Compressed *N*-Butane for treatment of commercial and non-commercial inulinases

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RESUMO – Inulinase from *Klyveromyces marxianus* NRRL Y-7571 presented an increase of 210 % in the residual activity using *n*-butane at 30 bar during 6 h exposure and with a depressurization rate of 20 bar/min. For commercial inulinase from *Aspergillus niger*, an increment of 142 % in the residual activity at 270 bar for 6 h treatment at the highest depressurization rate (100 bar/min) was observed. The use of compressed *n*-butane may be of technological relevance as a preceding, preparation step, towards improving enzyme activity, hence helping the development of new biotransformation processes.

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 %. 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 (Ettalibi *et al.*, 2001)

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 (Riss et al., 2012).

In recent years, many studies regarding the utilization of alternative solvents for biocatalysis have been presented in the literature. Considerable efforts have been reported in the literature towards green chemistry reactions, with emphasis on enzymatic reactions carried out in ionic liquids[7-10] and in sub- and supercritical fluids. 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 (Knez, 2009).

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. (Andrade *et al*., 2008).

Based on these aspects, the main focus of this study was to investigate the enzymatic activity of inulinases in compressed *n*-butane using a commercial immobilized inulinase from *Aspergillus niger* and a home-made immobilized inulinase from *Kluyveromyces marxianus* NRRL Y-7571. To our knowledge, no experimental data about the behavior of inulinases after treatment in compressed fluids are 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 (Silva et al., 2012)

2. EXPERIMENTAL

2.1. Chemical and Enzymes

The commercial inulinase was obtained from *Aspergillus niger* (Fructozyme, exo-inulinase (EC 3.2.1.80) and endo-inulinase (EC 3.2.1.7)) acquired of Sigma-Aldrich. Non-commercial inulinases were produced from *Kluyveromyces marxianus* NRRL Y-7571, flowing the methodologies described by Mazutti *et al*. (2010). The pure *n*-butane, propane (minimum purity of 99.5%) were purchased from White Martins S.A. 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 (wt% 4.8 wt%) and other minor constituents (methane, pentane, isopentane, etc.).

2.2. Inulinase immobilization

Firstly, a gel solution was prepared by adding 16.5 g of distilled water to 0.75 g of a food-grade sodium alginate (Vetec, Rio de Janeiro, Brazil), followed by heating at 55 °C for alginate dissolution and addition of 12.5 g of sucrose. After gel cooling, 5 mL of enzymatic solution, 3.5 mL

of glutaraldehyde and 0.75 g of activated carbon were added to the gel solution (Risso *et al*. 2012). A peristaltic pump was used for gel pumping and dropping into a solution of 0.2 mol/L of sodium chloride in 0.1 mL/L of sodium acetate buffer 0.1 M with 3.5% of glutaraldehyde. This procedure was performed at 10 °C under agitation.

2.3. High-pressure treatment of enzymes

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 immobilized enzyme 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 experimental condition that led the best results of activity residual were used to pre-treat to enzymes before the FOSs synthesis (Silva *et al*. 2012, 2013).

2.4 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 (Silva *et al.*, 2013) The evaluated variables for immobilized inulinases were pressure (30-275 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).

2.5 Measurement of the enzyme stability after compressed fluid treatment

The enzymes were incubated for 100 days at temperature 4 °C. The determination of enzyme activities were accomplished every 10 days, using inulin and sucrose as substrates.

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. 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 ($U.gds^{-1}$). The residual activity was defined as the ratio between the activities after and before treatment with pressurized fluid.

2.7 Scanning Electron Microscopy (SEM)

Textural characterization of supported enzymes was accomplished by AUTOSORB-1 (Quantachrome), while scanning electronic microscopy analysis was performed in a SEM SSZ 550 Shimadzu.

3.RESULTS AND DISCUSSION

Results obtained for the non-commercial inulinase from *Kluyveromyces marxianus* NRRL Y-7571 treated with pressurized *n*-butane are presented in Table 1, where it can be noticed that the compressed fluid treatment leads to a clear increase in enzyme activities. The highest increase (residual activity of 209.9 %) was observed for run 3, using 30 bar during 6 h and depressurization rate of 20 bar/min.

