FRUCTOOLIGOSACHARIDES PRODUCTION USING INULINASE OF *Aspergillus niger* AFTER TREATMENT WITH FLUID PRESSURIZED IN TWO DIFFERENT MEANS

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Abstract. The use of fluids pressurized in biochemical process had an increase in the last years, among their advantages as reaction medium stands out: reaction selectivity, high conversions, easy of separation of products and reagents and biocompounds extraction. Inulinases are 2,1-β-D frutano furohydrolase (EC 3.2.1.7.), what do inulin convert in fructose. Few studies approach the effects in the enzymatic activity of the inulinase after having submitted the high pressure. In this context, the present work seeks to investigate the influence of the pressure, time of exhibition and depressurization rate on the enzymatic activity of commercial inulinase of *Aspergillus niger*, in the immobilized form, in pressurized LPG, with the intention of increasing the catalytic power of the enzyme for production of FOS in organic/aqueous and aqueous media. The experiments were accomplished in the temperature of 40 °C, varying the pressure (30-270 bar), times of exhibition (1-6 h) and decompression rate (2-100 bar/min). The condition optimized for the increase of the enzymatic activity of the inulinase of *Aspergillus niger* treated with LPG was 30 bar, exposure time of 1 hour and decompression rate of 20 bar/min, getting an increase of 129% in relationship to the initial activities enzymatic, showing that treatment with pressurized fluids can increase the power of synthesis of the enzyme. For the synthesis of fructooligosacharides the obtained values of conversion with *Aspergillus niger* were 26,62% of kestose (GF2); 30,62% of nystose (GF3) and 8,47% of fructosylnystose (GF4) in medium organic/aqueous with sucrose, and the aqueous was them 75,97% of kestose (GF2) and 14,97% of fructosilnystose (GF4) in medium containing inulin.

Keywords: inulinase; compressed fluid, LPG, sucrose, inulin.

1. Introduction

Inulinases are enzymes potentially useful in the production of high fructose syrups (HFS) by enzymatic hydrolysis of inulin, affording a yield as high as 95% [1]. These enzymes are widely used for the production of fructooligosaccharides, compounds with functional and nutritional properties for use in low-calorie diets, stimulation of *Bifidus* and as a source of dietary fiber in food preparations [2,3].

The inulinases can be obtained from yeasts, fungus, bacteria and of plants [4]. The most studied are the enzymes produced by *Kluyveromyces marxianus* and *Aspergillus niger*, which possess good activity on the substrates sucrose and inulin [5].

The inulinases can be used in processes of hydrolysis of the inulin for obtaining of fructose syrup and of fructoligosacharides, and also in the oligosaccharides synthesis starting from the sucrose [2,3].

The oligosaccharides are carbohydrates with degree of polymerization from 2 to 10 which are presented as major components in many natural products, but also can be produced chemistry or enzymatically. Nowadays, enzymatically are produced starting from simple sugars, for transglycosylation, or for degradation of polysaccharides of vegetable origin, animal or microbial [6].

Among the several known oligosacchardes with prebiotic effect, the more investigated are the fructoligosaccharides, galactoligosachardes, oligosaccharides of the soy and some glucoligosaccharides [7].

To conduct enzyme-catalyzed reactions at high pressures, the enzyme behavior in compressed fluids is of primary importance, as the loss of enzyme activity may lead to undesirable poor reaction rates and low yields of target products. In fact, enzyme stability and activity may depend on the enzyme species, the characteristics of the compressed fluid, the water content of the enzyme/support and the process variables involved, which means that very distinct effects can be achieved depending on the characteristics of the system under investigation [8-10].

No study was found in the current literatura concerning the use of inulinase immobilized in activated carbon and sodium alginate for the production of FOS using aqueous and aqueous/organic media.

Based on these aspects, the main focus of this work was to investigate the production of FOS using immobilized inulinase of *Aspergillus niger*. For the experiments we have used as catalyst the free inulinase, immobilized with treatment in pressurized fluid and immobilized without treatment.

