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Fructooligosaccharides production from sucrose by *Aspergillus sp.* N74 in a hybrid bioreactor

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Abstract

Batch fructooligosacharides (FOS) production by fructosyltransferase from *Aspergillus sp.* N74 using whole cells was studied. The biomass production and enzymatic reaction were carried out in a hybrid reactor (airlift mechanically agitated). To evaluate the fructosyltransferase activity two biomass concentrations (6 and 9.5 g L^{-1}) of *Aspergillus sp* N74 mycelia were used. The reaction conditions were pH 5,5, 60 ºC and a initial sucrose concentration of 70% ($^{w}/_{v}$); in the reactor the superficial air velocity and impeller speed were 0.012 m s^{-1} and 450 r.p.m, respectively. For each biomass concentration the reaction time for the batch operation was 26h. FOS yield in batch operation for 6 g L^{-1} biomass concentration was 69%, while the highest FOS yield was 76% for 9.5 g L^{-1} biomass concentration in the first 4h of reaction. Results showed the design reactor and the native strain *Aspergillus sp.* N74 as an industrial alternative for the fructooligosaccharides production.

Keywords: Fructosyltransferase, Fructooligosaccharides, *Aspergillus sp*., Hybrid reactor

1. Introduction

Fructooligosaccharides (FOS) are oligosaccharides of fructose containing a single glucose moiety, they are produced by the action of fructosyl transferase (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). Among FOS, the ones with low polymeric grade show 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, non-cariogenic 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* (Sangeetha *et al.,* 2005a-b; Yun, 1996). Nevertheless, commercial FOS may contain glucose, fructose and sucrose in more than 500 g per kg of total FOS dry weight. Thus, the pursue of new potent transfructosylating–enzyme producers with their best reaction conditions are desirable in order to scale-up the process; in this study, the batch-FOS production from sucrose by whole cells of *Aspergillus sp* N74 in a mechanically agitated airlift reactor was evaluated at bench scale.

2. Materials and methods

2.1. Chemicals

1-Kestose, nystose, and 1-β-fructofuranosyl nystose used for standard in product analysis were purchased from Wako Pure Chemical Industries (Osaka, Japan). The sucrose was food–grade commercial products, 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 conditions were pH 5.5, 60ºC and 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) for 100 mL), diluted to a concentration of about $1x10^{7}$ spores ml^{-1} with sterilized water. The spore suspensions were kept at –20±1ºC and subcultured once a month.

2.3. Reactor description

The mycelium growth and the sucrose bioconversion were made in a concentric membrane–draft–tube bioreactor. The membrane was made in stainless steel with pore size of 20 μm and formed a filtration module with an irregular geometry that held the mycelium pellets in the fermentation vessel (Fig. 1). The total filtration surface was 1620 cm^2 for an area–operation volume ratio of 360 $cm^2 L^{-1}$.

At the centerline of the membrane–draft–tube the agitation and aeration system were placed. Agitation was made with two Rushton turbines and aeration was made through a perforated pipe sparger, the vertical distance between the lower turbine and the sparger was 3.5 cm (C_{ts}). The 6-

bleaded Rushton turbines were 7.5 cm in diameter (d_i) and every blade was 1.5 cm wide (w), for a w/d_i relation of 1/5. The vertical distance between the turbines was 5.0 cm (s_c) and the lower turbine was located 8.5 cm from the vessel bottom (C), for a s_c /d_i relation of 2/3. The bioreactor vessel was 16 cm in internal diameter (D) and 32 cm in overall height (H) with round bottom. The membrane–draft–tube, 15 cm length (h_m), was located 5.0 cm above the bottom of the vessel (C_l) and the raiser and downcomer relation area were 0.83 (A_r/A_d). The working volume and the overall bioreactor volume were 4.5 and 7 L, respectively. The initial static liquid height was 22.0 cm (h_L) and the clearance between the upper shape membrane–draft–tube and the liquid surface was 2.2 cm (C_u). Fig. 2 shows the reactor dimensions.

