SYNTHESIS OF FRUCTOOLIGOSACCHARIDES FROM *Aspergillus niger* COMMERCIAL INULINASE IMMOBILIZED IN POLYURETHANE FOAM AND PRE-TREATED IN PRESSURIZED LGP

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Abstract: Commercial inulinase from *Aspergillus niger* were immobilized in polyurethane foam and then treated in pressurized in liquefied petroleum gas (LPG). Firstly, the effects of system pressure, exposure time and depressurization rate, using liquefied petroleum gas (LPG), on the enzymatic activity were evaluated through central composite designs (CCD) 2^3. Residual activities of 145.1% were observed for LPG (30 bar, 6 hours and depressurization rate of 20 bar/min). The catalysts treated at these conditions were then used for the production of fructooligosaccharides using sucrose as substrates, in aqueous systems. The main objective of this step was to evaluate the yield and productivity in fructooligosaccharides, using alternatives for enhance the enzyme activity by means of pressurized fluids and also using low cost supports for enzyme immobilization, aiming at obtaining a stable biocatalyst to be used for synthesis reactions. Yields of 32% were achieved, with 5.42 % of kestose (GF2); 22.27% of nystose (GF3) and 3.52% of fructosylnystose (GF4), which shows the potential of this procedure.

Keywords: inulinase; LPG; fructooligosaccharides.

1. Introduction

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

Short-chain fructooligosaccharides (sc-FOS) are a mixture of 1- kestose (GF2), nystose (GF3) and 1F-fructofuranosylnystose (GF4), which have been regarded as prebiotics since the mid-1990s. They have important physiological functions due to their indigestibility in the upper gastrointestinal tract, which stimulate the selective growth of bifidobacteria in the large intestine [3, 4]. Sc-FOS have received GRAS status (generally recognized as safe), which has promoted their use as ingredients for both food and feed in East Asia, North America and Europe [5]. Currently, the main enzymes utilized by industrial FOS producers are invertases or b-fructofuranosidases (EC 3.2.1.26) from *Aspergillus*, which generally provide a mixture of 1F-FOS [6, 7].

The enzyme immobilizations reveals some advantages for industrial applications due to re-utilization, the increase in stability and the use of bioreactors make the separation of the products easier compared to soluble enzyme leading to economical bioprocess [8].

Polyurethane (PU) foam is a type of well known support due to its ability of confining or immobilizing biological materials [9]. The main advantages of this support are their elevated mechanical resistance and attack by organic solvents and microorganisms [10, 11] and their characteristics of biochemically inert [12, 13, 14]. PU is able to make foam with open cells, as a result of condensation of polycyanate (R-CNO) and polyol (R-OH). After polymerization, the carbon dioxide, to escape the matrix, leaves pores behind. Normally, porous matrix of PU increases not only the area but also minimize the diffusion limitation of the substrate and the product. A limited diffusion environment is a common disadvantage of polymers used for encapsulation, as acrylamide, alginate and carrageenan [12, 15]. To overcome such difficulties of diffusion, a variety of hydrophilic polyurethanes have been identified and tested for enzymes and cell immobilization [16, 17, 18]. Hypol 2000 and 3000 are also used as immobilization matrices for microbial cells for the biodegradation of toxic chemical products [18]. Fukushima [16] immobilized invertase using pre-polymers of urethane, as Hypol 2000. Cellulase immobilized in foam from Hypol 2002 was applied for cellulose degradation [19].

Several studies can be found in the recent literature about the use of pressurized solvents as a pre-treatment step for enzymes enhancement of activity and stability by employing clean and low-cost technologies [20, 21, 22, 23, 24, 25, 26]. In this sense, it is also worth noting that no reports were found in the open literature concerning the use of inulinase immobilized in polyurethane foam pre-treated in compressed liquefied petroleum gas.