2. Material and Methods

2.1 Chemical and enzyme

The commercial inulinase was obtained from the *Aspergillus niger* (Fructozyme, exo-inulinase (EC 3.2.1.80) and endo-inulinase (EC 3.2.1.7)) acquired of Sigma-Aldrich.

LPG (liquefied petroleum gas) was kindly donated by Petrobras and is constituted by a mixture of propane (50.3 wt%), *n*-butane (28.4 wt%), isobutane (13.7 wt%), ethane (4.8 wt%) and other minor constituents (methane, pentane, isopentane, etc.).

2.2 Inulinase immobilization

Inulinase were immobilized according to the methodology described by Risso et al. [2]. Initially, a gel solution was prepared containing 16.5 g of distilled water and 0.75 g of sodium alginate, and maintained under mild heating. After complete dissolution of the alginate, 12.5 g of sucrose were added, followed by 5 mL of the solution containing the recovered inulinase, 3.5 mL of glutaraldehyde and 0.75 g of activated carbon.

For sphere formation, the gel solution was pumped into a 0.2 M calcium chloride solution in sodium acetate buffer (0.1 M and pH=4.8) containing 3.5 wt% of glutaraldehyde, and stirred slowly at 10 °C. The immobilized inulinase was maintained at 4 °C for 24 h and then washed with sodium acetate buffer (0.1 M and pH=4.8). To maintain the structure, the immobilized spheres (around 0.005 m in diameter) were immersed in a 0.2 M calcium chloride solution in sodium acetate buffer (0.1 M and pH=4.8).

2.3 High-pressure treatment of enzyme

The experimental procedure adopted for enzymes treatment in pressurized fluid consisted, firstly, in adjusting the thermostatic bath to 50 °C, the temperature established in the present work for all experimental runs. Then, the enzymatic preparations (0.6 g) of enzyme in immobilized form were loaded into the cell. After this procedure, the system was submitted to pressurization keeping a constant pressurization rate (10 bar/min). The system was, finally, depressurized at different pre-established rates, according to the experimental design, by a programmed syringe pump piston displacement and the micrometric valve used at lower pressures, near the solvent saturation pressure. The enzymatic activity was determined before (initial activity) and after (final

activity) the treatment procedure with pressurized fluids. The best results of activity residual form used for synthesis of FOS [11,12].

2.4 Fructooligosaccharides synthesis

Synthesis was carried out in stirred reactors (150 rpm) with total volume of 10 mL. The operational conditions were aqueous medium: sodium acetate buffer 0.1 M (100% w/v), enzyme (5% w/v) and substrate (60% w/v to sucrose and 20% w/v to inulin) and aqueous/organic medium: sodium acetate buffer 0.1 M (70% w/v), ethyl acetate (25% w/v), enzyme (5% w/v) and substrate (60% w/v for sucrose and 20% w/v for inulin). The reaction time for the FOS synthesis was 24 h at 50 °C, and the kinds of FOS usually produced were: GF2 (kestose); GF3 (nystose), and GF4 (fructosyl nystose) [13].

2.5 Chromatographic analysis

The quantification was accomplished in system of liquid chromatography of high efficiency (HPLC) Agilient 1100 series, detector of refraction index, column model Luna NH2 (250 x 4,6 mm, 5 µm - Phenomenex - it USES), volume of injection of 5 µL, phase mobile acetonitrile/water (70:30), column and detector temperatures of 20 and 25 °C, respectively [13].

3. Results and Discussion

For the synthesis of FOS using the enzyme of *A. niger* in aqueous/organic (Table 1), the results obtained were found when the enzyme was submitted to the treatment with LPG and sucrose as substrate (GF2-26.62%; GF3-30.62%; GF4-8.47%). However, promising values were also obtained using the free enzyme and inulin as substrate (GF2- 77.19%; GF3-14.03%; GF4-0.07%).