Fig. 1 Upper **(a)** and lower **(b)** cross view and axial view **(c)** of the design filtration module.

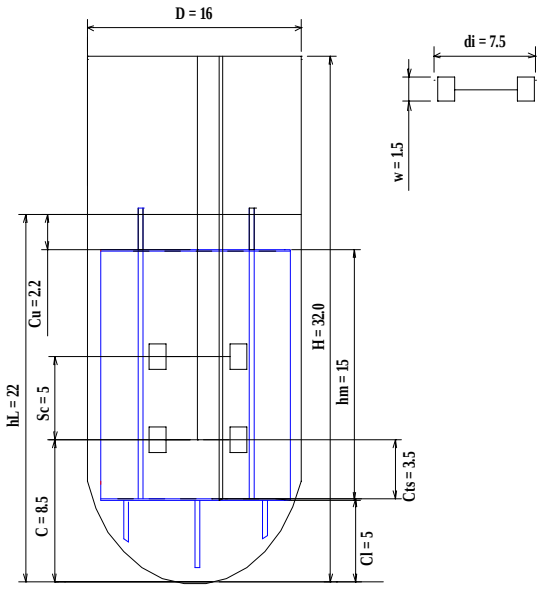

Fig. 2 Reactor dimensions (in centimeters).

2.4. Biomass Production

For every fermentation, a pre–inoculus was made in a 1L Erlenmeyer flask with 500 mL of culture medium (11% sucrose, 0.84% K_2HPO_4, 0.102% $MgSO_4.7H_2O$, 0.088% KCl, 0.007% $FeSO_4.7H_2O$, 0.085% NaNO3.4H2O, 2.0% yeast extract, 0.136% CaCO3, adjust to pH 5.5±0.1 with HNO3) inoculated with 600 µL of $1x10^7$ spores ml^{-1} shaken for 12h at 30±1ºC and 250 r.p.m. (New Brunswick C76). The inoculated culture medium (pH 5.5 and total volume 4.5L) was cultured for 24 or 48h, under the following conditions: 30ºC, 300 r.p.m and superficial aeration rate (U_{Gr}), 0.008 m s^{-1}. At the end of the growing time (24 or 48h), the culture medium was pumped out through the filtration module and the held biomass was washed several times with phosphate buffer (50 mM, pH 5.5) in order to remove the culture medium remnant. The biomass concentration was determinated at the end of the enzymatic reaction, after being washed with phosphate buffer (50 mM, pH 5.5) and dried for 48 h at 80°C.

To see the effect of the membrane filtration module over the biomass production, fermentations in the same reactor but without the membrane module under the same culture conditions and medium were made. Since, fermentations in submerged membrane bioreactors (MBRs) are used to get high cell density cultures (HCDC) (Chang e*t al.,* 1994).

2.4.1 Morphological characterization

During the culture, samples were taken for the fungal pellet morphology. It was characterized using image analysis (Casas López e*t al.,* 2005; Paul and Thomas, 1998). Prior to imaging, each sample was filtered and washed twice with 10 mL of distilled water. For each sample, 50 pellets were analyzed and measured the total pellet core diameter as the one-dimensional projected area. The image was captured with a CMOS camera (Evolution LC Color; Media Cybernetics Inc., Silver Spring, MD, USA) mounted on an inverted microscope (Leica DMIL) that used a 40x magnification.

2.5. Enzymatic Reaction

The enzymatic reaction was performed for both biomass concentrations in the hybrid reactor. The reaction volume and conditions were: 4.5 L, initial sucrose concentration 70% ($^{w}/_{v}$), pH 5.5, 60ºC, 350 r.p.m and superficial aeration rate, 0.012 m s^{-1}. The reactor was operated in batch for 26h; during the reaction were taken samples for the analysis of enzymatic activity and carbohydrates.

