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The Effect of Temperature, Pressure, Exposure Time, and Depressurization Rate on Lipase Activity in $SCCO_2$

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Abstract

We investigated the influence of temperature, pressure, exposure time, and decompression rate on lipase activity in high-pressure CO_2 medium. A high-pressure, variable-volume view cell was employed in the experiments, varying the temperature from 30 to 70°C in the pressure range of 70–250 bar at various high-pressure exposure times (60–360 min) and adopting several decompression rates (10–200 kg/[m^3·min]). The results obtained show that an increase in temperature and density led to an enhancement of enzyme activity losses while the decompression rates had a weak influence on enzyme inactivation.

Index Entries: Lipase activity; high pressure; decompression rate; CO_2; $SCCO_2$; exposure time.

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. The possibility of using a much less pollutant fuel (biodiesel) compared to diesel from petroleum and producing many chemical raw materials for food, pharmaceutical and cosmetic industries has motivated research efforts in the biotransformation of vegetable oils with the desired end result of high-value-added products or drastic reduction in environmental investments *(1)*.

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Enzyme-catalyzed reactions of vegetable oils in $SCCO_2$ seems to be a promising technique toward obtaining high-grade products—fatty acid ethyl esters, mono- and diglycerides—with very satisfactory reaction rates. Because biocatalysts have high specific activity and a low impact on the environment, they have become increasingly important for industry. For example, immobilized lipases are used as catalysts for reactions involving biomodification of triglycerides *(2)*. In this case, supercritical fluids, particularly CO_2, have currently received widespread attention as a possible medium for enzymatic reactions *(3)*. The main advantage of supercritical fluids over liquid solvents, such as *n*-hexane, is that the high diffusivity, low viscosity, and low surface tension of supercritical fluids can speed up mass transfer–limited enzymatic reactions *(4)*.

To conduct such reactions at high pressures, the enzyme behavior in $SCCO_2$ is of primary importance since the loss of enzyme activity may lead to undesirable poor reaction rates and reduction of desired product production. With respect to the stability of lipases, the high pressure and kind of medium are both interesting parameters from a theoretical and practical point of view *(5)*. Changes in protein structure may occur under extreme conditions, and the spatial structure of many proteins may be significantly altered, causing denaturation and consequent loss of 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 *(6)*.

Many enzymes are stable and catalyze reactions in supercritical fluids, just as they do in other non- or microaqueous environments *(7)*. Enzyme stability and activity may depend on the enzyme species, supercritical fluid, water content of the enzyme/support/reaction mixture, decompression rates, exposure times, and pressure and temperature of the reaction system.

To understand the potential of pressure application to enzyme processes and to help elucidate the reaction mechanism as well as a rational design of alcoholysis reactors for future scale-up, we investigated the influence of temperature, pressure, exposure times, and decompression rates on the activity of a commercial immobilized lipase (Novozym 435) activity in high-pressure CO_2 medium.

Materials and Methods

Chemicals

Lauric acid, ethanol, acetone, and other chemicals (analytical grade) were from Merck. CO_2 with a purity >99.99% was used as solvent in the high-pressure experiments.

Enzyme

The commercial lipase *Candida antarctica* (Novozym 435), immobilized on a macroporous anionic resin (0.12 U/g, 1.4% water, and diameter

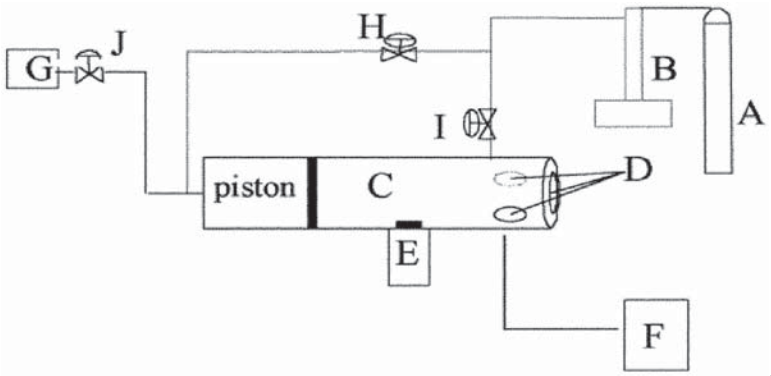

Fig. 1. Schematic diagram of high-pressure apparatus for enzyme activity tests. A, CO_2 cylinder; B, syringe pump; C, equilibrium cell; D, sapphire windows; E, magnetic stirrer; F, white light source; G, pressure transducer; H, ball valve; I, micrometering valve; J, relief valve.

in the range of 0.3–0.9 mm), was kindly supplied by Novozymes Brazil (Araucária, PR, Brazil).

Lipase Activity

Enzyme activity was determined as the initial rates in esterification reactions between lauric acid and propanol at a molar ratio of 1:3, a temperature of 60°C, and an enzyme concentration of 5 wt% 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 0.04 *N* NaOH. After the addition of the enzyme to the substrates, the mixture was kept at 60°C for 15 min. Then, lauric acid consumption was determined.

Apparatus and Experimental Procedure

The equipment used in all experiments consisted basically of a CO_2 cylinder, a 20-mL view cell with three sapphire windows for visual observations, an absolute pressure transducer (Smar LD 301) with a precision of ±0.012 MPa, a portable programmer (Smar HT 201) for pressure data acquisition, and a syringe pump (ISCO 260D). The equilibrium cell contained a movable piston, which permitted pressure control inside the cell. Figure 1 presents schematic diagram of the experimental unit.