A study of L'Hocine et al. [14] suggests that in the crude extract of *A. niger* an invertase (b-D-fructofuranosidase) exhibits only hydrolytic activity producing exclusively fructose and glucose from sucrose, while a separate enzyme fructosyltransferase catalyzes the fructosyltransferase reaction producing glucose and FOS. The work of Sirisansaneeyakul et al. [15], using a concentration of 60% (w/v) of sucrose, *Aspergillus niger* ATCC20611 and a glucose oxidase as catalyst, showed a FOS production of 0.44%; 0.47% and 0.91% for GF2, GF3 and GF4, respectively.

A research from Sangeetha et al. [16] pointed out that the use of liquid culture, cells and culture broth homogenate from *Aspergillus oryzae* CFR 202 and *A. pullulans* CFR 77 and different concentrations of sucrose as substrate, led to a maximum yield of FOS using *A. oryzae* CFR 202 liquid culture of 50– 54% after a reaction time of 12 h, at 55 °C, at sucrose concentrations of 55% (w/v) and 80% (w/v). The culture broth of *A. oryzae* CFR 202 resulted in 50–53% of FOS after 12 h of reaction when 55% (w/v) sucrose was used as substrate. Although the cells were not suitable for FOS production, the liquid culture and the culture broth homogenate of *A. pullulans* CFR 77 provided a reaction yield of up to 57% from sucrose at concentrations of 55% and 80% (w/v).

The application of enzymes to the catalytic processes in aqueous-organic systems has received a great deal of attention as a viable alternative to the chemical approach and due to its remarkable characteristics such as easier preparation, mild reaction conditions, and the easy separation of reaction products. Besides, upon placing an enzyme in an organic medium, the biocatalyst is subjected to a number of factors that can alter its native structure and function [17].

The production processes of FOS in aqueous systems are extensively studied and reviewed by several authors [16,18,19]. Santos and Maugeri [20] investigated the FOS synthesis in the aqueous system by inulinase from sucrose using stirred and packed reactors, in batch and continuous mode processes, with free and immobilized enzymes and results showed slight difference among the investigated processes.

For the synthesis of FOS using the enzyme of *Aspergillus niger* in aqueous medium (Table 1), the best results were obtained with the enzyme treated in LPG and using inulin as substrate (GF2 – 75.97; GF4 – 14.97%).

In the kestose synthesis starting from immobilized β-frutofuranosidase from *Aureobasidium* sp. ATCC 20524, the maximum production obtained by the authors Hayashi et al. [21] were 287 g of kestose starting from 40% (w/v) of sucrose solution in a time of 168 hours of reaction. The production of fructoligosaccharides obtained by Cruz et al. [22], starting from immobilized cells of *Aspergillus japonicus*, was 61.28% using a solution of sucrose of 65% (w/v) for 4 hours of reaction.

Lateef et al. [23] produced FOS with an obtained intracellular fructosyl transferase of *Aspergillus pullulans* CRF77, the reaction was accomplished in ultrasound system (20 W), in medium with sucrose, buffer citrate 0.1 M, pH 5 and temperatures varying from 15 to 55 °C, obtaining the maximum income of FOS (59%) in 9 minutes of reaction.

Table 1. Yield of FOS (%) using the enzyme of *Aspergillus niger* with inulin and sucrose as substrates.

Enzyme preparation	Substrates					
	Inulin			Sucrose		
	GF2	GF3	GF4	GF2	GF3	GF4
Aqueous Medium						
Immobilized enzyme with treatment in:						
LPG	75.97	-----	14.97	6.05	-----	-----
Aqueous Medium						
Immobilized enzyme with treatment in:						
LPG	16.41	21.17	----	26.62	30.62	8.47

In a study accomplished by Dominguez et al. [24] using experimental planning to optimize the synthesis of FOS for inulinase of *Aureobasidium pullulans* in medium containing sucrose, the authors obtained the maximum of income (64.1$g_{FOS}/g_{sucrose}$ (1-kestose 43.6$g_{kestose}/g_{sucrose}$ and 20.6$g_{nystose}/g_{sacarose}$) in the conditions of 390 rpm, 35 °C in 72 hours of reaction.

Figure 1 shows that the sucrose molecule was broken into fructose and glucose by the inulinase from *A. niger*. The mass balance confirms that only 48% of the sucrose was converted into fructose and glucose and only 0.71% of those products were used for the synthesis of FOS (GF2, GF3 and GF4).