Based on these aspects, the main interest of this work was to investigate the synthesis of fructooligosaccharides using a commercial inulinase from *Aspergillus niger* immobilized in polyurethane foam and pre-treated in pressurized LGP, using sucrose as substrate in aqueous medium.

2. Materials and methods

2.1 Chemicals and enzymes

LPG 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.).

The free commercial inulinase, obtained from *Aspergillus niger* (Fructozyme, mixture of exo-inulinase (EC 3.2.1.80) and endo-inulinase (EC 3.2.1.7)), was purchased from Sigma-Aldrich. Polyurethane foam was used as the low-cost support for the inulinase immobilization.

2.2 Inulinase immobilization

The polymerization synthesis of polyurethane was carried out following the methodology proposed by Silva [27], keeping a relationship polyol polyether to toluene diisocyanate of 1:1 (v/v). The polymerization reaction was carried out in a flask of 300 mL. The monomers (5 mL) were transferred to the glass with the help of a graduate plastic syringe disposable. After the addition of the monomers, the system was homogenized with a glass stick, during approximately 2 minutes, leaving the reaction to happen. Later the polymerization stage (5 minutes) the polyurethane was left in rest overnight for complete solidification of the foam. During the polymerization reaction, system temperature was monitored and recorded.

The immobilization step was performed using a volume of the extract glycolic containing the enzyme corresponding to 10% to the added volume of the monomers. For the immobilization, the glycolic enzymatic extract was added to the monomer polyol polyether and, later to the homogenization step isocyanate was added so as to start the polymerization reaction of polyurethane.

2.3 High-pressure treatment of enzymes

The commercial inulinases from *Aspergillus niger* immobilized in polyurethane foam were treated with pressurized LPG, using the experimental condition optimized by Kuhn [22], 150 bar, exposure time of 3.5 hours and depressurization rate of 60 bar/min, reaching a residual activity of about 400%.

2.4 Synthesis of fructooligossaccharides

The methodology used for the synthesis of FOS was previously described by Risso [28], with some modifications. The FOS synthesis was carried out in stirred reactors (100 rpm) with total volume of 40 mL. The operational conditions were sodium acetate buffer 0.1 M (100% w/v), immobilized pre-treated

inulinase (0.25% w/v) and substrate (15% w/v for sucrose). The reaction time for the FOS synthesis was 24 h at 50 °C.

2.5 Chromatographic analysis

The quantification of FOS 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), temperature of the column 20 °C and temperature of the detector 25 °C, following a methodology proposed and validated by our research group.

3. Results and Discussion

The inulinase immobilized in polyurethane, by the methodology previously described, maintained about 49% of its initial activity during 1008 hours and 24 reuse cycles for sucrose. The immobilized biocatalyst was pre-treated in pressurized LPG and, at the experimental conditions cited before, residual activities of about 400% were observed. The immobilized and pre-treated commercial inulinase was used as catalyst for synthesis of fructooligosaccharides using sucrose as substrate in aqueous system. A full discussion about the promising results described can be found in the works of Kuhn [29] and Silva [27].

Table 1 presents the results obtained for the production of fructooligosaccharides using sucrose as substrate. Figure 1(a) shows the chromatogram equivalent to the results of Table 1 and Figure 1(b) presents a typical chromatogram of the chemical standards (kestose (GF2), nystose (GF3) and fructosilnystose (GF4)) with the respective retention times.

Table 1. Production of FOS from commercial inulinase from *Aspergillus niger* immobilized in PU foam and pre-treated in pressurized LPG, using sucrose as substrate.

	FOS content (%)			
Commercial immobilized inulinase	1-Kestose	Nystose	1F-fructofuranosyl nystose	Total
Treated in LPG	5.42	22.27	3.52	31.21
Without treatment	5.36	17.49	3.70	26.55

From Table 1, we can observe that the total concentration of FOS was 31.21%, as a result of depletion of sucrose and formation of long chain FOS (GF3 and GF4) from kestose (GF2). Concentrations of FOS of 5.42, 22.27 and 3.52% were obtained for GF2, GF3 and GF4, respectively.

