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Fructooligosaccharides production using immobilized cells of *Aspergillus japonicus*

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Topic: Multi-scale and/or multi-disciplinary approach to process-product innovation.

Abstract

The fructooligosaccharides (FOS) production using immobilized cells of the fungus *Aspergillus japonicus* ATCC 20236 was evaluated. Polyurethane foam, stainless steel sponge, vegetal fiber sponge, pumice stones, zeolites and cork were tested as immobilization carrier during the fermentation under submerged conditions. Experiments were carried out in 500 ml Erlenmeyer flasks containing 1 g of carrier and 100 ml of sucrose medium (165 g/l) enriched with nutrient sources. The flasks were agitated in an orbital shaker at 160 rpm, 28°C, for 48 h. Samples were withdrawn during the fermentation to determine the consumption of sucrose, liberation of glucose and fructose to the medium, production of FOS (1-kestose (GF_2), 1-nystose (GF_3) and 1-β-fructofuranosyl nystose (GF_4)) and enzymatic activity of β-fructofuranosidase. At the fermentation end, the cell mass adhered to the carrier was quantified. The microorganism adhesion to the carrier varied to each tested material. Consequently, the FOS production and enzymatic activity also varied to each medium, due to the differences in the amount of free and immobilized cells present. The highest values of immobilized cells, FOS production and enzymatic activity were achieved by using vegetal fiber sponge as immobilization carrier, while cork gave the worst results.

1 Introduction

Fructooligosaccharides (FOS) are naturally occurring sugars that have beneficial effects as food ingredients, because of its low calorie, noncariogenic nature and ability to promote the growth of beneficial bifidobacteria-rich intestinal flora and immune system modulation. Although FOS is present in several plants, the concentration of them in these sources is low and choice of plants as a source of FOS is limited by seasonal conditions (Wang & Zhou, 2006). Hence, microbial FOS production by the action of fungal fructosyltransferase on sucrose is more feasible at industrial level (Sangeetha et al., 2005).

In the process of FOS production with fructosyltransferase, the main problem is that the activity of the enzyme is severely inhibited by glucose, which is generated as a byproduct. As a result, maximum conversion yield of sucrose to FOS ranges from 55 to 60% (w/w) due to unreacted sucrose and glucose. Since the produced FOS mixture contains considerable amounts of sucrose and glucose, its use has been limited (Yun, 1996). Therefore, the development of processes that permit the FOS production in industrial scale with higher yields, and lower necessity of purification of the final product, is necessary.

The use of immobilized enzymes or cells can be useful for the development of effective and economic methods for large-scale production of FOS. Nevertheless, the selection of the immobilization material is essential to design an effective system for this purpose. The aim of the present work was to evaluate the FOS production using immobilized cells of the fungus *Aspergillus japonicus* ATCC 20236. Polyurethane foam, stainless steel sponge, vegetal fiber

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sponge, pumice stones, zeolites and cork were tested as microorganism carrier during the production of FOS under submerged fermentation conditions.

2 Material and methods

2.1. Carrier's preparation

Six porous carriers were tested: Polyurethane foam, stainless steel sponge, and vegetal fiber sponge (Scotch Brite, 3M Spain, SA), pumice stones (Elite, purchased at a local department store), zeolites molecular sieves type 4A (BDH Chemicals Ltd., Poole, England), and cork. Polyurethane foam, vegetal fiber sponge and cork were 0.3 cm^3 cubes. Stainless steel sponge was used as cuttings of irregular size. Pumice stones were granules of 8–24 mesh (2.36 mm to 0.71 mm – sieve opening), and zeolites were approximately 0.1 cm in diameter and 0.4 cm length. To be used as immobilization carrier, all the materials were pre-treated by boiling for 10 min, washed three times with distilled water, and then dried overnight at 60 ºC. Prior to use, all the carrier materials were autoclaved at 121 ºC for 20 min.

2.2. Microorganism maintenance

The strain *Aspergillus japonicus* ATCC 20236 was used in the experiments. The strain was maintained on potato dextrose agar (PDA - Difco) plates at 4 ºC, and the spores were maintained mixed with glycerol solution in ultra-freezer at −80 °C. For the production of spores the strain was grown on PDA medium, at 25-30 ºC for 7–8 days.

2.3. Fermentation conditions

The fermentation experiments were carried out in 500 ml Erlenmeyer flasks containing 1 g of carrier and 100 ml of culture medium with the following composition (% w/v): sucrose 16.5, yeast extract 2.75, $NaNO_3$ 0.2, K_2HPO_4 0.5, $MgSO_4 \times 7H_2O$ 0.05, and KCl 0.05. Sterilization of the medium was carried out at 121 ºC for 20 min.