Figure 2 presents the products formed in the break of the inulin for synthesis of FOS for *Aspergillus niger*. It is clear from these figures that the inulinase does not get to break the inulin molecule totally, in spite of the good reached conversions.

From Figures 1 and 2 it is also possible to visualize the formation of other unidentified compounds, during the synthesis of FOS. For sake of clarity, Figure 3 presents the chromatogram of the standards of (a) fructose; (b) glucose; (c) sucrose; (d) kestose; (e) nystose and (f) fructosyl nystose.

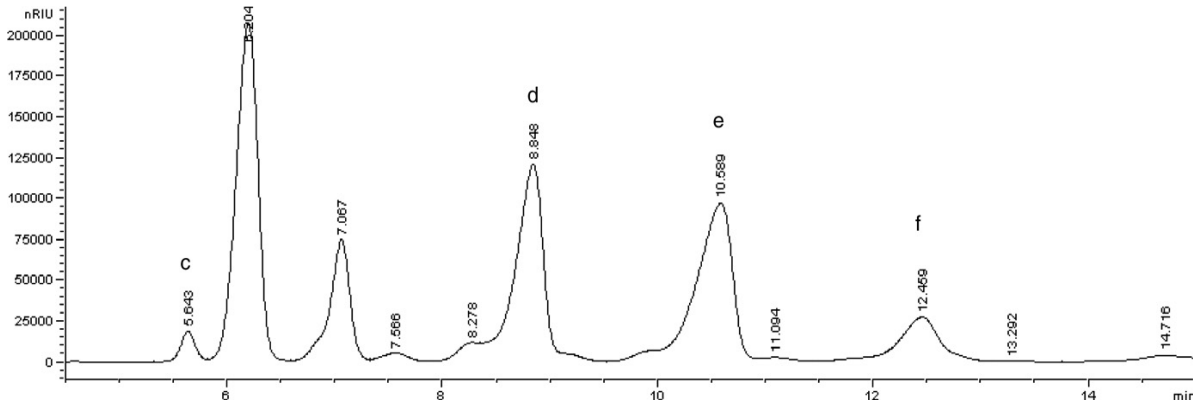

Figure 1. Compounds formed in the synthesis of FOS using *Aspergillus niger* treated in LPG and sucrose as substrate in aqueous/organic medium, (a) fructose; (b) glucose; (c) sucrose; (d) kestose; (e) nystose and (f) fructosyl nystose.

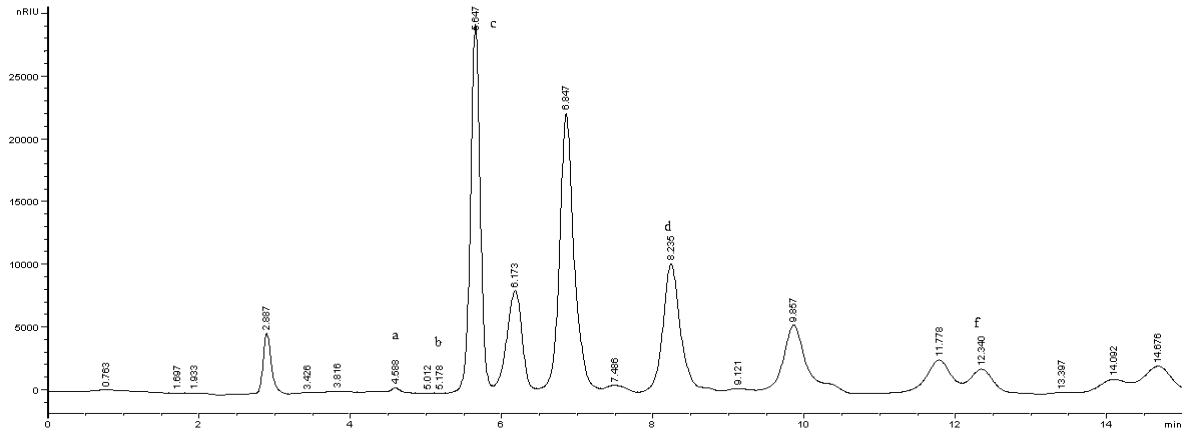

Figure 2. Compounds formed in the synthesis of FOS using *Aspergillus niger* treated in LPG and inulin as substrate in aqueous medium, (a) fructose; (b) glucose; (c) sucrose; (d) kestose; (e) nystose and (f) fructosyl nystose.

