight (Sangeetha *et al.*, 2005a-b; Yun, 1996).

FOS production with immobilized *Aspegillus japonicus* entrapped in calcium alginate (Cruz *et al.*, 1998) and gluten (Chien *et al.*, 2001) have been made. With the different immobilization techniques the possibility of continuous process for FOS production is being considered, using other microorganisms like *Aureobasidium pullulans, Aspergillus japonicus* (Sheu *et al.*, 2002) and *Penicillium citrium* (Park *et al.*, 2005), achieving positive results with respect to enzyme activity and a reaction time near to FOS production with free microorganisms (Kim *et al.,* 1996).

Thus, the searches of new potent transfructosylating–enzyme producers with their best reaction conditions are desirable in order to scale-up the process. In this study was evaluated the batch-FOS production from sucrose by immobilized cells of *Aspergillus sp* N74 in calcium alginate.

2. Materials and methods

2.1 Chemicals

Sodium alginate used for immobilization procedure was purchased to Sigma (USA). The sucrose was food–grade, while other chemicals were analytical grade.

2.2 Microorganism and spore production

The fungus *Aspergillus sp.* N74 was isolated from a sugar cane crop in La Peña (Colombia). In a previous study (Sánchez, 2006), this strain showed a high transfructosylating activity and the best sugar-bioconversion was at pH 5.5, 60ºC and initial sugar concentrations higher than 55% (w/v). The strain was cultivated on malt extract agar (MEA) plates at 30±1ºC for 7 days. To prepare spore suspensions, spores were scraped down from the MEA plates with a sterilized tensoactive solution (15% w/v glycerol, 0.1% w/v Tween 80 and acetate buffer 0.1M (pH 6.0) q.s.f. 100 mL) and diluted to a concentration of about 1×107 spores mL-1 with sterilized water. The spore suspensions were kept at –20±1ºC and subcultured once a month.

2.3 Biomass Production

For the different assays the biomass production was made in 250 ml shaker flasks with 100 mL of culture medium (11% sucrose, 0.84% K_2HPO_4, 0.102% $MgSO_4 \cdot 7H_2O$, 0.088% KCl, 0.007% $FeSO_4 \cdot 7H_2O$, 0.085% $NaNO_3 \cdot 4H_2O$, 2.0% yeast extract, 0.136% $CaCO_3$, adjust to pH 5.5±0.1 with HNO3) inoculated with 1000 µL of $1x10^7$ spores ml^{-1} shaken at 30±1ºC and 250 r.p.m. for 48h (New Brunswick C76). The obtained biomass,

it was filtered and washed twice with phosphate buffer 50 mM (pH 5.5) before immobilization.

2.4 Mycelia immobilization

For the immobilization procedure, mycelia of the strain *Aspergillus sp.* N74 was homogenized with 50 mL 2% (w/v) of sodium alginate. The gel produced was pump by a peristaltic pump (Cole-Parmer-77200). The drops from this falling into a 0.2 M $CaCl_2$ under constant and weak agitation, changed into calcium alginate beads. The beads were kept at 4ºC for 24 h before use.

2.5 Enzymatic Reaction

The enzymatic reaction was carried out at temperature range 45-60 ºC, pH range 5.0-6.0 and 30-80% ($^{w}/_{v}$) initial sucrose concentration at 150 r.p.m. for 5 h. During the reaction time supernatant samples were taken for sugar analysis. The reaction was stopped by heating each sample in boiling water for 10 min.

At the end of the reaction, the biomass concentration was determinated after washing the pellets with 7% (w/v) sodium citrate. The obtained biomass was washed with 50 mM phosphate buffer (pH 5.5) and dried for 48 h at 105°C (Dorta *et al.*, 2006; Cruz *et al.*, 1998).

The transfructosylating activity and the hydrolytic activity were determined by measuring both the glucose (G) and fructose (F) present in the reaction mixture. One unit of transfructosylating activity was defined as the amount of enzyme required to transfer 1 µmole of fructose min−1. One unit of hydrolytic activity was defined as the amount of enzyme required to release 1 µmole of free fructose min^{-1}.

The enzymatic productivity was calculated as the transfructosylating (U_{tE}) or hydrolytic (U_{hE}) activity per reaction volume, while the specific activity was calculated as the transfructosylating (U_{tS}) or hydrolytic (U_{hS}) activity per dried weighted biomass (Fernández et al., 2004; Hidaka et al., 1988; Nguyen et al., 1999). Transfructosylating (U_t) and hydrolytic (U_h) activities were calculated at a time interval by Eq. 1 and 2, respectively.

$$U_t = \frac{\left\{ \mu\text{mol Glucose}\big|_{t_i} - \mu\text{mol Glucose}\big|_{t_0} \right\} - \left\{ \mu\text{mol Fructose}\big|_{t_i} - \mu\text{mol Fructose}\big|_{t_0} \right\}}{(t_i - t_0)} \quad (1)$$

$$U_h = \frac{\mu\text{mol Fructose}\big|_{ti} \quad \mu\text{mol Fructose}\big|_{t0}}{(t_i - t_0)} \quad (2)$$

