EVALUATION OF THE INULINASE OF *Aspergillus niger* IMMOBILIZED AFTER TREATMENT IN PRESSURIZED FLUID

Marceli Fernandes Silva[1], Simone Maria Golunski[1], Diane Rigo[1], Vinícius Mossi[1], Graciele Kuhn[1], Marco Di Luccio[2], J. Vladimir de Oliveira[2], Marcus Vinícius Tres[1,*], Débora de Oliveira[2], Helen Treichel[3]

[1] Departamento de Engenharia de Alimentos
Universidade Regional Integrada do Alto Uruguai e das Missões
Avenida Sete de Setembro, 1621, 99700-000, Erechim, RS, Brasil

[2] Departamento de Engenharia Química e Engenharia de Alimentos
Universidade Federal de Santa Catarina
Caixa Postal 476, 88040-900, Florianópolis, SC, Brasil

[3] Universidade Federal da Fronteira Sul- Campus de Erechim

Email: marcusvtres@gmail.com

Abstract. The use of pressurized fluids at biochemical process had an increase in the last years. The advantages of the use of pressurized fluids at reaction medium are: reaction selectivity, high conversions, easiness separation of the products and the reagents and biocompounds extraction. The inulinases 2,1 -β - D frutano furohidrolase (EC 3.2.1.7.) makes inulin convert in fructose. Few studies approach the effects in the enzymatic activity of the inulinase after having submitted at high pressure. In this context, the present work shows an investigation about the influence of the pressure, time of exhibition and depressurization rate in the enzymatic activity of commercial inulinase of *Aspergillus niger* in the immobilized form, in pressurized LPG, propane and n-butane. The best evaluated conditions were 270bar, 6h, 100bar/min; 270bar, 6h, 100bar/min and 30bar, 1h, 20bar/min for n-propane, butane and LPG, respectively. After the treatment, the enzymes were maintained at -4°C by 100 days in a medium containing sucrose and inulin with substrate. When it stored in a medium containing sucrose the enzyme treated in LPG gets to maintain 100% of its initial activity. For the treatments with propane and n-butane the enzymes maintain 97 and 70% of their initial activities, respectively, after 70 days. In the medium containing inulin, the enzymes treated with pressurized fluids maintain 52% of its initial activity after 100 days.

Keywords : Inulinase, pressurized fluids, sucrose, inulin, *Aspergillus niger.*

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].

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 [2,3].

In recent years, many studies regarding the utilization of alternative solvents for biocatalysis have been presented in the literature [4,5]. Considerable efforts have been reported in the literature towards green chemistry reactions, with emphasis on enzymatic reactions carried out in ionic liquids [6-9] and in sub- and supercritical fluids [10,11]. 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.

Supercritical carbon dioxide has special characteristics, such as low toxicity, working temperature compatible with the optimal temperature for enzymes and favorable transport properties that can accelerate mass-transfer-limited enzymatic reactions [10]. Nevertheless, carbon dioxide is not the only gas whose properties seem to be adequate for biocatalysis as the comparable dielectric constant of propane and n-butane to carbon dioxide support a firm belief that such pure gases and their mixtures may also be suitable as reaction media for enzyme-catalyzed bioconversions [12].

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 [13-15].

Based on these aspects, the main focus of this study was to investigate the enzymatic activity of inulinase in compressed propane, n-butane and LPG (using a commercial immobilized inulinase from *Aspergillus niger*. 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 [16].

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.