The enzymatic activity was determinated by the total yield of fructooligosaccharides (Y_{FOS}) (Madlová *et al.,* 1999), which was calculated from the yield of 1-kestose (Y_{GF2}) nystose (Y_{GF3}) and 1-β-fructofuranosyl nystose (Y_{GF4}). While the selectivity was calculated by Eq. 5.

$$Y_{GF_2} = \frac{2[GF_2]}{[S]_0} \quad (1)$$

$$Y_{GF_3} = \frac{3[GF_3]}{[S]_0} \quad (2)$$

$$Y_{GF_4} = \frac{4[GF_4]}{[S]_0} \quad (3)$$

$$Y_{FOS} = Y_{GF_2} + Y_{GF_3} + Y_{GF_4} \quad (4)$$

$$S_{FOS} = \frac{2[GF_2] + 3[GF_3] + 4[GF_4]}{2[GF_2] + 3[GF_3] + 4[GF_4] + [F]} \quad (5)$$

Where, $[GF_2]$, $[GF_3]$, $[GF_4]$, $[S_0]$ and $[F]$ are the molar concentrations of 1-kestose, nystose, 1-β-fructofuranosyl nystose, initial sucrose and fructose respectively.

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 e*t al.,* 2004; Hidaka *et al.,* 1988; Nguyen *et al.,* 1999). The transfructosylating (U_t) and hydrolytic (U_h) activities were calculated by Eq. 6 and 7, respectively.

$$U_t = \frac{\left\{ \mu\text{mol Glucose}\big|_{t_i} \quad \mu\text{mol Glucose}\big|_{t_0} \right\} \quad \left\{ \mu\text{mol Fructose}\big|_{t_i} \quad \mu\text{mol Fructose}\big|_{t_0} \right\}}{(t_i \quad t_0)} \quad (6)$$

$$U_h = \frac{\mu\text{mol Fructose}\big|_{ti} - \mu\text{mol Fructose}\big|_{t0}}{(t_i - t_0)} \quad (7)$$

Eq. 8 and 9 were used to evaluate the specific activity and Eq. 10 and 11 the volumetric productivity of the enzyme.

$$U_{tS} = \frac{U_t}{mg \text{ dried biomass}} \quad (8)$$

$$U_{hS} = \frac{U_h}{mg \text{ dried biomass}} \quad (9)$$

$$U_{tE} = \frac{U_t}{\text{Reaction volume}} \quad (10)$$

$$U_{hE} = \frac{U_h}{\text{Reaction volume}} \quad (11)$$

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 an injection valve with a 20 μL loop.

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

A Shodex® column was used for 1-kestose, nystose and 1-β-fructofuranosyl nystose identification and quantification. The chromatographic conditions were: column temperature, 65° C; mobile phase, water-acetonitrile (72:28) at a flow rate of 1.0 cm^3 min^{-1} and RI detector temperature, 45°C (Sánchez, 2006).

3. Results and discussions

3.1 Biomass production and enzyme activity

The dried biomass produced in the agitated membrane airlift reactor at 24 and 48h were 27 and 43g, respectively; ~45% higher than the dried weighted biomass gotten in the same reactor but without the membrane filtration module. This increase in the biomass weight could be due to the fact the internal cell retention that allows it to increase (Chang e*t al.,* 1994).

Fig. 3 illustrates pH, dissolved oxygen (D.O), dried biomass concentration and apparent viscosity during the 48h microorganism culture in the hybrid reactor. The D.O and biomass profiles show a common behaviour for aerobic fermentations, where the oxygen tension falls down while the biomass concentration increases. In the same way, the apparent viscosity increased with the biomass concentration; a similar behaviour was reported for a submerged culture of filamentous fungi (*Aspergillus terreus*) by Rodríguez-Porcel e*t al.* (2005) and Casas López *et al.* (2005). The pH profile was kept in the range of 5.50±0.06.

