ABSTRACT

The ethanolysis of sunflower oil catalyzed by Pseudomonas cepacia lipase was carried out at 40 °C using dense carbon dioxide as reaction medium. At 130 bar, around 45% w/w of CO2 was dissolved in the reaction mixture (ethanol + sunflower oil). The kinetic behavior was studied by sampling at different periods of time. The ethanolysis reaction in CO2-expanded media was compared with the reaction performed without CO2 in a sealed flask at ambient pressure, at the same temperature, same charge of substrates and using the same batch of immobilized lipase. In both cases, the biocatalyst showed similar degree of deactivation after the respective ethanolysis reactions. The experimental data obtained for the respective reactions was correlated using a simplified kinetic model.

Keywords: ethanolysis, CO2 expanded media, sunflower oil, Pseudomonas cepacia.

RESUMEN

Se llevó a cabo la etanolisis de aceite de girasol catalizada por lipasa de Pseudomonas cepacia a 40 °C usando dióxido de carbono denso como medio de reacción. En la mezcla de reacción (etanol + aceite de girasol) se disolvió 45% p/p de CO2 a 130 bar de presión. Se estudió el comportamiento cinético a través de muestreo a diferentes períodos de tiempo. Se comparó la reacción de etanolisis en medio expandido con CO2 con la reacción llevada a cabo en un recipiente cerrado herméticamente sin CO2, a presión ambiente, a la misma temperatura, la misma carga de sustratos y la misma cantidad de lipasa inmovilizada que en la primera. En ambos casos, el biocatalizador mostró similar grado de desactivación después de las respectivas reacciones etanolisis. Los datos experimentales obtenidos para las respectivas reacciones se correlacionaron utilizando un modelo cinético simplificado.

Palabras claves: etanolisis, medio expandido con CO2, girasol, Pseudomonas cepacia.
1. INTRODUCTION

During the last two decades, the utilization of supercritical carbon dioxide (SC-CO₂) as a reaction medium in biocatalysis has been receiving increased attention as potential clean chemical synthesis due to environmentally-compatibility, zero chemical residue in the product, total replacement of organic solvent, high catalytic efficiency and considerable processing flexibility. Biocatalysts have the benefit of substrate specificity and SC-CO₂ has several advantages over liquid solvents such as high solute diffusivities and low viscosity, what can accelerate mass transfer -limited enzymatic reactions¹⁻⁴. However, the major drawback of enzymatic SC-CO₂ reactions carried out in a single supercritical phase is the high pressures required to ensure the entire (low) solubility of many organic compounds in CO₂.

Lipase-catalyzed reactions in SC-CO₂ have been reported by numerous investigators⁵⁻⁷ and several excellent reviews summarize research activities in this area⁸⁻⁹. Related with lipid-type substrates, King et al has conducted synthesis to make simple esters¹⁰, transesterifications to produce methyl esters¹¹, patented a glycerolysis process¹² and performed randomization of fats/oils¹³ in SC-CO₂. Concerning the enzymatic alcoholysis of vegetable oils in SC-CO₂, very little experimental data has been reported in the literature. An example is the comparison of palm kernel oil ethanolysis taking place in both SC-CO₂ and in n-hexane solvents, as described by Oliveira¹⁴.

When the substrates are liquid substances an alternative practice is dissolving CO₂ in the reaction mixture by moderately increasing pressure. Thus, a liquid expanded homogeneous medium can be achieved and again viscosity decreases improving diffusion of reactants and products. The challenge in developing catalysis in CO₂-expanded media is explained on the basis of the high solubility of CO₂ in many organic liquids¹⁵. Particularly, if the substrates are partially miscible the addition of CO₂ can considerably enhance their mutual solubility.

In this work the ethanolysis of sunflower oil catalyzed by Pseudomonas cepacia lipase was carried out at 40 °C using CO₂ as solvent. A variable volume view cell of 120 cm³ capacity, equipped with a magnetic stirrer and cold end light, was employed to observe the course of the reaction. The kinetic behavior during 5 h of the ethanolysis reaction was studied. Samples were collected at different intervals of time and pressure was maintained during sampling by means of a manual hydraulic pressurization system.

The high pressure CO₂- expanded ethanolysis reaction was compared with that performed without CO₂ at 40 °C, ambient pressure and using the same batch of immobilized lipase. In this case, the reaction was carried out in a sealed flask placed in an orbital shaker, following the procedure described by Torres¹⁶. In both cases, at the end of the respective ethanolysis reactions, the lipase employed was recovered and the remaining activity of the biocatalyst was analyzed. The experimental data obtained was fitted to a simplified version of the kinetic model recently presented, for lipase-catalyzed ethanolysis reactions using Pseudomonas cepacia lipase¹⁶.

2. MATERIALS AND METHODS

2.1 Chemicals

Sunflower oil, with less of 0.5 % w/w of humidity, according to seller specifications, was used in the present study. All solvents used were HPLC grade from Lab scan (Dublin, Ireland). Ethanol absolute (water content < 0.1 % w/w) was obtained from Panreac (Barcelona, Spain) and was dried with molecular sieves 4 Å from Sigma-Aldrich (St. Louis, MO, USA). The rest of the materials were used without further purification. The lipase Pseudomonas cepacia (PS) was obtained from Amano (Lombard, IL).

