ASSESSMENT OF THE EFFECTS OF TEMPERATURE AND PRESSURE ON LIPASE ACTIVITY IN $SCCO_2$

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This work investigates the influence of temperature, pressure, exposure times and decompression rate on lipase (Novozym 435) activity in high-pressure CO_2 medium. For this purpose, a high-pressure variable-volume view cell was employed in the experiments, varying the temperature from 30–70^oC, in the pressure range of 70-250bar, at some high-pressure exposure times (60-360min) and adopting several decompression rates (10-200$kgm^{-3}min^{-1}$). Enzyme activity was determined as the initial rates in esterification reactions between lauric acid and propanol at a molar ratio of 1:3, temperature of 60^oC and enzyme concentration of 5wt% in relation to the substrates. The results obtained show that an increase in temperature and density leads to an enhancement of enzyme activity losses while the decompression rates had a weak influence on the enzyme activity loss.

INTRODUCTION

Bioconversion of vegetable oils through the use of enzymes as catalysts in supercritical medium is undoubtedly a matter of great scientific and technological interest nowadays. The possibility of using a much less pollutant fuel when compared to diesel from petroleum and producing many chemical raw materials for food, pharmaceutical and cosmetic industries has motivated the biotransformation of vegetable oils to result in high-value added products or drastic reduction in environment investments [1]. Enzyme catalyzed reactions of vegetable oils in $SCCO_2$ seems to be a promising technique towards obtaining high grade products – fatty acid ethyl esters, mono and diglycerides, with very satisfactory reaction rates. In fact, numerous studies have shown recently that many enzymatic reactions can be advantageously conducted with $SCCO_2$ over organic solvents, resulting in much higher rates and selectivity [2]. To conduct such reactions at high pressures, the enzyme behavior in $SCCO_2$ is of primary importance as the loss of enzyme activity may lead to undesirable poor reaction rates and reduction of desired products production. With respect to the stability of lipases, the high pressure and kind of medium, are both interesting parameters from the theoretical and practical point of view [3]. Changes in protein structure may occur under extreme conditions. The spatial structure of many proteins may be significantly altered, causing denaturation and consequent loss in activity. If conditions are less adverse, protein structure may largely be retained. Minor structural changes may induce an alternative active protein state, which may possess altered activity, specificity and stability [4]. Many enzymes are stable and catalyze reactions in supercritical fluids, just as they do in other non- or micro-aqueous environments [5]. Enzyme stability and activity depend on the enzyme species, the supercritical fluid, the water content of the enzyme/support/reaction mixture, the decompression rates, the exposure times and on the pressure and temperature of the reaction system. To understand the potential of pressure application to enzyme processes, the influence of temperature, pressure, exposure times and decompression rates on a commercial immobilized lipase (Novozym 435) activity in high-pressure CO_2 medium was studied.

MATERIALS AND METHODS

Enzyme

The commercial lipase used in this work was kindly supplied by NOVOZYMES Brazil (Araucária, PR, Brazil): *Candida antarctica* (Novozym 435) immobilized on a macroporous anionic resin (0.12U/g, 1.4% water and diameter in the range of 0.3-0.9mm).

Lipase activity

The enzyme activity was determined as the initial rates in esterification reactions between lauric acid and propanol at a molar ratio of 1:3, temperature of 60^{o}C and enzyme concentration of 5wt% in relation to the substrates. At the beginning of the reaction, samples containing the mixture of lauric acid and propanol were collected and the lauric acid content was determined by titration with NaOH 0.04N. After the addition of the enzyme to the substrates, the mixture was kept at 60^{o}C for 15 min. Then, the lauric acid consumption was determined.

Chemicals

Lauric acid, ethanol, acetone and other chemicals (analytical grade) were from Merck. Carbon dioxide with purity higher than 99.99% was used as solvent in the high-pressure experiments.

Apparatus and Experimental Procedure

The equipment used in all experiments consists basically of a CO_2 cylinder, a high-pressure pump (ISCO 260D), a 10mL reactor, an absolute pressure transducer and a thermostatic bath. The lipase (approximately 1.0g) was charged into the reactor and the temperature established in the experimental design was reached. Afterwards, the system was pressurized and maintained at constant temperature and pressure for a pre-established exposure time. Typically, the pressure come up time was less than 0.5min and accordingly was not included in the pressure holding time because of its relatively small time compared to longer holding times. Then, at the decompression rates (10-200kgm^{-3}min^{-1}) defined, the system was depressurized and the lipase activity was measured. The loss in the lipase activity was defined as the difference between the activity at the beginning and at the end of the process.

Experimental design

A Taguchi experimental planning with two levels and four variables (temperature, exposure time, decompression rate and reduced density) was adopted. The experimental planning, covering the variable ranges commonly used for transesterification reactions [1], is presented in Table 1. The experiments were accomplished randomly, and duplicate runs were carried out for all experimental conditions. The activity loss was then modeled by an empirical model in order to determine the influence of the variables.

RESULTS AND DISCUSSION

The experimental results obtained are presented in Figure 1. From this figure one can observe that the temperature, reduced density and exposure time influenced positively the activity loss while the decompression rate had a weak negative effect. It is also relevant to consider the effect of cross interaction parameters.

Table 1: Taguchi experimental planning: Experimental conditions.