It was noticed that for the culture conditions (stirring rate 300 r.p.m and superficial gas velocity 0.008 m s^{-1}), the pellet size after the first 6 hours of cultured kept a mean core diameter of 2200±100 μm (Fig. 4). Similar behaviour and pellet size were gotten by Casas López e*t al.* (2005) whom worked the morphological and rheology characterization of *Aspergillus terreus* cultures at the same stirring rate.

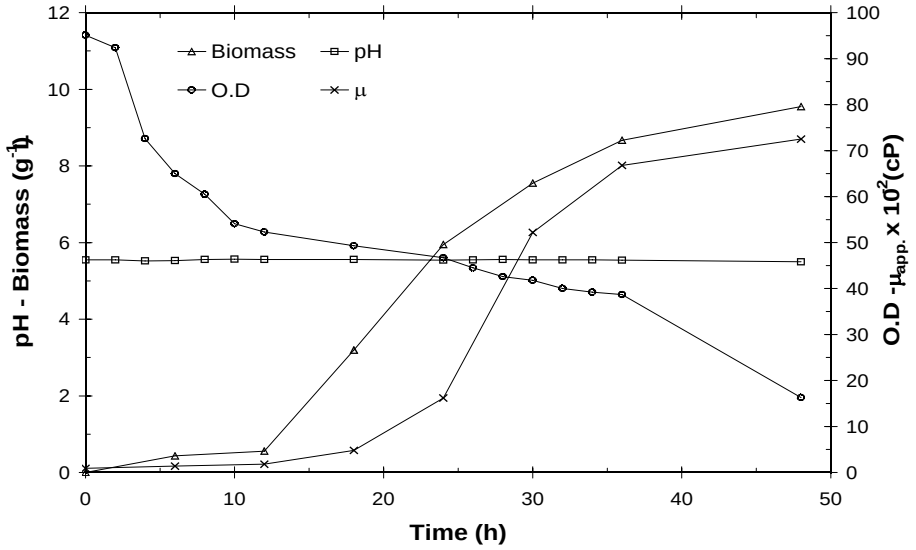

Fig. 3 pH, D.O., apparent viscosity and dried weight biomass profiles during the fungi culture.