2.2 Analytical methods

Analysis of the reaction product was carried out by gas chromatography. The samples (100 µL) were mixed with 2 mL of chloroform ethanol 2:1 v/v and immediately filtered with a 0.45 m Sartorius (Goettingen, Germany) nylon syringe filter. Samples were then dried with sodium sulfate. Aliquots of the final transparent solution (250 µL) were diluted with 750 µL of hexane. One µL of the diluted sample was injected into an Agilent (Avondale, PA) gas chromatograph (6890N Network GC System) coupled to an
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autosampler (Agilent 7683B). The capillary column was a 30 m HP-88 (Avondale, PA) (0.25 mm i.d.) . The temperatures of the injector and detector were both 220 and 250 °C, respectively. The temperature program was as follows: starting at 100 °C and then heating to 180 °C at 20 °C/min; followed by heating from 180 to 220 °C at 15 °C/min. The final temperature (220 °C) was held for 30 minutes. Identification of the various free fatty acids was based on a PUFA No 3 standard (#4-7085) obtained from Supelco.

2.3 Study of lipase stability

Once the enzymatic reaction was completed, the lipase was separated from the product mixture by vacuum filtration during 5 minutes. The solid obtained was then washed once with chilled acetone and vacuum filtered again. In order to obtain comparable results, the same procedure was utilized with fresh lipase that was previously submerged in the reaction mixture for 5 minutes. The activity of both fresh and recovered lipase was determined as follows: 150 mg of oleic acid was mixed with 75 mg of butanol and 40 mg of the lipase in 10 ml of isoctane previously saturated with water at 20 °C. The reaction was allowed to proceed for 60 minutes in an orbital shaker (200 rpm). The product mixture was solved up to 50 mL of chloroform. The final transparent solution was analyzed by HPLC to determine the percentage of oleic acid consumed. The residual activity of the recovered lipase, in percentage, was determined as the ratio of the oleic acid consumed in the reaction with the recovered enzyme and that of the fresh enzyme.

2.4 Apparatus and experimental procedure

Ambient pressure ethanolysis reaction: 10 g of a mixture of sunflower oil containing 8 % (w/w) of hexadecane (internal standard) and 1.5 g of ethanol were placed in a 120 mL flask and mixed by swirling. After 500 mg of the immobilized lipase PS was added, the flask was stoppered and placed in an orbital shaker (200 rpm) at 40 °C. Samples (100 µL) were withdrawn periodically, and the flasks were resealed after each sampling. Reaction was allowed to proceed for 6 h. For a detailed explanation of the procedure the reader is referred to Torres et al.16.

Ethanolysis reaction in CO2-expanded media: a variable volume cell was employed to carry out the high pressure ethanolysis reaction (Figure 1). The cell has a maximum capacity of 120 cm3 and comprises a cylinder and a piston. The movable piston has a double seal (Polypak® from Parker) to separate the equilibrium chamber from the pressurizing circuit. The piston is driven by a manual pressure generator (HIP model 62-6-10), using ethanol as the pressurizing fluid. Inside the cell a teflon-coated magnetic bar is placed, which is driven by an external alternating magnetic field and provides vigorous stirring inside the cell.

The cylinder is surrounded by a 20 mm thick aluminium jacket, externally heated by two electrical resistances connected to a temperature controller (Glas-Col). The temperature is measured by a 100 ohm Pt resistance thermometer, placed inside a groove in the aluminium jacket, and is controlled to within 0.1 K. A gauge transducer (Barksdale) coupled with a digital indicator (Redlion) is connected to one of the three capillary lines. The estimated accuracy of the pressure measurements is ± 0.2 bar. The rest of capillary lines are employed to feed the substrates, the CO2 solvent and to sample.

Figure 1. C: variable volume cell; P: piston; MPG: manual pressure generator; MS: magnetic stirring bar; W: glass window; HR: heating resistances; MFG: magnetic field generator; TS: temperature sensor; TC: temperature controller; PS: pressure sensor; PI: pressure indicator; ER: ethanol reservoir; CO2R: CO2 reservoir; V: on-off valve; MSV: micrometering sampling valve; PG: pressure gauge.
To start a reaction the lipase (500 mg) was placed inside the cell together with 10 g of sunflower oil containing 8 % (w/w) of hexadecane (internal standard). When the desired temperature was stabilized inside the cell, 1.5 g of ethanol was fed together with 10 g of CO₂ (previous purging). Then, pressure was raised by means of the manual pressure generator, until the vapor phase almost completely disappeared and a liquid phase was observed. The liquid CO₂-expanded phase was observed at pressures higher than 130 bar. Around 15 minutes elapsed from the ethanol feed until the desired liquid phase was observed inside the cell. It has to be point out that during this period of time the ethanolysis reaction proceed in some extend (although no stirring was effected). When the pressure inside the cell attained the desired value, the first sample was withdrawn from the reaction mixture. Then, stirring was turned on, and several samples were collected at different intervals of time during 5 hours. The magnetic stirred maintained the biocatalyst dispersed in the liquid bulk phase. The volume of the reactive system was almost twice than the volume of the substrates introduced into the cell.