Run	Temperature (T) [^{o}C]	Initial pressure [bar]	Exposure time (t) [min]	Decompression rate (R) [kgm^{-3}min^{-1}]	Reduced density (RD)
1	40	80.0	60	10	0.60
2	40	130.7	60	200	1.60
3	40	130.7	360	10	1.60
4	40	80.0	360	200	0.60
5	70	255.5	60	10	1.60
6	70	107.2	60	200	0.60
7	70	107.2	360	10	0.60
8	70	255.5	360	200	1.60
9	55	132.0	210	105	1.10

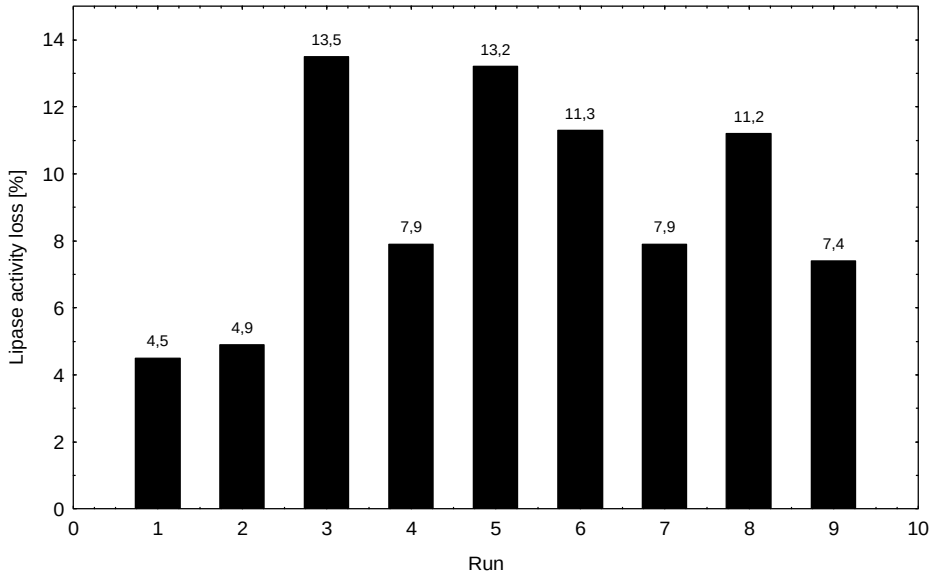

Figure 1: Activity loss obtained in the experimental planning.

The influence of temperature, exposure time, decompression rate and reduced density as well as the cross-interactions exposure time-decompression rate and temperature-exposure time were investigated. In order to allow a direct comparison of each variable effect, the independent variables were normalized in the range of –1 to +1. The "-1" level represents the inferior limit, while the "+1" level represents the superior limit of each variable. A statistical modeling technique was used to obtain an empirical model able to represent the experimental data. Empirical models were built by assuming that all variable interactions were significant, estimating the parameters related to each variable interaction and main variable effects, and discarding the meaningless parameters considering a confidence level of 95%, by using the Student's t-test.

Table 2 presents the results obtained in the statistical modeling. From this table it can be observed that temperature and reduced density had a pronounced effect on enzyme activity loss, both showing a positive effect. At this point, it is important to mention that the cross-interaction temperature-exposure time had a significant negative effect. In the range investigated (10-200kgm^{-3}min^{-1}), the decompression rate had a weak negative effect on enzyme activity loss. The same effect was observed with regard to the exposure time (60-360min).

Table 2: Regression results for the system Novozym 435-$SCCO_2$.

Model: Activity loss= a0+a1*T+a2*t+a3*R+a4*RD+a5*T*R+a6*T*t+a7*T*T
R=0.99957

Parameter	a0	a1	a2	a3	a4	a5	a6	a7
	7.36	1.59	0.83	-0.48	1.39	0.86	-2.18	1.94
Standard deviation	0.28	0.099	0.099	0.099	0.099	0.099	0.099	0.295

From the results obtained, it can be observed that, in all experimental conditions, the enzyme activity loss was lower than 15%. When compared to the work presented by Habulin and Knez [6] this activity loss is considered low. Some works available in the literature are pointing the use of $SCCO_2$ as a satisfactory medium to inactivate some enzymes [6, 7]. It is important to mention that all these works used enzymes in native form and the results presented here are related to an immobilized enzyme (Novozym 435). On the other hand, the results obtained here are in agreement with those obtained by Castellari et al. [7] for the system containing a polyphenoloxidase present in grape musts. In that work, at pressures from 300 to 900MPa, the residual activity after the high pressure treatment for 10 minutes was approximately 90%.

CONCLUSION

From this work, one can conclude that a commercial immobilized lipase from *Candida antarctica* (Novozym 435) was stable in $SCCO_2$ for all experimental conditions investigated. Based on the results obtained here and comparing with the results obtained by other authors, it can be concluded that the magnitude of pressure, temperature, decompression rate and exposure time needed to inactivate the enzyme strongly depends on the nature and the source of enzyme and, mainly, whether the enzyme is in its native or immobilized form. For the purpose of using this enzyme to catalyze the transesterification reaction of vegetable oils so as to produce esters, the results obtained in this work are relevant, since we can use the immobilized lipase with low activity loss at typical conditions of temperature and pressure employed in many biotransformation of raw materials.

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