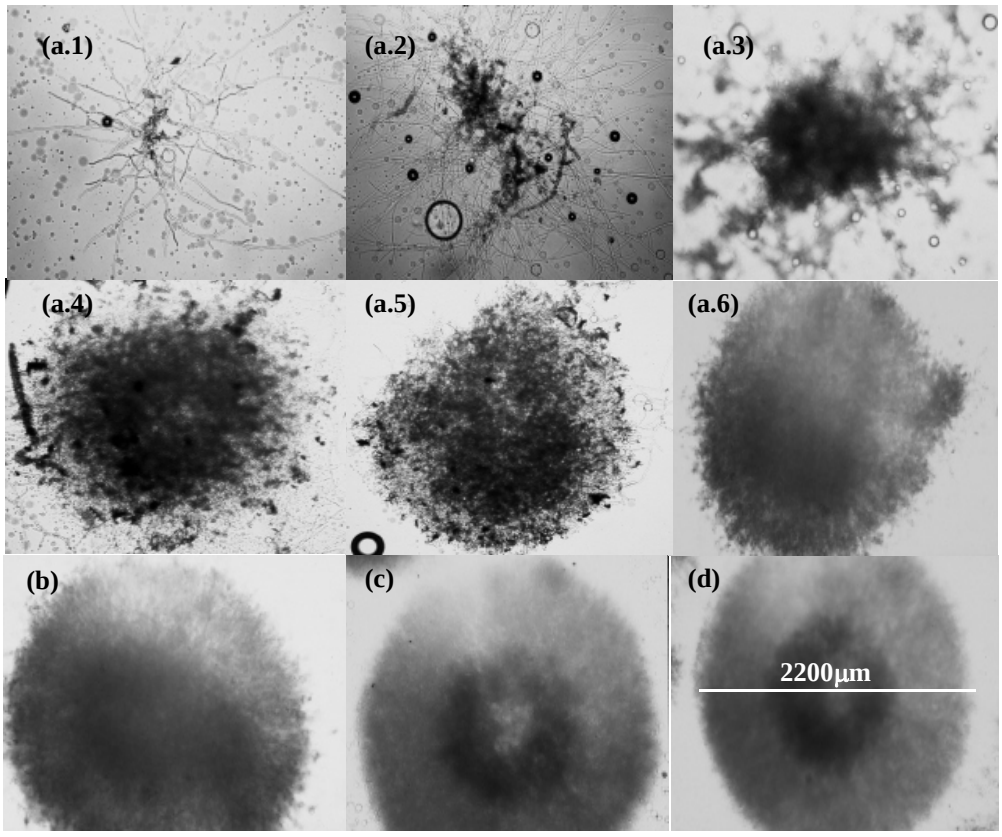

Fig. 4 Pellet formation sequence at the first 6h of culture (a.1-a.6). Pellet core size follow–up during the culture time: 6h (a-6), 18h (b), 30h (c) and 48h (d) (40x magnification).

Fig. 5 shows the enzymatic activity for the 48h microorganism culture. It is observed, that the specific transfructosylating activity presents a peak after 24h of culture becoming a plateau, although the transfructosylating volumetric productivity does not show a peak. Also, is detected a maximum in the hydrolytic activity about after 12h of culture. This phenomenon may be due to the fact that before that time there is not a high FTase production, since there is a low biomass concentration and invertase enzyme production is enhanced under lower biomass and FOS concentration (Fernandez e*t al.,* 2004).

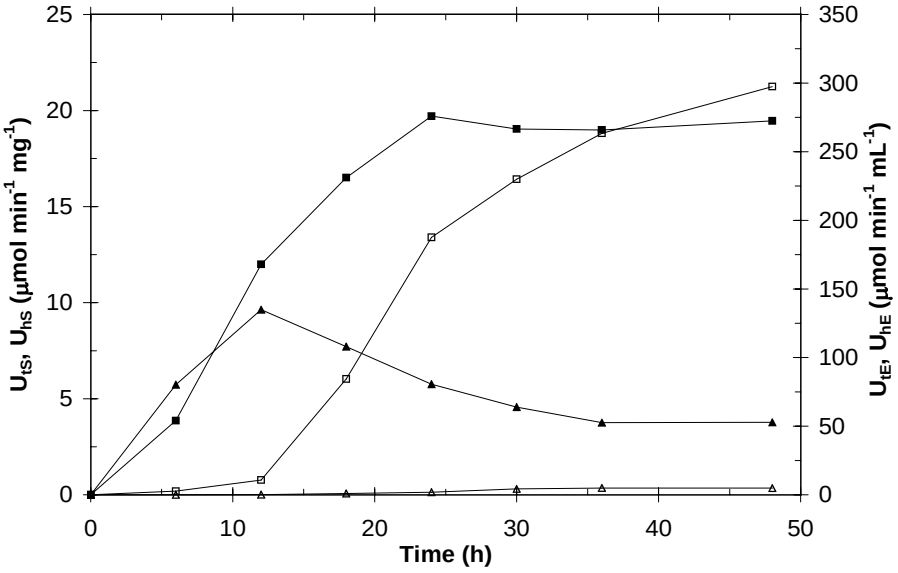

Fig. 5 Enzymatic activity profiles (U_{tS} ■, U_{tE} □, U_{hS} ▲, U_{hE} Δ) gotten during the biomass production.