Specific activity and volumetric productivity of the enzyme were evaluated through Eq. 3-4 and Eq. 5-6, respectively.

$$U_{tS} = \frac{U_t}{mg \text{ dried biomass}} \quad (3)$$

$$U_{hS} = \frac{U_h}{mg \text{ dried biomass}} \quad (4)$$

$$U_{tE} = \frac{U_t}{\text{Reaction volume}} \quad (5)$$

$$U_{hE} = \frac{U_h}{\text{Reaction volume}} \quad (6)$$

2.6 Analysis of sugars

The analysis of sugars was performed by high performance liquid chromatography (HPLC). The HPLC equipment consisted of a pump Waters 515 with an on line degasser, a refractive index (RI) detector Waters 410 and injection valve with a 20 μL loop.

A Sugar-Pak^{TM} (Waters) column was used for sucrose, glucose and fructose identification and quantification. The chromatographic conditions were: column temperature, 84 ºC; mobile phase, water at flow rate of 0.4 cm^3 min^{-1} and RI detector temperature, 40 ºC (Sánchez, 2006).

3. Results and discussions

Sucrose was rapidly converted into FOS and glucose. The results showed that increasing the initial sucrose concentration is increased the FOS production. Likewise results are reported by Hidaka *et al.* (1988), who observed the higher synthesis of FOS in concentrated sucrose solutions, although they tested sucrose concentrations up to 50%. Park and Almeida (1991), verified a considerable increase in FOS production and a decrease in the content of free fructose in the middle of the reaction when the sucrose concentration was increased from 30% to 60%, which was explained by the competition among water and substrates used as acceptors in the reactions catalyzed by the β-fructosyltransferase. Fernández *et al.,* (2004), Beker *et al.,* (2002), Yun *et al.,* (1990), Hidaka *et al.,* (1988), have reported the same performance.

The initial sucrose concentration and pH increased favored the transfructosylating activity and transfrutosylating volumetric productivity. The maximum specific transfructosylating activity and consequently volumetric productivity was obtained at pH 6.0 and 60ºC, (Fig. 1-2) but at this condition was obtained the highest hydrolytic activity and volumetric productivity. The remnant sucrose was no higher than 15% at the end of 5h but no significant differences in the activities were found between the first hour and the end of the reaction (5h). Likewise Hayashi *et al.,* (1992) and Cruz *et al.,* (1998), obtained the same perform for the enzyme produced by *Aspergillus japonicus*. This probably could be by the higher product intraparticle transportation time that favors the hydrolytic activity of the enzyme.

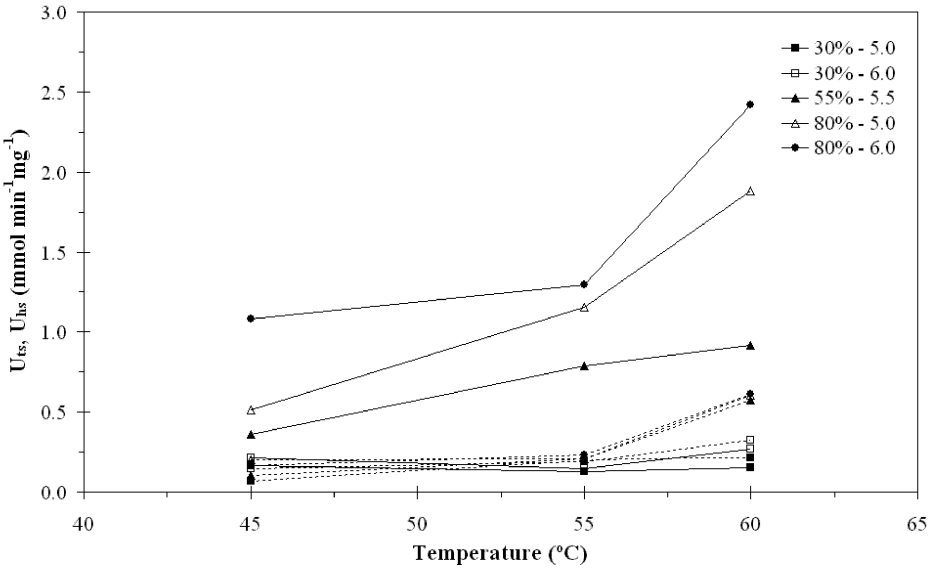

Figure 1. *pH, temperature and initial sucrose concentration effect on the specific transfructosylating (—) and hydrolytic (---) activity of the FTase produced by the immobilized mycelium of Aspergillus sp. N74 in calcium alginate.*