Lipase (approx 1.0 g) was charged into the cell, and the temperature established in the experimental design was reached. Afterward, the system was pressurized and maintained at a constant temperature and pressure for a preestablished exposure time. Typically, the pressure elevation time was <0.5 min and was not included in the pressure holding time because of its comparatively short duration. Then, at the decompression rates (10–200 kg/[m^3·min]) defined, the system was depressurized and the lipase activity was measured. The loss of lipase activity was defined as the difference between the activity at the beginning and at the end of the process.

Table 1
Taguchi Experimental Plan Conditions

Run	Temperature (T) (°C)	Initial pressure (bar)	Exposure time (t) (min)	Decompression rate (R) (kg/[m^3·min])	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

Experimental Design

A Taguchi experimental plan with two levels and four variables (temperature, exposure time, decompression rate, and reduced density) was adopted. The experimental plan, 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 leading to an average reproducibility better than 5%. The activity loss was then modeled empirically in order to determine the influence of the process variables on main and cross-interaction parameters.

Results and Discussion

The experimental results obtained are presented in Fig. 2. One can observe from Fig. 2 that temperature, reduced density, and exposure time influenced positively the activity loss whereas decompression rate had a weak negative effect.

The influence of temperature; exposure time; decompression rate; and reduced density; as well as the cross-interaction variables exposure time–decompression rate and temperature–exposure time were investigated. 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.

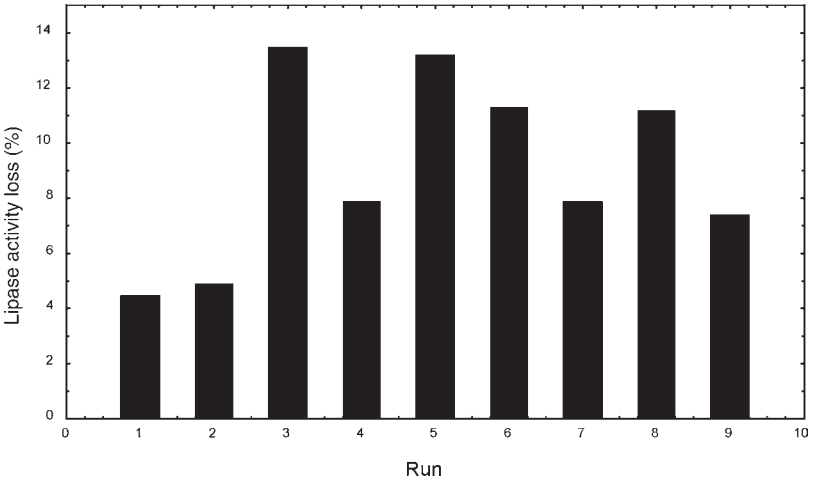

Fig. 2. Loss of enzyme activity obtained in experimental planning.

Table 2
Regression Results for Novozym 435–$SCCO_2$

Model:
Activity loss = $a_0 + a_1 \times T + a_2 \times t + a_3 \times R + a_4 \times RD + a_5 \times T \times R + a_6 \times T \times t + a_7 \times T \times T$
$R = 0.99957$

Parameter	a_0	a_1	a_2	a_3	a_4	a_5	a_6	a_7
	7.36	1.590	0.830	–0.480	1.390	0.860	–2.180	1.940
SD	0.28	0.099	0.099	0.099	0.099	0.099	0.099	0.295

a_0, a_1, a_2, a_3, a_4, a_5, a_6, a_7, model parameters.

Table 2 presents the results obtained in the statistical modeling. 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 (10–200 kg/[m^3·min]), the decompression rate had a weak negative effect on loss of enzyme activity. The same effect was observed with the exposure time (60–360 min).

The results show that in all experimental conditions the enzyme activity loss was lower than 15%. When compared to the work presented by Habulin and Knez *(8)*, this activity loss is considered low. Some works available in the literature point to the use of $SCCO_2$ as a satisfactory medium to inactivate some enzymes *(8,9)*. It is important to mention that all these works used enzymes in native form, and the results presented herein are related to an immobilized enzyme (Novozym 435). On the other hand, our results are in perfect agreement with those obtained by Castellari et al. *(9)* for the system containing a polyphenoloxidase present in grape musts.

In that work, at pressures from 300 to 900 MPa, the residual activity after the high-pressure treatment for 10 min was approx 90%.

A series of enzyme-catalyzed reactions recently conducted in both conventional and supercritical fluid medium has shown that while no loss of enzyme activity was experimentally observed for the conventional medium, the same was no longer valid for supercritical CO_2 systems *(1,4,10,11)*. For instance, Steinberger and Marr *(12)* have pointed out that the stability of an enzyme in supercritical CO_2 depends on both its tertiary structure and several parameters during exposure to high-pressure fluid. They argued that high temperatures, the water content in CO_2 and pressurization/depressurization steps might cause enzyme inactivation.

Another interesting aspect verified by Oliveira and Oliveira *(1,4)* and by Cernia et al. *(13)* is that the reaction rate generally exhibits an unusual behavior in the range of 6–25 MPa. A rise in pressure leads to an increase in the reaction rate, and then a dramatic decrease occurs after a certain pressure limit inducing modifications in both structure and function of enzyme.

Conclusion

We conclude that a commercial immobilized lipase from *C. antarctica* (Novozym 435) was stable in $SCCO_2$ for all experimental conditions investigated. Based on the results obtained here and comparison of them with the results obtained by other investigators, 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, primarily, 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 in order to produce esters, the results obtained herein are relevant, because the immobilized lipase can be used with low activity loss at typical conditions of temperature and pressure employed in many biotransformations of raw materials.

Acknowledgments

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