LIPASE-CATALYZED ETHANOLYSIS OF SUNFLOWER OIL IN CO_2-EXPANDED MEDIA

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Abstract

The ethanolysis of sunflower oil catalyzed by *Pseudomonas cepacia* lipase was carried out at 40 °C using dense carbon dioxide (CO_2) as reaction medium. At 130 bar a liquid phase was observed containing ca. 45% w/w of CO_2 dissolved in the reaction mixture. The kinetic behavior was studied by sampling at different periods of time. The ethanolysis reaction in CO_2-expanded media was compared with the reaction performed without CO_2 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.

1. Introduction

During the last two decades, the utilization of supercritical carbon dioxide (SC-CO_2) 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 synthesized product, total replacement of organic solvent, high catalytic efficiency and considerable processing flexibility. Biocatalysts have the benefit of substrate specificity and SC-CO_2 has several advantages over liquid solvents such as high solute diffusivities and low viscosity, what can accelerate mass transfer-limited enzymatic reactions[1-4]. However, the major drawback of enzymatic SC-CO_2 reactions carried out in a single supercritical phase is the high pressures (on the order of hundreds of bars) required to ensure entire solubility of many organic compounds in CO_2.

Lipase-catalyzed reactions in SC-CO_2 have been reported by numerous investigators[5-7] and several excellent reviews summarize research activities in this area[8, 9]. King et al. has conducted synthesis to make simple esters[10], transesterifications to make methyl esters[11], patented a glycerolysis process[12] and performed randomization of fats/oils[13] in SC-CO_2. Concerning the enzymatic alcoholysis of vegetable oils in SC-CO_2, very little experimental data have been reported in the literature. An example is the comparison of palm kernel oil ethanolysis taking place in both SC-CO_2 and in n-hexane solvents, as described by Oliveira et al.[14].

When the substrates are liquid substances an alternative practice is dissolving CO_2 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 CO_2-expanded media is explained on the basis of the high solubility of CO_2 in many organic liquids[15]. Particularly, if the substrates are partially miscible the addition of CO_2 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 CO_2 at high pressure as solvent. A variable volume view cell of 120 cm^3 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 CO_2-expanded ethanolysis reaction was compared with that performed without CO_2, at the same reaction conditions but ambient pressure, and using the same batch of immobilized lipase. In this case, the ethanolysis of sunflower oil was carried out in a sealed flask placed in an orbital shaker, following the procedure described by Torres et al.[16]. 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* lipase[16].

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 μm 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 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 follow: 150 mg of oleic acid was mixed with 75 mg of butanol and 40 mg of the lipase in 10 ml of isooctane previously saturated with water at 20 ºC. 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 cm^3 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 CO_2 solvent and to sample.

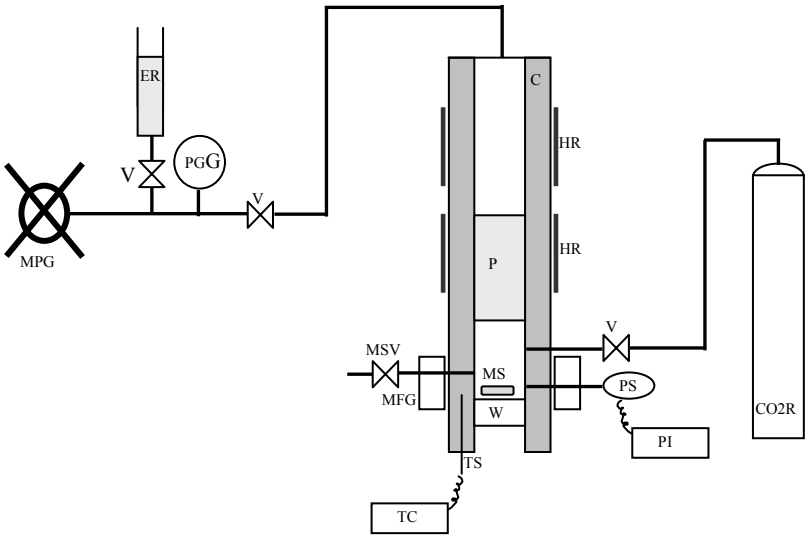

Figure 1. C: variable volume cell; P: piston; MPG: manual pressure generator; MS: magnetic stirring bar; W: glass window; HR: heating resistances; MFG: magnetic field generator; TS: temperature sensor; TC: temperature controller; PS: pressure sensor; PI: pressure indicator; ER: ethanol reservoir; CO2R: CO_2 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 feed together with 10 g of CO_2 (previous purging). Then, pressure was raised by means of the manual pressure generator, until the vapor phase disappeared and a liquid phase was observed. The liquid CO_2-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 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