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

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 experiments involving the immobilized inulinase were performed in a laboratory-scale unit similar to that employed by Kuhn et al. [16], which consists basically of a solvent (propane, n-butane and 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 Figure 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 (0.7 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 of enzymes was determined before (initial activity) and after (final activity) the treatment procedure with pressurized fluids, as previously described.
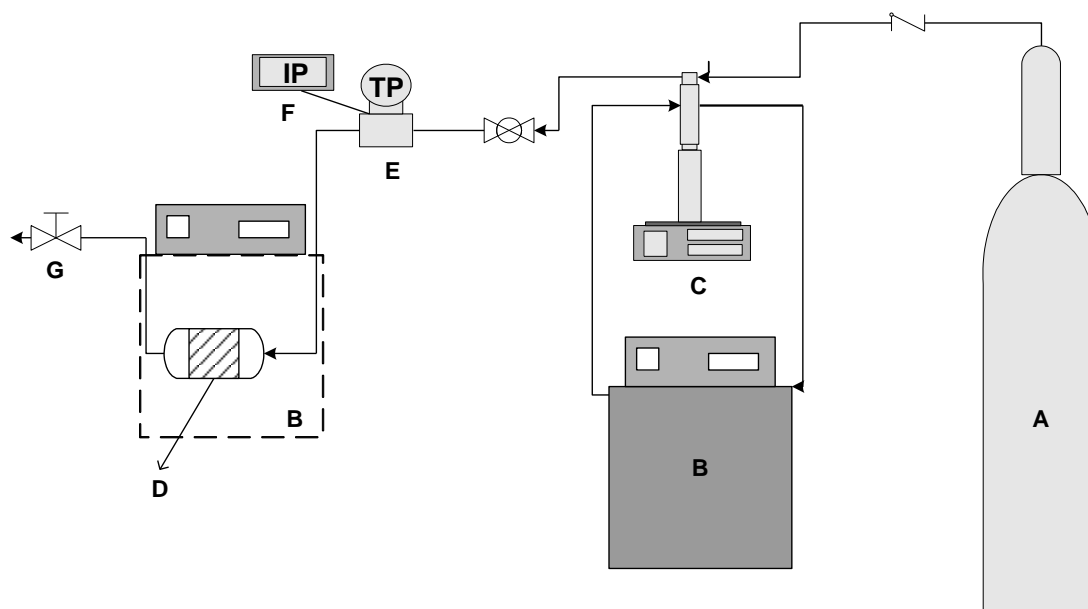

Figure 1. Schematic diagram of the apparatus for treatment of solid enzyme with compressed solvents. A-solvent reservoir; B-thermostatic bath; C-syringe pump; D-treatment vessel; E-pressure transducer; F-pressure indicator; G-micrometric valve.

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 [14,15]. The evaluated variables for immobilized inulinase were pressure (30-270 bar), depressurization rate (20-100 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 stability after fluid treatment

After determining the conditions that led to the highest increase in enzyme activity, 40 g of enzyme were treated and incubated to – 4°C for further determination of the storage stability. Samples were stored for 100 days, and the inulinase activity measured every 10 days using both sucrose and inulin as substrates. All experiments were carried out at least in duplicate. The mean experimental error was always below 5%.

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% of substrate (sucrose or inulin) 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 [17]. 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.

3. Results and Discussion

Table 1 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 1, it is verified that among the studied variables and their interactions, only the depressurization rate showed a negative significant effect ($p < 0.05$) in the treatment with propane (Figure 2a). In the treatment with *n*-butane (Figure 2b) the depressurization rate and the interaction between pressure and depressurization rate presented a positive significant effect ($p < 0.05$). In Figure 2c, treatment with LPG, the depressurization rate presented a negative significant effect, however its interaction with pressure showed a positive significant effect ($p < 0.05$). The presented results confirm that the depressurization rate is one of the factors that can promote alterations in the enzymatic cellular structure and consequently loss or gain of enzyme activity.

Table 1. Relative residual activity[*] (%) of the commercial immobilized inulinase from *Aspergillus niger* after treatment in pressurized fluids. Initial activity=100U/mL.

Run	P (bar)	t (hours)	R (bar/min)	propane (%)	*n*-butane (%)	LPG*** (%)
1	-1 (30)	-1 (1)	-1 (20)	153.6	96.9	129.4
2	- 1 (30)	-1 (1)	1 (100)	113.7	113.6	91.3
3	-1 (30)	1 (6)	-1 (20)	152.6	116.2	119.5
4	-1 (30)	1 (6)	1 (100)	123.3	109.1	97.4
5	1 (270)	-1 (1)	-1 (20)	136.1	66.0	105.2
6	1 (270)	-1 (1)	1 (100)	130.2	125.2	96.0
7	1 (270)	1 (6)	-1 (20)	176.8	90.9	98.5
8	1 (270)	1 (6)	1 (100)	178.2	141.6	100.4
9	0 (150)	0 (3.5)	0 (60)	162.4	123.7	97.5
10	0 (150)	0 (3.5)	0 (60)	164.6	123.4	93.6
11	0 (150)	0 (3.5)	0 (60)	163.2	123.1	97.6

P=pressure, t=exposure time and R=depressurization rate. [*] Residual activity defined as absolute value of (Final activity/ Initial activity) x 100. ** initial activity of the enzyme immobilized without treatment *** Liquefied gas of petroleum

The values of residual activity using LPG treatment can be considered very promising, since this fluid is constituted by a mixture and is commercially used as cooking gas and therefore has a much lower cost compared to carbon dioxide and mainly to propane and *n*-butane. The LPG composition of around 50 wt% of propane and 40 wt% of *n*-butane+isobutane justifies the similar values found for the residual enzymatic activity when compared to those verified when the enzyme was treated with pure propane and *n*-butane.

