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Effect of compressed fluids treatment on the activity of inulinase from *Kluyveromyces marxianus* NRRL Y-7571 immobilized in montmorillonite

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ABSTRACT

This work is aimed at assessing the influence of pressurized fluids treatment on the enzymatic activity of inulinases from *Kluyveromyces marxianus* NRRL Y-7571 immobilized in natural montmorillonite. The effects of system pressure, exposure time and depressurization rate, using propane and liquefied petroleum gas (LPG) on the enzymatic activity were evaluated through central composite designs (CCD) 2^3. In general, results showed that the enzyme activity changes significantly depending on the experimental conditions investigated, allowing the selection of proper operating conditions for advantageous application of this biocatalyst in hydrolysis reactions. Further, the stability of the enzyme after high-pressure pre-treatment was also experimentally monitored, and results demonstrated that the activity of the biocatalyst was always higher than the non-treated one. Results obtained here might be useful as a basis for the selection of appropriate process conditions, so that the catalyst can be applied in biotransformation reactions.

1. Introduction

Inulinases are 2,1-β-D-fructan-furohydrolases (EC 3.2.1.7), which hydrolyzes inulin, yielding fructose moieties [1]. The hydrolysis (acid-catalyzed or enzymatic) produces fructose oligomers that are named fructo-sugars, fructooligosaccharides (FOS), or simply oligofructoses [2]. According to catalytic mechanisms, inulinases can be included in the transferase group (EC 2.4.1), when it yields difructose anhydrides, or in the group of hydrolases (EC 3.2.1), when it hydrolyses polyfructans based on a process of endo or exodegradation [3].

The microbial inulinases are classified according to their way of action. Exo-inulinases (2,1-β-D-frutano-fructohydrolase; EC 3.2.1.80) are specific for inulin hydrolysis and break the bonds between the fructose moieties located far from the ends of the polymer chain, to produce oligosaccharides. The endo-inulinases (β-D-frutano-frutanohydrolase; EC 3.2.1.7) act randomly on the inulin molecule, yielding inulo-triose, inulo-tetraose and inulo-pentaose as main products [4–7].

To date, the high cost of enzyme production has been probably one of the major obstacles to commercialization of enzyme-catalyzed processes. For this reason, recent advances in enzyme technology, such as the use of solvent-tolerant and/or immobilized inulinases, which make possible the re-utilization of the catalyst, have been made to develop cost-effective systems [8,9].

In recent years, many studies regarding the utilization of alternative solvents for biocatalysis have been presented in the literature [10,11]. Considerable efforts have been reported in the literature towards green chemistry reactions, with emphasis on enzymatic reactions carried out in ionic liquids [12–15] and in sub- and supercritical fluids [16–18]. The use of compressed fluids as solvents (normally gaseous solvents) for chemical reactions may be a promising route to completely eliminate solvent traces from reaction products. In addition, manufacturing processes in near-critical fluids can be advantageous in terms of energy consumption, easier product recovery, adjustable solvation ability, and reduction of side reactions.

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 [19,20]. In fact, enzyme stability and activity may depend on the enzyme, 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 [21–24].

Based on these aspects, the main focus of this study was to investigate the enzymatic activity of inulinase in compressed propane and LPG using a home-made enzyme from *Kluyveromyces marxianus* NRRL Y-7571 immobilized in montmorillonite. After treatment in compressed fluids, the immobilized inulinases were

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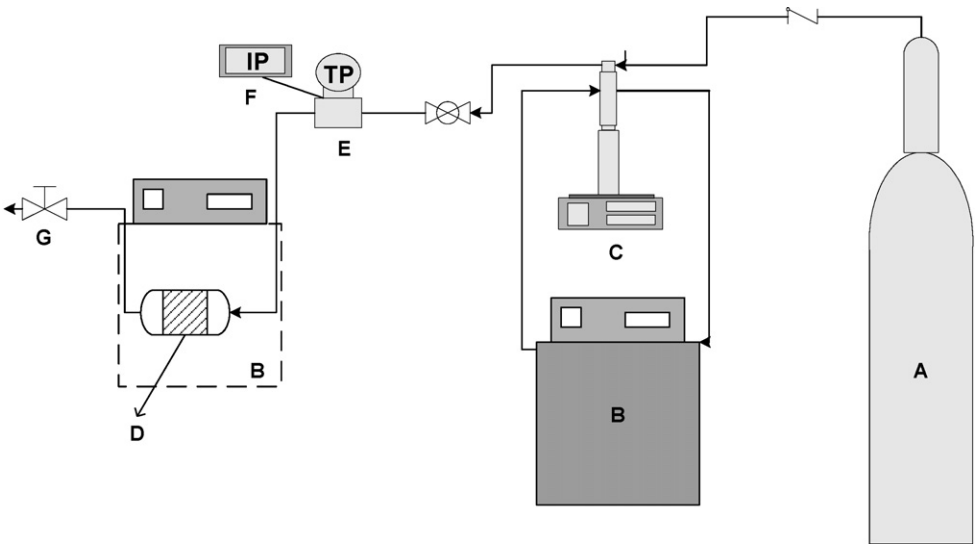

Fig. 1. Schematic diagram of the apparatus for treatment of solid enzyme with compressed solvents. (A) Solvent reservoir; (B) thermostatic bath; (C) syringe pump; (D) treatment vessel; (E) pressure transducer; (F) pressure indicator; (G) micrometric valve.

evaluated in terms of stability at low temperatures. To our knowledge, no experimental data about the behavior of inulinases after treatment in compressed fluids were found in the current literature. The present report is part of a broader project and reflects our efforts to help developing new enzyme-catalyzed processes in alternative fluid media [19,20,25–29].