Table 1 shows the composition of the samples collected at different intervals of time for both ethanolysis reactions carried out at ambient pressure (without CO_2) and at high pressure (with CO_2 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 1. Concentration of total ester bonds (TEB), ethanol and fatty acid ethyl esters (FAEE) of the samples collected during the lipase catalyzed ethanolysis reaction of sunflower oil at 40 °C.

ambient pressure reaction				reaction in CO2-expanded media			
time (h)	concentration (mM)			time (h)	concentration (mM)		
	TEB	ethanol	FAEE		TEB	ethanol	FAEE
0.0	2374.8	2470.9	0.0	0.0	1150.5	1203.5	2.3
0.3	2336.4	2432.5	38.4	0.3	1113.6	1164.9	38.5
0.5	2300.6	2396.7	74.1	0.5	1109.0	1160.3	43.2
1.0	2227.5	2323.6	147.2	1.0	1097.5	1148.8	54.7
2.0	2106.9	2203.0	267.9	2.0	1079.3	1130.6	72.9
3.0	2000.5	2096.6	374.3	3.0	1066.6	1117.9	85.5
4.0	1905.8	2001.9	469.0	4.0	1046.8	1098.1	105.4
5.0	1823.3	1919.4	551.5	5.0	1026.6	1077.8	125.6
6.0	1754.3	1850.4	620.4				

Figure 2 shows the progress of both ethanolysis reactions as a function of time. Figure 2(a) shows TEB conversion:

$$\text{TEB conversion}\,(\%) = 100 \times \frac{TEB_{(t=0)} - TEB_{(t)}}{TEB_{(t=0)}} \qquad (1)$$

and Figure 2(b) shows the TEB conversion related to the lipase load (mg lipase / mL reaction mixture). According to Figure 2(a) the ethanolysis in CO_2-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 CO_2 was almost twice than the volume of the reactive mixture without CO_2, similar behavior can be deduced if the TEB conversion is referred to the amount of lipase available per unit of reaction volume (see Figure 2(b)).

The experimental TEB conversions obtained were correlated by using a simplification of the kinetic model recently presented by Torres et al.[16]. Since the results of the lipase deactivation study indicated that the residual activity of the immobilized lipase was ca. 97 % in both cases, the term considering lipase inactivation was neglected. Additionally, only the direct reaction of the mechanisms proposed was considered (no improvement in the correlation of data was achieved when inverse reaction or inhibition effects were included) and the ethanol

concentration in the reaction mixture was considered to be equal to TEB concentration. Thus, the rate of disappearance of TEB resulted expressed as a second order reaction:

$$-\frac{d[TEB]}{dt} = K_1[TEB]^2 \qquad (2)$$

were K_1 is the 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 2.

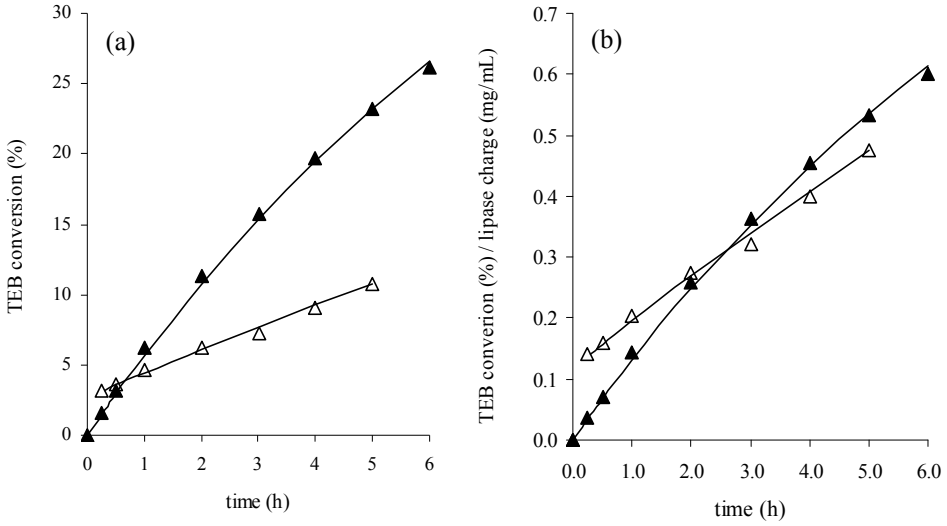

Figure 2. TEB conversion (a) and TEB conversion referred to lipase charge (b) as a function of time. (▲) ambient pressure ethanolysis reaction; (△) ethanolysis reaction in CO_2-expanded media. Solid lines: values calculated using Eq. (2).

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 stechiometric 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.

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