3. RESULTS AND DISCUSSION

Table 1 shows the composition of the samples collected at different intervals of time for both ethanolysis reactions carried out at ambient pressure (without CO₂) and at high pressure (with CO₂ dissolved). The concentration of total ester bonds (TEB) (defined as the ester bonds remained in mono-, di- or triacylglycerols molecules) is given in the table, together with the concentration of ethanol and fatty acid ethyl esters (FAEE).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ambient Pressure Reaction</th>
<th>Reaction in CO₂-expanded Media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TEB (mM)</td>
<td>Ethanol (mM)</td>
</tr>
<tr>
<td>0.0</td>
<td>2374.8</td>
<td>2470.9</td>
</tr>
<tr>
<td>0.3</td>
<td>2336.4</td>
<td>2432.5</td>
</tr>
<tr>
<td>0.5</td>
<td>2300.6</td>
<td>2396.7</td>
</tr>
<tr>
<td>1.0</td>
<td>2227.5</td>
<td>2323.6</td>
</tr>
<tr>
<td>2.0</td>
<td>2106.9</td>
<td>2203.0</td>
</tr>
<tr>
<td>3.0</td>
<td>2000.5</td>
<td>2096.6</td>
</tr>
<tr>
<td>4.0</td>
<td>1905.8</td>
<td>2001.9</td>
</tr>
<tr>
<td>5.0</td>
<td>1823.3</td>
<td>1919.4</td>
</tr>
<tr>
<td>6.0</td>
<td>1754.3</td>
<td>1850.4</td>
</tr>
</tbody>
</table>

The experimental TEB conversions obtained were correlated using the kinetic model recently presented by Torres et al. Several TEB conversions can be calculated using the following equation:

$$\text{TEB conversion} (\%) = 100 \cdot \frac{\text{TEB} \text{ at } t = 0 - \text{TEB} \text{ at } t}{\text{TEB} \text{ at } t = 0}$$

Figure 2 shows the progress of TEB conversion.

For both ethanolysis reactions as a function of time. Figure 2 shows TEB conversion related to the lipase load (mg lipase / mL reaction mixture). According to Figure 2 the ethanolysis in CO₂-expanded media proceed much slowly than the ambient pressure reaction. Nevertheless, since the amount of lipase loaded in both cases was the same (500 mg) but the volume of the reactive mixture with CO₂ was almost twice than the volume of the reactive mixture without CO₂, similar behavior can be deduced if TEB conversion is referred to the amount of lipase available per unit of reaction volume (see Figure 3). With respect to enzyme activity, the lipase deactivation study indicated that the residual activity of the immobilized lipase was ca. 97 % in both cases. The experimental TEB conversions obtained
simplifications were considered taking into account the following:
- The results of the lipase deactivation study indicated high residual activity of the immobilized lipase in both cases. Thus, the term considering lipase inactivation was neglected.
- Only the direct reaction of the mechanisms proposed was considered, since no improvement in the correlation of data was achieved when inverse reaction or inhibition effects were included.
- Since ethanol and sunflower oil were load in steechiometric ratio, the ethanol concentration in the reaction mixture was considered to be equal to TEB concentration (see Table 1).

Thus, the rate of disappearance of TEB resulted expressed as a second order reaction:

\[- \frac{dTEB}{dt} = K_1 [TEB]^2 \quad (2)\]

were \(K_1\) is the second order kinetic constant, which resulted to be 0.0255 mol\(^{-1}\)h\(^{-1}\) for the ethanolysis reaction carried out at ambient pressure (without CO\(_2\)) and 0.0162 mol\(^{-1}\)h\(^{-1}\) for the high pressure CO\(_2\)-expanded ethanolysis reaction. Satisfactory correlation of experimental data could be achieved in both cases as can be observed in Figure 3.

**CONCLUSIONS**

The lipase-catalyzed ethanolysis of sunflower oil was carried out at 40 °C using dense CO\(_2\) as solvent and employing ca. 20 mg of lipase per mL of reaction mixture. The CO\(_2\)-expanded reaction was compared with the ethanolysis at ambient pressure, without any solvent and with a lipase concentration of 40 mg/mL. In both cases a steechiometric oil/ethanol ratio was employed.

The CO\(_2\)-expanded ethanolysis rate was almost half of the ethanolysis performed without CO\(_2\), as can be deduced from the second order kinetic constant regressed from the experimental data. Nevertheless, taking into account that the lipase concentration in the reaction at ambient pressure was twice than the concentration in the CO\(_2\)-expanded reaction, similar behavior can be presumed. Additionally, very low and similar lipase deactivation was determined for both reactions. Thus, the dissolution of CO\(_2\) at high pressure seems not to affect *Pseudomonas cepacia* lipase activity and further investigations increasing lipase concentration, exploring different temperatures and different ethanol/oil ratios are promising.

**REFERENCES**

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