According to Knez the depressurization is one of the factors that more affect the enzymatic activity [18]. The pressurized fluid enters in contact with the tertiary structure of the enzyme in a slow way. When the system is depressurized quickly there is a fast expansion of the fluid, causing a higher flowing pressure in the

enzyme than in the system, which can provoke an owed cellular fissure due to the overpressure, causing an unfolding in the structure of the enzyme, increasing or reducing its activity and selectivity.

Protein denaturation induced by pressure has been an important research topic in recent years and, from some existing examples, the denaturation seems to be a reversible process, different from denaturation caused by temperature [19]. Conversely, some previous studies show that solvents with low dielectric constant, such as propane, could keep or even enhance enzyme activity and stability [6,14, 20]. Since the solvent properties affect the specific interaction with the enzymes, different effects may be obtained depending on the enzyme studied [20, 21]. Additionally, as propane presents relatively low solubility in water, it can be speculated that it might be acting as a piston fluid, enhancing the pressure over the enzyme. Regarding the effect of hydrostatic pressure on enzyme stability, the literature pointed out that pressure values around those used in this work cause a small effect on enzyme activity [22].
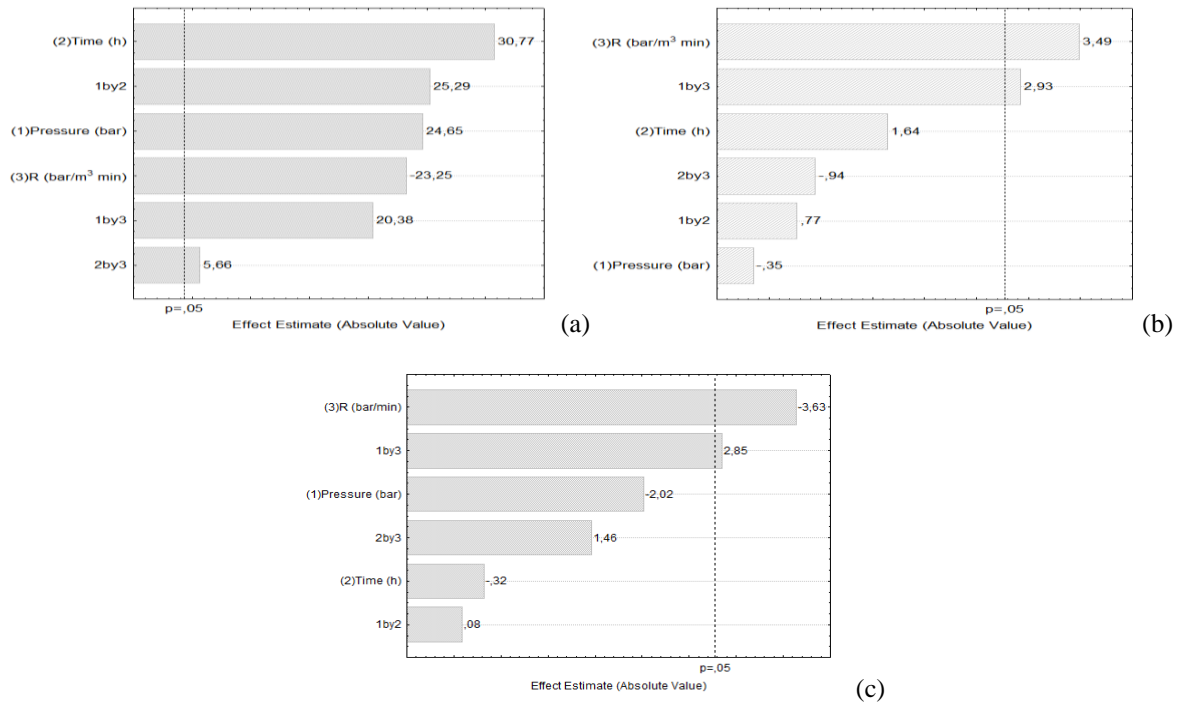
(a)

(b)

(c)