Flasks were inoculated with 1.0 ml of a spore suspension containing around 1.8×10^7 spores/ml, which was prepared by scrap down the spores from the PDA plates with a sterilized solution of 0.1% w/v Tween 80, and counted in a Neubauer chamber. The inoculated flasks were incubated in a rotary shaker at 28 °C and 160 rpm for 48 h. Cells were immobilized in situ in the flasks by natural adsorption.

Samples for analysis were collected at regular intervals and filtered, using 0.2 μm filters to separate the cell pellets from the culture fluid. In the filtered broth, FOS (1-kestose, 1-nystose, and 1-β-fructofuranosyl nystose), residual concentration of other sugars (sucrose, fructose, and glucose), pH and extracellular enzyme activity were measured. The concentrations of free and immobilized cells were determined at the fermentation end.

All the fermentation experiments were conducted in duplicate and the average values are reported. The relative standard deviation was less than 5%.

2.4. Analytical methods

Cell mass concentration. Free cell mass concentration was determined by dry weight per volume (g/l). At the end of the fermentation, the biomass was collected by vacuum filtration of the fermentation broth through pre-weighed 0.45 μm membranes, washed with distilled water and dried at 105 °C to constant weight.

The amount of biomass attached to the carriers was determined after washing the support material with distilled water for three times, and drying at 105 °C to constant weight. The biomass dry weight was determined from the difference between the cells plus carrier and the carrier itself.

Enzymatic activity: Samples of the fermentation media were filtered (through 0.2 μm membranes) to remove the cell mass and the filtrate was utilized as extracellular enzyme

source. The β-fructofuranosidase (FFase) activity was determined by measuring the amount of glucose produced from sucrose (Yoshikawa et al., 2006). The reaction mixture contained 100 ml of the crude FFase extract, 300 mmol of sucrose and 50 mmol of sodium acetate buffer (pH 5.0) in a total volume of 1 ml. After incubation for 20 min at 30 ºC, the reaction was stopped by heating for 5 min at 100 ºC. After cooling, the amount of glucose released into the supernatant was measured by high-performance liquid chromatography (HPLC, see below). One unit (U) of the FFase activity was defined as the amount of enzyme required to release 1 µmol of glucose per min, from sucrose under the above conditions.

Transfructosylating and hydrolyzing activities. The reaction was carried out for 180 min using 0.5 U/ml of FFase in the reaction mixture described above (Yoshikawa et al., 2006). Transfructosylating activity (Ut) and hydrolyzing activity (Uh) were determined by measuring the concentrations of 1-kestose and fructose by HPLC, respectively. One unit of transfructosylating activity was defined as the amount of enzyme required to transfer 1 µmol of fructose per min. One unit of hydrolyzing activity was defined as the amount of enzyme required to release 1 µmol of free fructose per min.

Sugars and FOS concentrations. FOS (1-kestose, 1-nystose, and 1-β-fructofuranosyl nystose) and other residual sugars (sucrose, glucose, and fructose), were directly analyzed by high performance liquid chromatography (HPLC) on an equipment LC-10 A (Jasco, Japan) with a Prevail Carbohydrate ES column (5 um, 250 x 4.6 mm, Alltech) at room temperature, and a refractive index detector. The response of the refractive index detector was recorded and integrated using the Star Chromatography Workstation software (Varian). A mixture of acetonitrile and 0.04% ammonium hydroxide in water (70/30 v/v) was used as mobile phase at a flow rate of 1.0 ml/min. Before injection, the samples were filtered through 0.2 µm filters and diluted with Milli-Q water when needed. The sugars and FOS concentrations were determined from standard curves made with known concentrations of each compound. The total yield of FOS (Y_{FOS}) was calculated as the sum of 1-kestose (Y_{GF2}), 1-nystose (Y_{GF3}), and 1-β-fructofuranosyl nystose (Y_{GF4}), to initial sucrose concentration.

3 Results and discussion

3.1. Immobilized cells concentration

Concentration of cells immobilized in the different carriers is given in Table 1. It can be noted that all the evaluated materials were able to immobilize cells, but with different capacities. Among them, pumice stones, cork and zeolites gave the lower immobilized cells concentration. A major quantity of cells was adhered into polyurethane foam, but the highest cell mass was adhered into stainless steel and vegetal fiber sponges, with the result of vegetal fiber sponge being 10% higher than that of stainless steel sponge.