The increase in the hydrolysis potential of sucrose by inulinase, evaluated by the enzyme activity, after treatment with pressurized *n*-butane was verified, though the mechanism of such increment is still not clear. Kamat *et al.*,(2000) suggest that enzymes exposed to supercritical fluid conditions can suffer a change in its molecular and conformational structure and the interaction of the enzyme with the solvent could cause an increase in the activity originally presented by the enzyme.

Table 1. Relative residual activity* (%) of non-commercial immobilized inulinase of *Kluyveromyces marxianus* NRRL Y -7571 after treatment in pressurized *n*-butane at 40 ^{o}C. The initial activity of the immobilized enzyme without treatment was 66 U/g.

Run	P (bar)	t (hours)	R (bar/min)	Residual activity (%)
1	-1 (30)	-1 (1)	-1 (20)	140.6
2	- 1 (30)	-1 (1)	1 (100)	138.8
3	-1 (30)	1 (6)	-1 (20)	209.9
4	-1 (30)	1 (6)	1 (100)	151.4
5	1 (270)	-1 (1)	-1 (20)	116.6
6	1 (270)	-1 (1)	1 (100)	143.5
7	1 (270)	1 (6)	-1 (20)	111.5
8	1 (270)	1 (6)	1 (100)	129.0
9	0 (150)	0 (3.5)	0 (60)	148.4
10	0 (150)	0 (3.5)	0 (60)	152.1
11	0 (150)	0 (3.5)	0 (60)	153.9

Regarding the effect of hydrostatic pressure on enzyme stability, the literature points out that pressure values around those used in this work can cause a small effect on enzyme activity (Treichel *et al.*, 2011).

After statistical analysis of the experimental data presented in Table 1 it is possible to observe (Figure 1a) that among independent variables evaluated, after treatment using *n*-butane, system pressure and the interaction between pressure and time presented a negative effect and the interaction between pressure and depressurization rate presented a significant positive effect ($p<0.05$).

Table 2 presents the residual enzymatic activity values obtained after treatment of the commercial inulinase by *Aspergillus niger*. After the statistical treatment of the data presented in Table 2, it is verified that among the studied variables and their interactions (Figure 1b) the depressurization rate and the interaction between pressure and depressurization rate presented a positive significant effect ($p <0.05$).

Table 2. Relative residual activity* (%) of the commercial immobilized inulinase from *Aspergillus niger* after treatment in pressurized *n*-butane at 40 ^{o}C. The initial activity of the immobilized enzyme without treatment was 100 U/g.

Run	P (bar)	t (hours)	R (bar/min)	Residual activity (%)
1	-1 (30)	-1 (1)	-1 (20)	96.9
2	- 1 (30)	-1 (1)	1 (100)	113.6
3	-1 (30)	1 (6)	-1 (20)	116.2
4	-1 (30)	1 (6)	1 (100)	109.1
5	1 (270)	-1 (1)	-1 (20)	66.0
6	1 (270)	-1 (1)	1 (100)	125.2
7	1 (270)	1 (6)	-1 (20)	90.9
8	1 (270)	1 (6)	1 (100)	141.6
9	0 (150)	0 (3.5)	0 (60)	123.7
10	0 (150)	0 (3.5)	0 (60)	123.4
11	0 (150)	0 (3.5)	0 (60)	123.1

After depressurization, the water content of the treated enzyme was measured immediately by Karl Fischer titration and the activity was measured after storage in a freezer. Figure 1a shows the stability of the inulinases from *Kluyveromyces marxianus* NRRL Y-7571 and *Aspergillus niger* submitted to the treatment in *n*-butane using sucrose and inulin as substrates. When analyzing the experimental data obtained in this work, it can be noted from Figure 1b that the enzyme from *Aspergillus niger* kept its relative residual activity at 50 % when inulin is used as substrate and 60 % for sucrose.

In Figure 1a it can be observed that the best values of relative residual activity for inulinase from *Kluyveromyces marxianus* NRRL Y-7571 was obtained until the 20 day, showing a sharp decline from the 30 day of storage using inulin as substrate. However, when observing

the behavior of the inulinases (Figure 1b) in sucrose, it is verified that both possess the same behavior, reaching a residual activity of 55 % after 100 days of storage.