Figure 2. Pareto chart of effects of pressure, exposure time and depressurization rate on residual activities of inulinase from *Aspergillus niger*: (a) propane, (b) *n*-butane and (c) LPG.
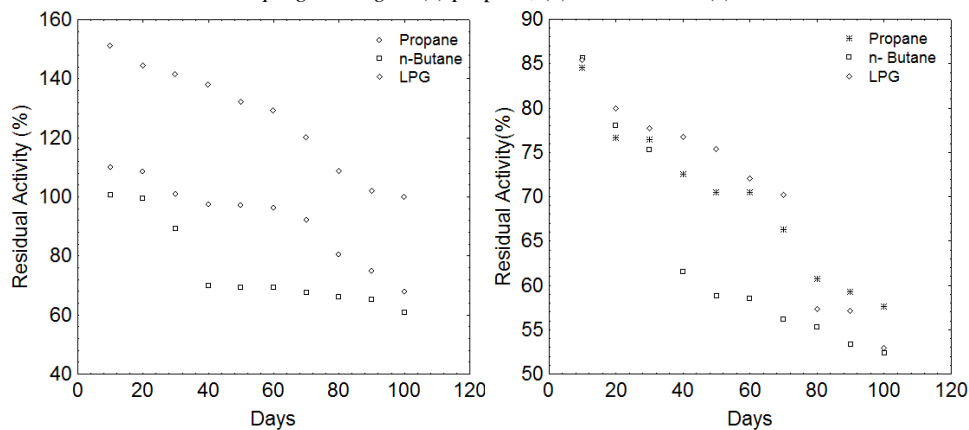
Figure 3. Storage stability of inulinase after submitted to the pressurized fluid using inulin and sucrose as substrate.

Figure 3 shows the storage stability of the immobilized inulinases from *Aspergillus niger* submitted to the treatment in propane. Data analysis reveals that the *Aspergillus niger* enzyme kept its relative residual activity of 67% when inulin was used as substrate and 57% with sucrose. When treated with LPG maintained its relative residual activity at 100% when inulin was used as substrate and at 52% for sucrose. When submitted to the treatment in *n*-butane using sucrose and inulin as substrates, its relative residual activity at 50 % when inulin is used as substrate and 60 % for sucrose.

Pressure and temperature can also directly affect enzyme stability and reaction parameters, including the rate constant [23,24]. Pressure can affect the reaction rate by changing the concentrations of reactants and products in solution because the partitioning of reaction components between the two phases depends on pressure. Changes in pressure can progressively alter the enantioselectivity of enzymatic reactions [24,25].

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 propane, *n*-butane and LPG depends significantly on the structural nature of the enzyme and the experimental conditions imposed, 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 propane, *n*-butane and LPG may be of technological relevance as a preceding, preparation step, to improve enzyme activity, hence helping the development of new biotransformation processes.

Acknowledgements

References

[1] M. Ettalibi, J.C. Baratti, Sucrose hydrolysis by thermostable immobilized inulinases from *Aspergillus ficuum*. Enz. Microb. Technol., 28 (2001) 596-601.

[2] F.V. Risso, M.A. Mazutti, H. Treichel, F. Costa, F. Maugeri Filho, M.I. Rodrigues, Comparative studies of the stability of free and immobilized inulinase from *Kluyveromyces marxianus* NRRL Y-7571 in an organic medium. Braz. J. Chem. Eng., 27 (2010) 507-516.

[3] F.V. Risso, M.A. Mazutti, H. Treichel, F. Costa, F. Maugeri Filho, M.I. Rodrigues, Comparison Between Systems for Synthesis of Fructooligosaccharides from Sucrose Using Free Inulinase from *Kluyveromyces marxianus* NRRL Y-7571. Food. Bioproc. Technol., (2010), DOI: 10.1007/s11947-009-0272-1, in press.

[4] H. Zhao, O. Olubajo, Z. Song, A.L. Sims, T.E. Person, R.A. Lawal, L.A. Holley, Effect of kosmotropicity of ionic liquids on the enzyme stability in aqueous solutions. Bioorg. Chem., 34 (2006) 15-25.

[5] Z. Knez, M. Habulin, Compressed gases as alternative enzymatic-reaction solvents: A short review. J. Supercrit. Fluid, 23 (2002) 29-42.

[6] W.Y. Lou, M.H. Zong, Y.Y. Liu, J.F. Wang, Efficient enantioselective hydrolysis of D-L phenilglycine methyl ester catalyzed by immobilized *Candida antarctica* lipase B in ionic liquid containing systems. J Biotechnol., 125 (2006) 64-69.

[7] K.W. Kim, B. Song, M.Y. Choi, M.J. Kim,. Biocatalysis in ionic liquids: Markedly enhanced enantioselectivity of lipase. Organic Lett., 10 (2001) 1507-1512.