3.2 Biomass production and enzyme activity

The bioconversion of sucrose and FOS produced showed a dependence on the reaction time and biomass concentration (Fig. 6 and 7). In fact, 1-kestose and nystose were the FOS produced for the 6 g L^{-1} biomass concentration, there was no evidence of the 1-β-fructofuranosyl nystose production (Fig. 6). The highest sucrose bioconversion was registered at 24h of reaction, leaving sucrose and fructose remnant of 13.5 and 5.5%, respectively. The FOS profiles showed a constant behaviour after 18h of reaction with 1-kestose and nystose production of 251.5±14.0 and 99.8±23.6g, respectively; for a total FOS production of 351.2±35.7g. About, a 54% $^{w}/_{w}$ of sucrose bioconversion. The FOS profiles showed a normal behaviour in the transformation from sucrose to 1-kestose and from 1-kestose to nystose. At the end of reaction time (26h) the reached FOS yield (Y_{FOS}) was 69% (43% 1- kestose and 26% nystose).

When the enzyme reaction was carried out with the biomass concentration in a dried basis of 9.5 g L^{-1} (Fig.7), 94% of the initial sucrose concentration was biotransformed in the first 4 hours of reaction, with a remnant of 5.2 and 4% of sucrose and fructose, respectively. But at the end of the

reaction time (26h), the sucrose and fructose remnant was 8.2 and 10.1%, respectively. This behaviour could be due to the fact that the fructosyltransferase enzyme has a Ping-pong mechanism, which can produce sucrose from FOS (Crittenden and Playne, 1996; Yun, 1996). Among the FOS produced at this biomass concentration was the 1-β-fructofuranosyl nystose. The FOS profiles showed a high biotransformation of nystose from 1-kestose in the second hour, nevertheless the biotransformation of 1-β-fructofuranosyl nystose from nystose was not high and kept constant. In the first 4 hours of reaction, the Y_{FOS} was 76% (43% 1-kestose, 29% nystose and 4% 1-β-fructorianosyl nystose), while at the end of the reaction time the Y_{FOS} was 57% (18% 1-kestose, 33% nystose and 6% 1-β-fructofuranosyl nystose). The FOS yield decayed possibly by the fact that after the first 4 hours of reaction the high glucose concentration in the medium inactivated the FTase increasing the hydrolytic activity over the FOS produced, this behaviour led to an increase in the free fructose and sucrose concentration in the medium (Hirayama *et al.,* 1989; Song and Jacques, 1999). It is noticed, that at 9.5 g L^{-1} biomass concentration is important to control reaction time, in order to get a considerable sucrose bioconversion to FOS and to handle their composition.

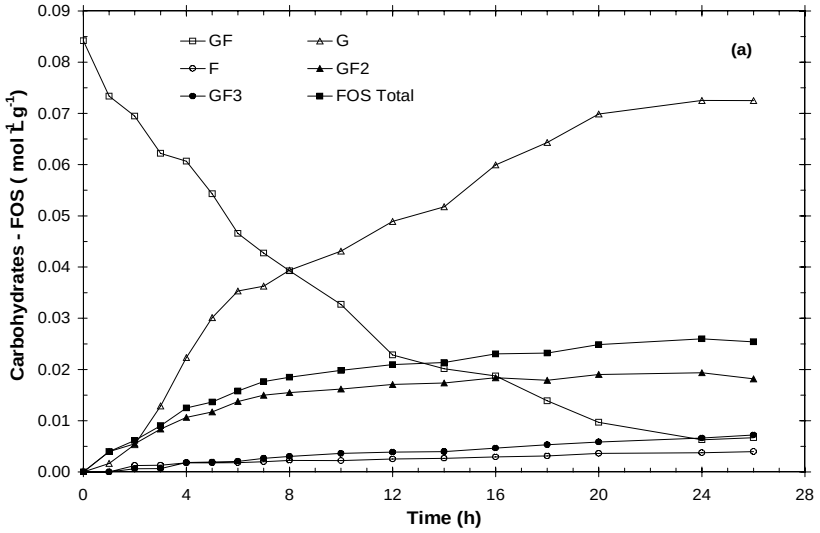

Fig. 6 Sugar profiles in a batch process, with a biomass concentration of 6.0 g L^{-1}. Nomenclature: GF, sucrose; G, glucose; F, fructose; GF_2, 1-kestose; GF_3, nystose; GF_4, 1-β-fructofuranosyl nystose and FOS_{Total} the total FOS production.