2. Material and methods

2.1. Chemicals and enzyme

The pure gas propane (minimum purity of 99.5%) was purchased from White Martins S.A. 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 low-cost support used for the immobilization of enzyme was a natural montmorillonite kindly donated by Colorminas SA.

Inulinases were produced from *K. marxianus* NRRL Y-7571. The extract containing extracellular inulinase was obtained by solid-state fermentation using sugarcane bagasse as substrate. The medium composition was optimized in a previous work by our research group as following: 2 kg of sugarcane bagasse supplemented with pre-treated cane molasses 15 wt%, corn steep liquor (CSL) 30 wt%, and soybean bran 20 wt%. The moisture content was set to 65 wt% and autoclaved at 121 °C for 20 min. The fermentation runs were started with the inoculation of an optimized volume corresponding to a cell mass of 14 g. All experiments were carried out for 24 h [30]. After fermentation, the enzyme was extracted from the sugarcane bagasse by adding sodium acetate buffer 0.1 mol L^{-1} pH 4.8 in a solid/liquid ratio of 1:10, following incubation at 50 °C and 150 rpm for 30 min [31].

2.2. Inulinase immobilization

Enzyme immobilization was performed using 2 g of the support natural montmorillonite in 60 mL of acetate buffer 0.1 M and pH 4.8 at enzyme to support mass ratio of 3:10 and submitted to preferential immobilization by physical adsorption on support. Immobilization was carried out with magnetic stirring in an ice cooler during 10 min and after this time, the solution was filtered under vacuum and was kept in desiccators for about 48 h.

2.3. Catalyst structural characterization

Catalyst samples were analyzed with respect to structure through nitrogen adsorption at 77 K (Autosorb-1 equipment, Quatachome, 2200e series). Before analysis, samples were treated under vacuum at 373 K for complete drying and then submitted to liquid N_2. Average specific superficial area was determined by the BET method while the average porous diameter was obtained using the BJH (Barret, Joynere, Halenda) technique.

2.4. High-pressure treatment of enzymes

The experiments involving the immobilized inulinases were performed in a laboratory-scale unit similar to that employed by Kuhn et al. [27], which consists basically of a solvent (propane, or liquefied petroleum gas – LPG) reservoir, two thermostatic baths, a syringe pump (ISCO 260D), a stainless steel vessel (cell) with an internal volume of 3 mL, an absolute pressure transducer (Smar, LD301) equipped with a portable programmer (Smar, HT201) with a precision of ±0.37 bar, as schematically represented in Fig. 1. All lines of the experimental setup consisted of 1/16″ OD tubing of stainless steel (HIP) and between the pump and solvent reservoir a check (one way) valve (HIP 15-41AF1-T 316SS) was positioned to avoid pressurization solvent back flow to the head of solvent cylinder. Two additional micrometering valves (HIP 15-11AF2 316SS) completed the experimental apparatus, one located after the syringe pump, at the entrance of high-pressure cell, to allow solvent loading and the other just after the cell to perform solvent discharge. The high-pressure cell was submerged into the water bath and was supported by a simple device while the micrometering valves were located outside the bath.

The experimental procedure adopted for enzymes treatment in pressurized fluid consisted, firstly, in adjusting the thermostatic bath to 40 °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 under different exposure times, according to pre-established conditions following an experimental design, 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, as previously described.

2.5. Experimental conditions

Aiming at evaluating the effects of process variables on the activities of immobilized inulinase after treatment with pressurized fluid, a central composite design 2^3 was adopted. The experimental planning was conceived to cover, at the same time, the variable ranges commonly used for enzyme-catalyzed reactions in compressed fluids, the optimum range of activity of each enzyme and the equipment operating limits [22–24]. The evaluated variables for immobilized inulinases were pressure (30–270 bar), depressurization rate (10–200 bar/min) and exposure time (1–6 h). Each run of the experimental design was carried out randomly, including a central point condition performed in triplicate, for experimental error evaluation. The analysis was performed using the software Statistica® 6.1 (Statsoft Inc., Tulsa, OK, USA).

2.6. Inulinase activity assay

An aliquot of 0.5 g of the enzyme source, softened, was incubated with 4.5 mL of 2 wt%/v% sucrose solution in sodium acetate buffer (0.1 mol L^{-1} pH 5.5) at 50 °C. Reducing sugars released were measured by the 3,5-dinitrosalicylic acid method [32]. A separate blank was set up for each sample to correct the non-enzymatic release of sugars. One unit of inulinase activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of sucrose per minute under the mentioned conditions (sucrose as a substrate). Results were expressed in terms of inulinase activity per gram of dry solids (U g ds^{-1}). The residual activity was defined as the ratio between the activities after and before treatment with pressurized fluid. Following the same procedure described above, the inulinase activity using inulin as