Table 1. Free and immobilized cells concentration during FOS production by *A japonicus* immobilized in different materials.[a]

Immobilization carrier	Immobilized cells (g/g carrier)	Free cells (g/l)
Pumice stones	0.13	20.83
Zeolites	0.19	19.36
Stainless steel sponge	1.13	2.59
Polyurethane foam	0.48	6.44
Vegetal fiber sponge	1.25	5.09
Cork	0.14	9.97

[a] Values after 48 h of fermentation

All the experiments presented free cells in the medium besides those immobilized. Pumice stones, cork and zeolites, which gave the lowest immobilized cells concentration, presented the major amounts of free cells; while stainless steel sponge and vegetal fiber sponge, which immobilized the major proportion of cells, resulted in the lowest free cells concentration.

3.2. Sucrose consumption and FOS production with immobilized cells

Fermentation with the different carriers had similar behaviors of sucrose consumption and FOS production, with an initial lag phase of approximately 12 h where few substrate was consumed (Figure 1A). As a consequence, practically was not observed FOS production during this period (Figure 1B). Nevertheless, in the subsequent 12 h the process attained the maximum FOS production for practically all the carriers evaluated, as result of the almost depletion of sucrose from the media. 1-Kestose was the main FOS formed in the media at this time, followed by 1-nystose and 1-β-fructofuranosyl nystose that required a larger fermentation time to be formed. On an average, maximum 1-kestose concentration was observed in 24 h of fermentation; 1-nystose concentration was maximum around 36 h of fermentation, while the concentration of 1-β-fructofuranosyl nystose required approximately 48 h to attain the highest value. Similar behavior was observed by Cruz et al. (1998) during the FOS production by *Aspergillus japonicus* immobilized in calcium alginate. According to these authors, the fructooligosaccharide synthesis was always sequential in the sense $GF \rightarrow GF_2 \rightarrow GF_3 \rightarrow GF_4$ as a consequence of the increasing *Km* values for such products presented by the transfructosylase. Thus, high concentrations of the preceding oligosaccharide are always necessary for the synthesis of its homologue with one more fructose unit. This would also explain why the content of 1-kestose is higher at the beginning of the enzymatic reaction.

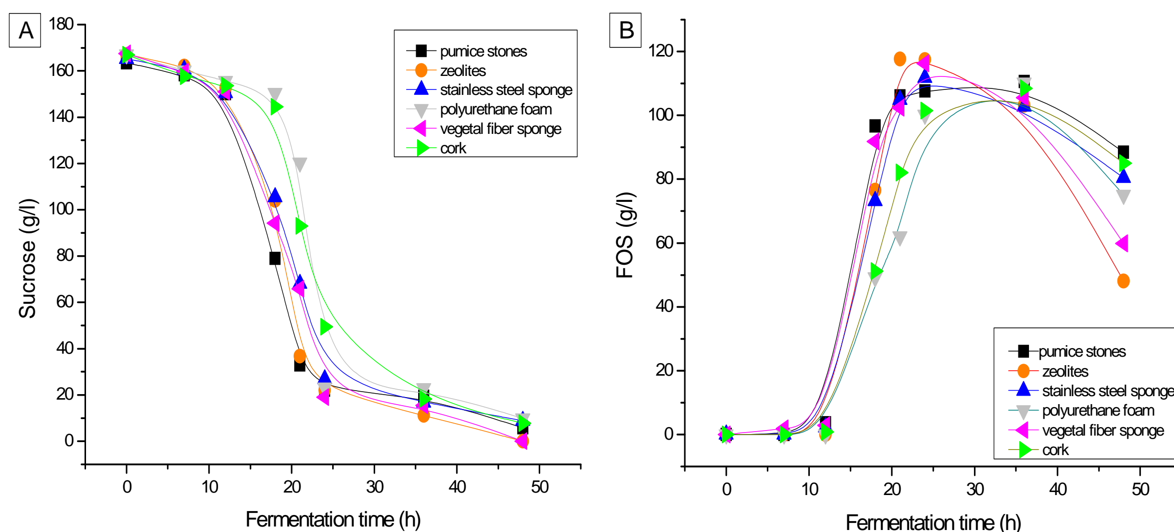

Figure 1. Sucrose consumption (A) and FOS production (B) using *A. japonicus* immobilized in different carriers.

After the initial 24 h fermentation, due to the sucrose exhaustion from the media, the microorganism passed to consume 1-kestose, and therefore, even being observed formation of the other two FOS, the total FOS concentration started to decrease in all media, as can be noted in Figure 1B. Consequently, when the reaction time was extended to over 48 h, changes in the total FOS content occurred, with an increase in GF_3 and GF_4 contents, and a corresponding loss of 1-kestose.