Yoshikawa et al. [25] produced FOS from sucrose using crude enzyme preparations of b-fructofuranosidases from *A. pullulans* DSM 2404 and obtained a maximum yield of 62%. When inulin was used as substrate, the maximum inulooligosaccharide yield reported was about 72% (w/w), using free endoinulinase from *Pseudomonas* sp. [26].

Mutanda et al. [27] investigated the batchwise production of inulooligosaccharides from pure chicory inulin using a commercial endoinulinase preparation (Novozyme 960) that was isolated from *A. niger* and obtained a maximum yield of 54% after 72 h of reaction with 5% of inulin, at 45 °C and enzyme activity of 5 U/g of substrate.

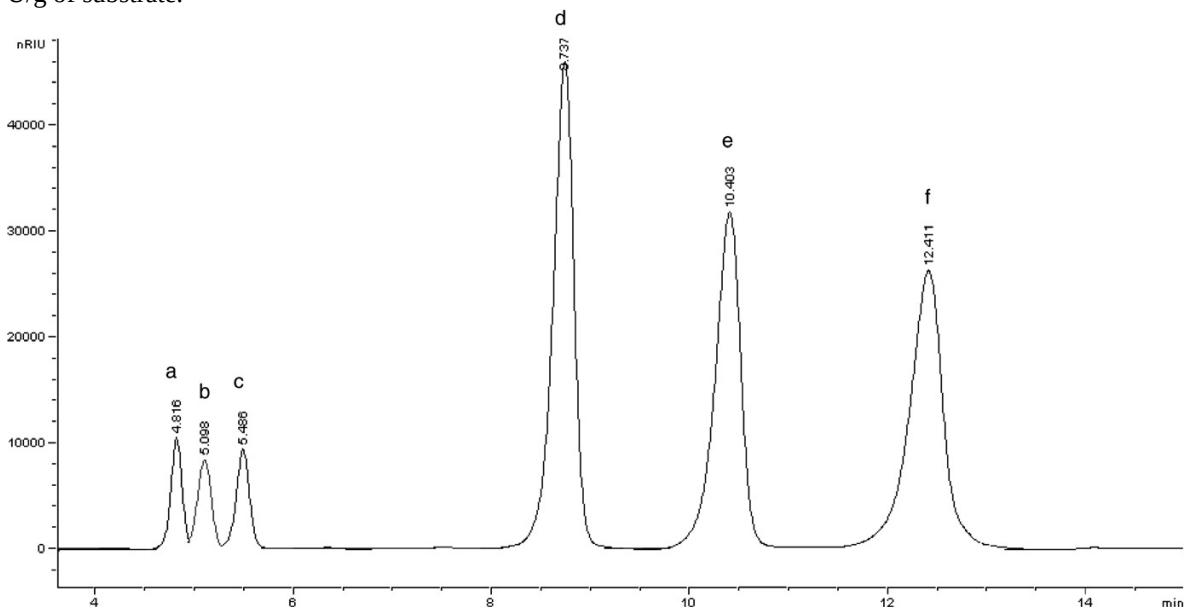

Figure 3. Chromatogram of the standards of (a) fructose; (b) glucose; (c) sucrose; (d) kestose; (e) nystose and (f) fructosyl nystose.

4. Conclusions

Production of microbial FOS using sucrose and inulin as substrates constitute an innovative and profitable alternative to the industry, while satisfying the growing needs of the population for healthy foods with bioactive compounds. Considering the important implications of the results obtained in the present work, further studies are currently underway in our laboratory to improve FOS production from *A. niger* inulinase.

Acknowledgements

The authors thank CNPq, CAPES and FAPERGS for the financial support of this work and scholarships.

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