[8] R.M. Lau, F.V. Rantwijk, K.R. Seddon, R.A. Sheldon, Lipase-catalyzed reactions in ionic liquids. Organic Lett., 26 (2000) 4189-4196.

[9] M. Erbeldinger, A.J. Mesiano, A.J. Russell, Enzymatic catalysis of formation of Z-aspartame in ionic liquid - an alternative to enzymatic catalysis in organic solvents. Biotechnol. Prog., 16 (2006) 1129-1134.

[10] R. Kumar, S. Madras, J. Modak, Enzymatic synthesis of ethyl palmitate in supercritical carbon dioxide. Ind. Eng. Chem. Res., 43 (2004) 1568-1573.

[11] J.V. Oliveira, D. Oliveira, Kinetics of enzymatic alcoholysis of palm kernel oil in SC-CO_2. Ind. Eng. Chem. Res., 39 (2000) 4450-4455.

[12] F.J. Kao, S.A. Ekhorutomwen,, S.P. Sawan,. Residual stability of lipase from *Candida rugosa* in hexane, supercritical CO_2, and supercritical SF_6. Biotechnol. Tech., 11 (1997) 849-852.

[13] S. Kamat, E.J. Beckman, A.J. Russell, Control of enzyme enantioselectivity with pressure changes in supercritical fluoroform. J. Am. Chem. Soc., 115 (1993) 8845-8851.

[14] D. Oliveira, A.C. Feihrmann, C. Dariva, A.G. Cunha, J.V. Bevilaqua, J. Destain, J.V. Oliveira, D.M.G. Freire, Influence of compressed fluids treatment on the activity of *Yarrowia lipolytica* lipase. J. Mol. Catal. B Enz., 39 (2006) 117-121.

[15] D. Oliveira, A.C. Feihrmann, A.F. Rubira, M.H. Kunita, C. Dariva, J.V. Oliveira, Assessment of two immobilized lipases activity treated in compressed fluids. J. Supercrit. Fluids, 38 (2006) 127-133.

[16] G. Kuhn, M. Marangoni, D.M.G. Freire, V.F. Soares, M.G. Godoy, A.M. Castro, M. Di Luccio, H. Treichel, M.A. Mazutti, D. Oliveira, J.V. Oliveira, Esterification activities of non-commercial lipases after pre-treatment in pressurized propane. J. Chem. Technol. Biotechnol. 85 (2010) 839-844.

[17] G.L. Miller, Use of dinitrosalisylic acid reagent for determination of reducing sugar**.** Anal. Chem. 31 (1959) 426-428.

[18] [18] Z. Knez, High pressure process technology- Quo vadis?, Chem. Eng. Res. Des. 82 (2004) 1541-1548.

[19] M.A. Jackson, F.J. Eller, Isolation of long-chain aliphatic alcohols from beeswax using lipase-catalyzed methanolysis in supercritical carbon dioxide. J. Supercrit. Fluids, 37 (2006) 173-177.

[20] A.T. Fricks, D.P.B. Souza, E.G. Oestreicher, O.A.C. Antunes, J.S. Girardi, D. Oliveira, C. Dariva, Evaluation of radish (*Raphanus Sativus* L.) Peroxidase activity after high-pressure treatment with carbon dioxide. J. Supercrit. Fluids, 38 (2006) 347-353.

[21] V. Kasche, R. Schlothauer, G. Brunner, Enzyme denaturation in supercritical CO_2: stabilizing effect of S-S bonds during the depressurization step. Biotechnol. Lett., 10 (1998) 569-573.

[22] L.R. Castilho, C.M.S. Polato, E.A. Baruque, G.L. Sant'Anna Jr., D.M.G. Freire, Economic analysis of lipase production by *Penicillium restrictum* in solid-state and submerged fermentations. Biochem. Eng. J., 4 (2000) 239-247.

[23] H. Ishikawa, M. Shimoda, A. Yonekura ,Y. Osajima, Inactivation of enzymes and decomposition of a-helix structure by supercritical carbon dioxide microbubble method. J. Agr. Food. Chem., 44 (1996) 2646–2650.

[24] C. Dalla Rosa, M.B. Morandim, J.L. Ninow, D. Oliveira, H. Treichel, J.V. Oliiveira, Continuous lipase-catalyzed production of fatty acid ethyl esters from soybean oil in compressed fluids, Bioresour. Technol. 100 (2009) 5818–5826.

[25] T. Matsuda, T. Harada, K. Nakamura, T. Ikariya, Asymmetric synthesis using hydrolytic enzymes in supercritical carbon dioxide. Tetrahedron-Asymmetr ., 16 (2005) 909–915.