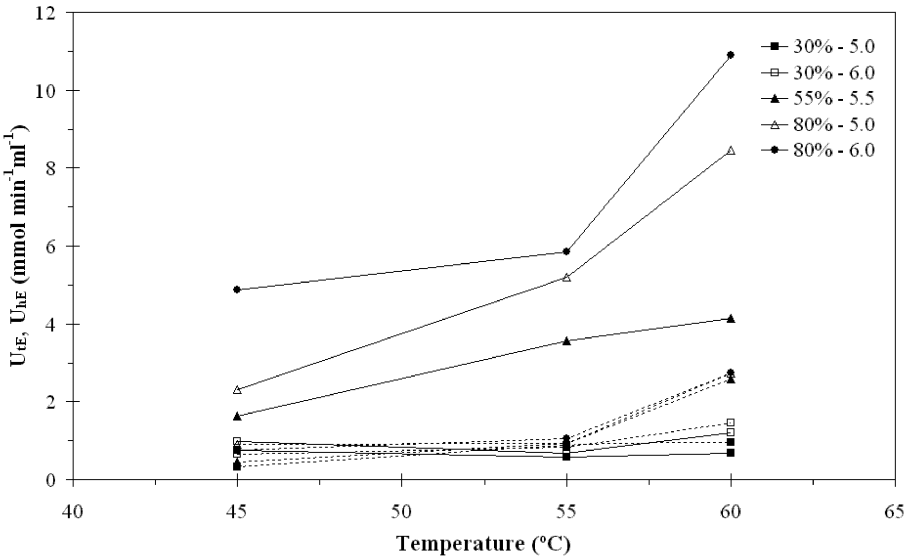

Figure 2. *pH, temperature and initial sucrose concentration effect on the volumetric transfructosylating (—) and hydrolytic (---) productivity of the FTase produced by the immobilized mycelium of Aspergillus sp. N74 in calcium alginate.*

Cruz *et al.,* (1998) have reported a transfructosylating activity and hydrolytic activity ratio from 1.94 to 8.91 for *Aspergillus japonicus* immobilized in calcium alginate at 60ºC, 60% initial sucrose concentration, pH 5.0. In this study, were obtained the better ratios (from 3.78 to 5.62) at pH 5.0, 55ºC and 55-80 % initial sucrose concentration. The result displayed an ezyme activity loss at 60°C due to the diminution on the U_t:U_h ratio (Table 1).

Table 1. U_t:U_h ratio for tested conditions

Initial sucrose (%) and pH	U_t:U_h ratio Temperature (°C)		
	45	55	60
30 %, pH 5.0	2.36	0.64	0.71
30 %, pH 6.0	1.47	0.79	0.83
55 %, pH 5,5	3.58	3.78	1.59
80 %, pH 5,0	2.53	5.62	3.10
80 %, pH 6,0	5.49	5.52	3.97

The best conditions for FOS production by the immobilized *Aspergillus sp.* N74 cells were like for the free cells reported by Sánchez (2006). At pH 5.0 and 60ºC with initial sucrose concentrations higher than 55%$^{w}/_{v}$, the FOS production was about a 50%$^{w}/_{w}$.

Several authors who have worked with immobilized cells (like *Penicillium citrinum* KCCM11663, *Aspergillus japonicus* and *Aureobasidium pullulans*) or immobilized enzyme (purified or commercial like Pectinex Ultra SP-L) have reported optimal values of pH, temperature and initial sucrose concentration, 5.0-6.0, 50-60ºC and 50-80%, respectively (Park *et al.,* 2005; Tanriseven and Aslan, 2005; Chien *et al.,* 2001; Yun *et al.,* 1990).

The enzymatic activity and productivity are influenced by the cell entrapping in calcium alginate. Compared the obtained enzyme activity and productivity for the free (Sánchez, 2006) and immobilized cells, there was a decrease of the calculated values U_{tS}, U_{hS}, U_{tE} and U_{hE} for all essay conditions with the immobilized microorganism. The loss of enzymatic activity could be due to the enzyme leakage from the permeabilized cells, which were entrapped in the alginate beads and chemical interactions between the polymeric matrix and the cells, which affected the enzyme production (Chien *et al* 2001).

Additionally, comparing FOS concentrations gotten in both cases, an increase in reaction time is observed to get a similar FOS production using free cells. This delay in the reaction time was probably due to the mass transfer limitation in the immobilized particles where contribution of diffusion resistance in the alginate matrix to the enzymatic reaction was evident. This behaviour is very similar to the results reported by Chien *et al.* (2001) and Yun *et al.* (1990), who carried out the FOS production using immobilized cells of *A. japonicus* in gluten and *Aureobasidium pullulans* entrapped in calcium alginate beads, respectively.

4. Conclusions

The *Aspergillus sp.* N74 immobilized in calcium alginate present a favorable performance for the fructooligosaccharides production. The specific transfructosylating activity showed a dependence on the reaction pH, temperature and initial sucrose concentration. The highest transfructosylating activity was found at 60ºC, pH 5.0 and a initial sucrose concentration higher than 55%$^{w}/_{v}$ with a Ut/Uh from 3.78 to 5.62.

The immobilization of the native strain *Aspergillus sp.* N74 could be an appropriated alternative for the study of scaled-up process of commercial FOS.

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