Figure 1. Pareto chart of effects of pressure, exposure time and depressurization rate on residual activities of (a) non-commercial inulinase from *Kluyveromyces marxianus* NRRL Y 7571 and (b) inulinase from *Aspergillus niger* both in *n*-butane.

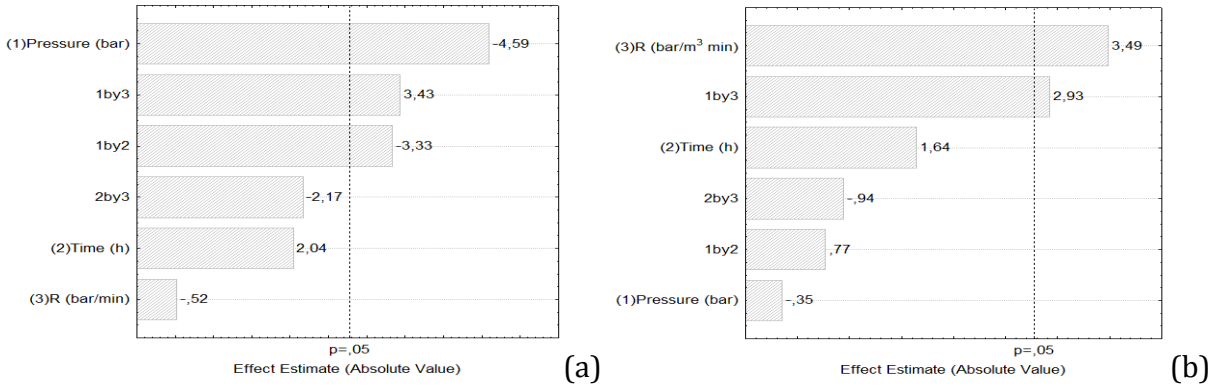
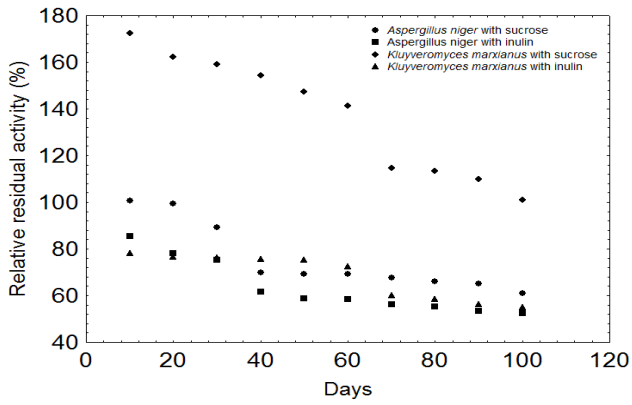


Figure 2. Stability of inulinase after submitted to the pressurized *n*-butane in inulin and sucrose.



For the use of biocatalysts in dense gases the knowledge about their stability in such media is very important. Since different parameters, such as temperature, pressure, water activity, nature of the solvent, etc. are influencing the stability and activity of enzymes at supercritical conditions, no prediction whether an enzyme is stable under these conditions can be made (Celia *et al.*, 2005).

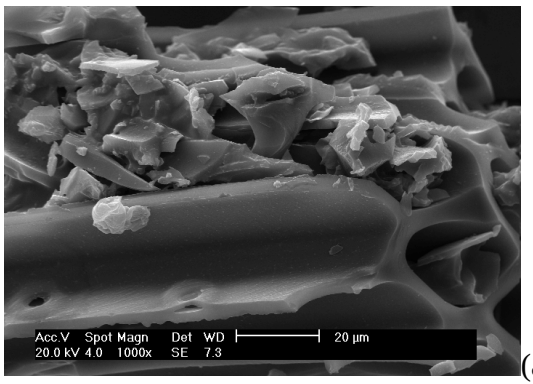
It is well known that pressure has a disruptive effect on the tertiary and quaternary structure of most globular proteins (Knez, 2009). Non-covalent bonds of large protein molecules are altered, whereas amino acids and vitamins are unaffected by high pressure (Knez, 2009) Treatments at high pressures, higher than 200 MPa produce, at pH 8, important changes of