Although the kinetic behaviors of sucrose consumption and FOS production have been similar for all the assays, the fermentative parameters were different to each one of them,

due to the differences in residual sucrose content and total FOS produced, probably as a consequence of the different values of free and immobilized cells. As can be observed in Table 2, the medium containing zeolites gave the best results, but cells in this medium were mostly free (only few amounts were immobilized in the carrier – see Table 1). The media containing vegetal fiber or stainless steel sponges as carriers gave fermentative parameters values for FOS production close similar to those achieved with zeolites, but with an important advantage that in these cases the carrier was able to immobilize the large amounts of cells (Table 1). This means that using vegetal fiber or stainless steel sponges as carrier, it was possible to immobilize large amounts of cells and produce FOS with elevated yields. The medium containing cork as carrier resulted in the lowest values of FOS concentration, $Y_{P/S}$, and Q_P.

Table 2. Fermentative parameters of FOS production by immobilized *A. japonicus*.

Immobilization carrier	Maximum FOS (g/l)	Fermentation time (h)	$Y_{P/S}$ per total substrate (g/g)	$Y_{P/S}$ per consumed substrate (g/g)	Q_P (g/l.h)
Pumice stones	110.55	36	0.68	0.76	3.07
Zeolites	117.45	24	0.70	0.81	4.89
Stainless steel sponge	111.80	24	0.68	0.81	4.66
Polyurethane foam	110.26	36	0.66	0.76	3.06
Vegetal fiber sponge	116.26	24	0.69	0.78	4.84
Cork	108.47	36	0.65	0.73	3.01

3.3. Enzyme activity

Results for the FFase activity by *A. japonicus* immobilized in the different carriers are shown in Figure 2. The best value of enzyme production (42.92 U/ml) was found after 36 h with cells immobilized in vegetal fiber sponge. These values can be favorably compared to those obtained during FOS production by other fungus species. For example, under optimized cultivation conditions, *A. japonicus* JN-19 yielded the highest FFase activity of 55.42 U/ml at 96 h fermentation (Wang and Zhou 2006). In the present study, the maximum FFase activity was achieved after only 36 h fermentation, giving thus a much higher productivity.

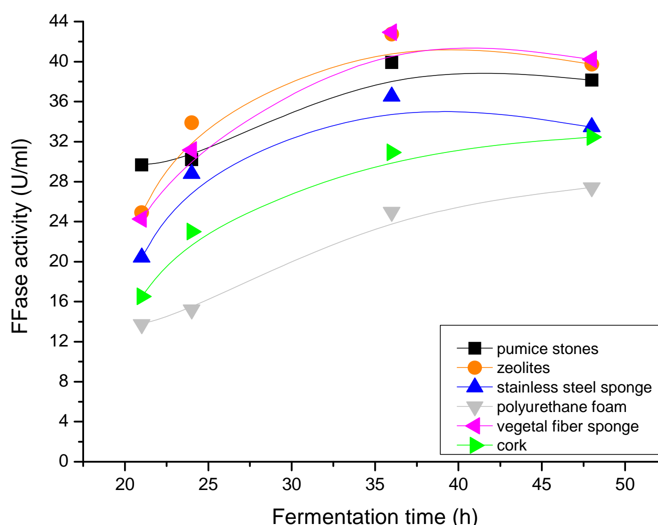

Figure 2. FFase activity by *A. japonicus* cells immobilized in different carriers.

FFases commonly possess both hydrolytic (Uh) and transfructosylating (Ut) activities. Nevertheless, for an efficient FOS production it is preferable to have a high Ut/Uh ratio (Chen & Liu, 1996). Table 4 shows the Ut/Uh, ratio of FFase obtained for *A. japonicus* immobilized in the different carriers. Maximum values were achieved at 48 h fermentation using vegetal fiber sponge as immobilization carrier.

Table 3. Ratio between the transfructosylating and hydrolyzing activities of FFase during FOS production by immobilized *A. japonicus*.

Immobilization carrier	Ut/Uh ratio at 48 h fermentation
Pumice Stones	4.31
Zeolites	3.12
Stainless steel sponge	2.93
Polyurethane foam	4.15
Vegetal fiber sponge	4.82
Cork	2.34

Conclusions

Among the six different materials evaluated for immobilization of the fungus *Aspergillus japonicus* during FOS production, vegetal fiber sponge proportioned the best fermentation results, being able to immobilize the major amount of cells, and giving the highest values of FOS production and FFase activity. Such material was thus selected for use in subsequent experiments aiming to maximize the FOS production by *Aspergillus japonicus* ATCC 20236.

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