The direct comparison of the results from Table 1 (about 31% FOS) with those obtained by Risso et al. (2004) [30] (17% FOS), which used a non-commercial inulinase from *Kluyveromyces marxianus* NRRL Y-757, permit us to verify that very promising results were obtained.

Ignacio, Luis and Domingo [31] suggest that, when a support has little pore size comparing to the enzyme dimensions, the adsorption occurs only in the surface, as the case of clays, diatomaceous earth and other similar materials. However, when the support presents higher pores in comparison to the dimensions of the protein, it is possible to find accession into the pores. This situation occurs in materials as activated coal and polyurethane foam.

This fact, related to the different way of trapping the inulinase into the support, can be responsible for the different yields obtained. When montmorillonite was used as support for inulinases immobilization, conversions of until 18% were achieved [29]. Using polyurethane foam as support, very promising conversions were observed, showing the strong correlation between the support of immobilization and the substrates.

The production of oligosaccharides using mainly fructofuranosidases and fructosyltransferase enzymes has been studied by a number of researchers in the last decades. These studies investigated all kinds of organisms, from lactic acid and ethanol producing bacteria to moulds, yeasts and vegetables.

Chen and Liu [32] studied the b-fructofuranosidase from *Aspergillus japonicus*, an enzyme showing both hydrolytic and transfructosilating activities, optimum activity being achieved at pH 5,0-6,0 and temperatures between 55 and 65 ºC for fructose transfer, and at pH 4,0 and 65 ºC for the hydrolytic activity.

Whole cells of *Bifidobacterium bifidum* are also used for the synthesis of galactooligosaccharides from lactose, reaching a conversion rate of 35%. The optimum activity was achieved at 40 ºC and pH 6.8-7.0 [33]. *Rhodotorula* yeasts were found to produce extracellular invertase, b-glucosidases and levanases, their activity on cellobiose and lactose resulting in gluco- and galactooligosaccharides with yields from

36 to 38%. The optimum pH and temperature for the enzymes of *Rhodotorula minuta* were pH 4.0-6.0 and a temperature of 70 ºC [34, 35].