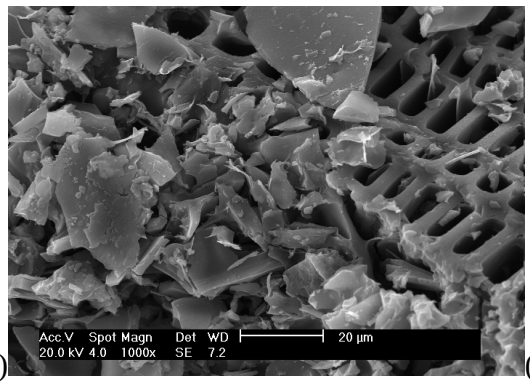
secondary structure in proteins: evolution to a more disordered structure with protein aggregation, especially in the 11S fraction (Knez, 2009)

Figure 3 (a and b) and 4 (a and b) show the structure of the inulinases from *Aspergillus niger* and *Kluyveromyces marxianus* NRRL Y-7571, respectively, before and after treatment with pressurized *n*-butane.

Figure 3a shows that the structure of inulinase from *Aspergillus niger*, without treatment, has a deformed structure, what cannot protect the properties of the enzyme. On the other hand, the structure of the enzyme after treatment (Figure 3b) shows a more orderly structure propitiating a possible protection to the inulinase from *Aspergillus niger*, increasing its residual activity.

Figure 3. SEM of the structure of the obtained enzyme of *Aspergillus niger*, immobilized in sodium alginate and activated coal, before (a) and after (b) treatment in pressurized *n*-butane.

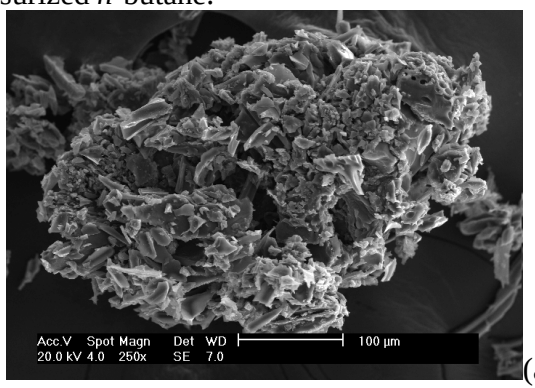


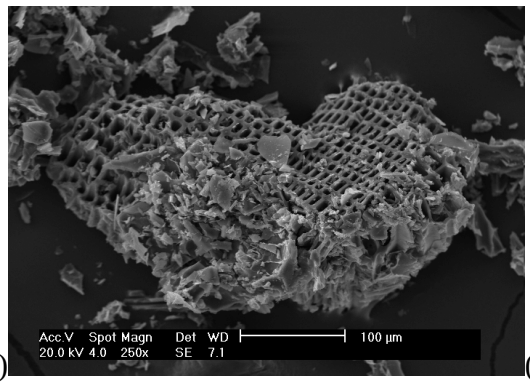
(a)


(b)

Figure 4a shows the disordered structure of the non-commercial immobilized inulinase from *Kluyveromyces marxianus* NRRL Y-7571. The treatment with pressurized *n*-butane (Figure 4b) seems to give a more ordered structure, promoting a favorable environment to the enzyme and accordingly to its catalytic power.

Figure 4. SEM of the structure of the obtained enzyme of *Kluyveromyces marxianus* NRRL Y-7571, immobilized in sodium alginate and activated coal, before (a) and after (b) treatment in pressurized *n*-butane.



(a)


(b)

4. CONCLUSIONS

Based on the results obtained in this work, in a general sense, one may infer that the enzyme activity after treatment with pressurized *n*-butane depends significantly on the structural nature of the enzyme and the experimental conditions investigated, i.e., exposure time, depressurization rate and system pressure. It was experimentally observed for immobilized inulinase gains of enzyme activities, under several experimental conditions, thus allowing the selection of optimal operating conditions for the advantageous application of these treated biocatalysts in many important reactions of food interest. Thus, the use of compressed fluids, such as *n*-butane may be of technological relevance as a preceding, preparation step, to improve enzyme activity, hence helping the development of new biotransformation processes.

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