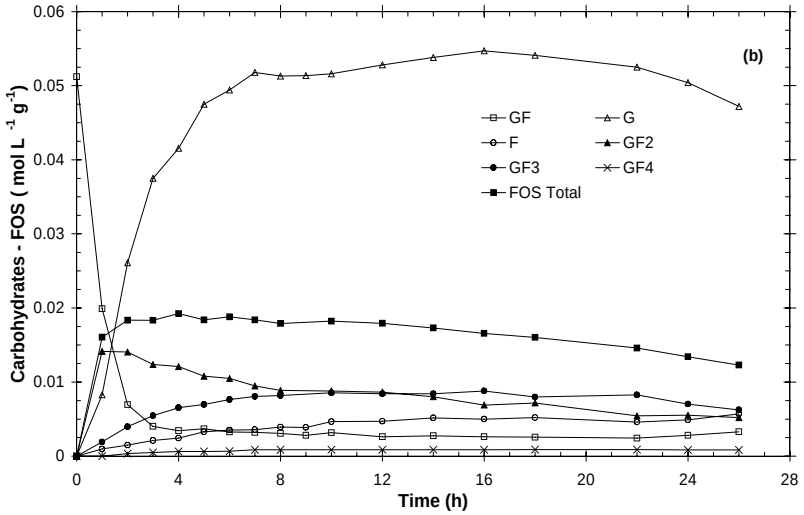

Fig. 7 Sugar profiles in a batch process, with a biomass concentration of 9.5 g L^{-1} **(b)**. Nomenclature: GF, sucrose; G, glucose; F, fructose; GF_2, 1-kestose; GF_3, nystose; GF_4, 1-β-fructofuranosyl nystose and FOS_{Total} the total FOS production.

Few studies at bench scale have been reported. Yun e*t al.* (1990) made a semibatch production of FOS in a one–liter reactor using *Aureobasidium pullulans*, the reaction solution was 77% ($^{w}/_{w}$) sucrose, pH 5.5 and at 55ºC. They got a FOS conversion of 57 and 55% for the free and immobilized cells, respectively. Sangeetha *et al.* (2005b) scaled up the FOS production using a 10L reactor and *Aspergillus orizae* CFR 202 reaching a FOS yield of 52% at the following conditions: sucrose 60% ($^{w}/_{w}$), pH 5.5 and at 55ºC for a reaction time of 18h.

The results suggest that by means of the fructosyltransferase from the native strain *Aspergillus sp.* N74 in the design reactor is an alternative to study the FOS production in a large scale or even in an industrial scale.

4. Conclusion

The designed reactor (submerged membrane airlift reactor agitated mechanically), allowed achieving high cell density cultures of the native strain *Aspergillus sp*. N74, ~ 45% higher than the biomass reached without the filtration module.

The FTase specific transfructosylating activity showed a dependence on the biomass concentration and the reaction time, getting a plateau after 24h of culture. Although, the volumetric productivity showed dependence on the biomass concentration did not present a peak.

The composition of the produced FOS depends on the biomass concentration and the reaction time. Therefore, for a biomass concentration of 9.5 g L^{-1} was synthesized the 1-β-fructofuranosyl nystose, while for the biomass concentration of 6.0 g L^{-1} it was not.

The best reaction time for the bath FOS production with a biomass concentration of 6.0 and 9.5 g L^{-1} was 24 and 4h, respectively. For each case the FOS concentration was 378 and 427 g L^{-1}, respectively.

The designed reactor and fructosyltransferase from the native strain *Aspergillus sp*. N74 can be considered as an industrial alternative for the fructooligosaccharides production.

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