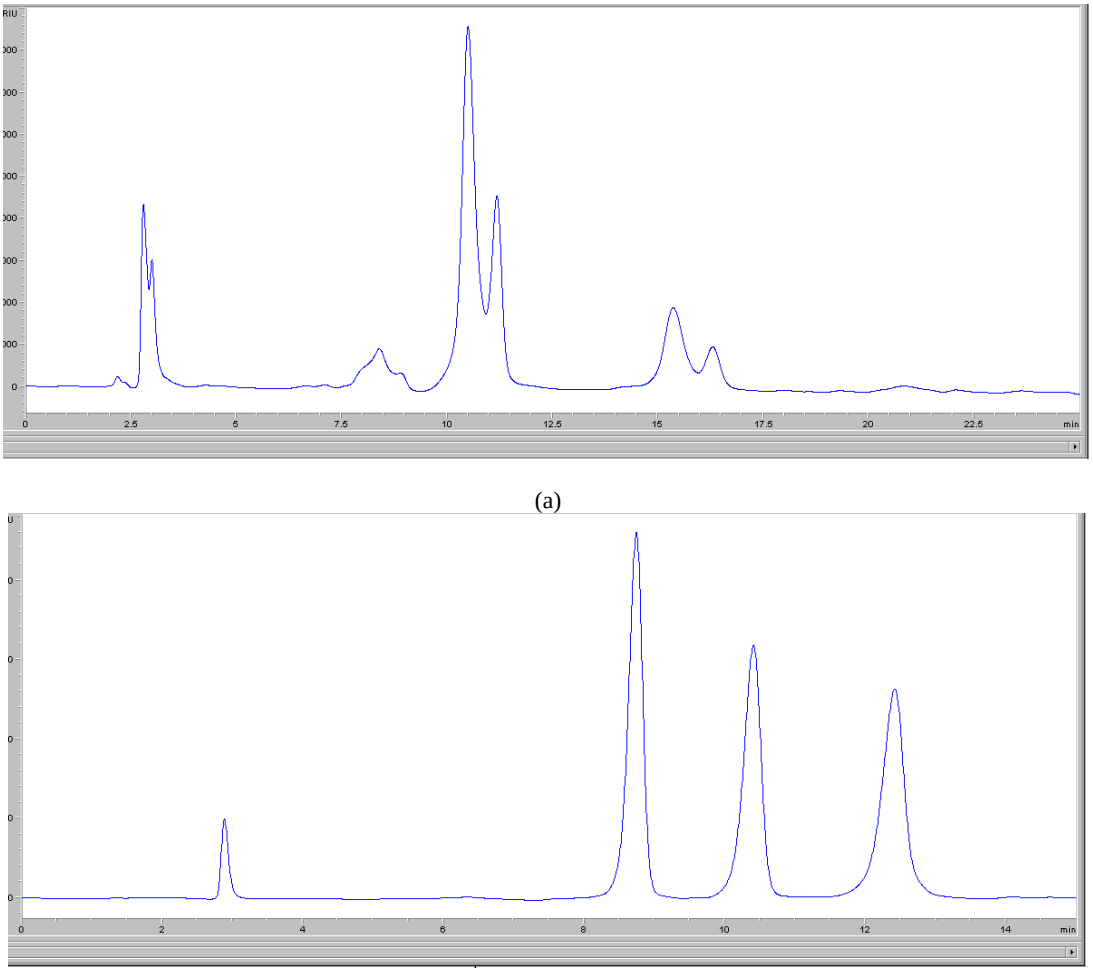

Figure 1. HPLC quantitative analysis for the synthesis of FOS in aqueous medium, using sucrose as substrate and *Aspergillus niger* immobilized inulinase after treatment in pressurized LPG (a) and chemical standards (kestose (GF2), nystose (GF3) and fructosilnystose (GF4) (b).

Silva [36] reached FOS yields of until 65 % using commercial inulinases from *Aspergillus niger* immobilized in sodium alginate and activated coal.

Concerning the aqueous system for the FOS synthesis from sucrose using inulinase as catalyst, Santos and Maugeri [37] found FOS yielding (approximately 10% w/w) in stirred reactor with free inulinase from *K. marxianus* ATCC 16045. Yoshikawa [38] produced FOS from sucrose using crude enzyme preparations of β-fructofuranosidases from *Aureobasidium ullulans* DSM 2404 and they obtained a maximum yielding of 62%. When inulin was used as substrate, the maximum inulooligosaccharide yielding reported was about 72% (w/w) with free endoinulinase from *Pseudomonas* sp. [39]. Mutanda [40] investigated the batchwise production of inulooligosaccharides from pure chicory inulin with a commercial endoinulinase preparation (Novozyme® 960) that has been isolated from *A. niger* and obtained a maximum yielding of 54% after 72 h of reaction with 5% inulin, at 45 °C, and 5 U g^{-1} substrate.

The results obtained here, in terms of product yield, compared to the literature, could be considered promising. Based on these results and on the potentialities of the proposed methodology, further studies on the optimization of process variables for the fructooligosaccharides production using inulinases immobilized in polyurethane foam are undergo by our research group.

4. Conclusions

This work describes a methodology for the fructooligosaccharides production using commercial inulinases immobilized in a very low cost support, polyurethane foam, and pre-treated, in relatively low

pressure, using pressurized LPG. The proposed methodology showed as a potential technology for synthesis of fructooligosaccharides using low cost purposes, since yields of about 31% were obtained. Further studies are currently underway in our laboratory to improve FOS production from commercial inulinases from *Aspergillus niger* immobilized in polyurethane foam.

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