BIOENZIMATICREACTION OF OLEIC ACID IN SUPERCRITICAL CO_2

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Abstract

In this work, oleic acid, a fatty acid occurring in most vegetable oils, has been transformed into fatty acid esters by reaction with ethanol catalyzed by immobilized lipase Lipozyme® RM IM (Rhizomucor miehei) using carbon dioxide as supercritical media in order to investigate the feasibility of the biotransformation process. The reaction has been carried out in a high pressure reactor of 3.5 L, at pressures of 12 and 21.4 MPa, temperatures of 313 K, enzyme concentration of C_{Enzyme} = 0.0132 g.mL^{-1} and 0.0228 g.mL^{-1}, and substrate concentrations of $C_{Substrate}$ = 0.1325 g.mL^{-1} and 0.2282 g.mL^{-1}. The process feasibility has been investigated by analyzing the conversion of oleic acid into ethyloleate and the quality of the desired product (fatty acid esters) as a function of reaction time and state conditions. The chemical identification of fatty acid ester has been performed in terms of Acid Index. The experimental data of chemical kinetics show that the highest conversion of oleic acid into ethyloleate was 94.83% at 313 K, 12 MPa, and fatty acid/alcohol molar ratio of 1:2.5.

Keywords: Bioreactions, Oleic Acid, Fatty Acid Esters, Supercritical CO_2.

1. Introduction

Chemical transformation of fatty acids into fatty acid esters in supercritical media catalyzed by enzymes have been applied as an alternative method to produce esters free of reagent residuals as well as undesired products, thus making it possible not only to perform selective separations of non reacted substrate and reaction products in supercritical media but also to obtain the desired product of biotransformation at a high purity[1,2,3]. In this process the reaction products and reagents are distributed in the coexisting liquid and gaseous phases, whereas they are selectively separated by differences in dissolution within the supercritical media. Esterification of fatty acids catalyzed by enzymes using carbon dioxide as reaction media has been investigated in the last years by many authors, as found elsewhere in the literature as follows.

Knez *et al* (1998), studied the esterification reaction of oleic acid with oleyl alcohol (cis-9-Octadecen-1-ol), catalyzed by *Rhizomucor miehei* (LipozymeIM), using supercritical carbon dioxide as a reaction media. The authors reported that because of process limitations as well as the fact that CO_2 is a non polar solvent, dissolving preferentially hydrophobic molecules, solvents other than carbon dioxide have also been used as a reaction media (n-butane, n-propane, and a mixture n-propane/n-butane). In addition, the lipase activity in supercritical CO_2 media has been also investigated (T = 323 K, P = 30 MPa, τ = 4 – 24 hours), as well as using supercritical n-butane and a mixture of n-propane/n-butane (T = 318 K, P = 10 MPa, τ = 36 hours), showing that by those state conditions, the lipase activity has almost been unchanged.

Novak *et al* (2003), investigated the application of two lipases, *Candida rugosa* and *Porcine pancreas*, immobilized into aerogels of silica (sol-gel), as biocatalysts in the esterification reaction of fatty acids, using CO_2 and propane as reaction media at 313 K and 10 MPa. The reaction rate with propane has been increased approximately two to three times compared to those using free lipases, that is, non immobilized lipases. It was observed that use of CO_2 has shown an influence on the deactivation of non immobilized lipase, while for the immobilized enzyme, the conversion rate was 35 %. By using propane, the reaction rates have been increased twenty times compared to those carried out in aqueous media.

Srivastava *et al* (2003), investigated the esterification of myristic acid catalyzed by *Crude Hog Pancreas* (HPL), a commercial lipase. The bioreactions have been carried using supercritical CO_2 and acetronitrile as a reaction media as well as in solvent free basis. Conversions up to 76 % have been achieved in solvent free basis, while by using supercritical CO_2 and acetronitrile, conversions could only achieve 37 % and 4 % respectively.

In this work, the influence of state conditions and fatty acid/alcohol ratios on the esterification reaction catalyzed by immobilized lipase Lipozyme® RM IM (*Rhizomucor miehei*) using CO_2 as reaction media has been investigated to analyze the feasibility of the biotransformation process.

2. Materials and Methods

2.1 Materials

Carbon dioxide 99.95 % [vol./vol.] pure was supplied by Gáspará S/A, Belém-PA, Oleic Acid with a purity of 96.0 [wt.%] was supplied by Vetec Química Fina Ltda (Brazil), Absolute Ethanol with a purity of 99.5 [wt.%] was supplied by Merck (Germany), Potassium Hydroxide in granular form (ACS) of analytical grade was supplied by Merck (Germany), Sodium Hydroxide in granular form with a purity of 97.0 [wt.%] was supplied by Merck (Germany), and the immobilized enzyme *Rhizomucor miehei* was supplied (Donation) *by* Novozymes A/S (Bagsvaerd-Denmark).

2.1.1 Oleic Acid

The chemical structure of Oleic Acid is shown in Figure 1. This compound is mainly used for the impregnation of textiles, manufacture of esters, lubricants, cosmetics, salves, nutrition fat and feeding stuff additives. It can be also transformed into Stearic Acid by hydrogenation.

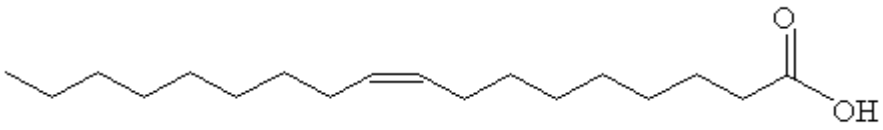

Figure 1: Chemical structure of Oleic Acid.

2.2 Esterification of Fatty Acids Catalyzed by Lipases

The reaction scheme below shows the general biochemical transformation of fatty acids into fatty acid esters catalyzed by lipases, as depicted by Figure 2.

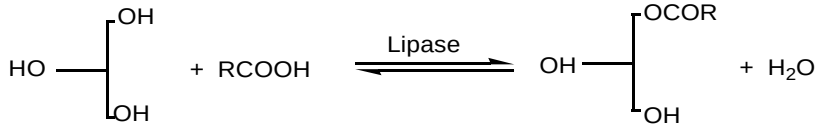

Figure 2: Reaction scheme of esterification reaction.

2.3 Experimental Set-up and Procedures

2.3.1 Supercritical Extraction Unit

A schematic diagram of the extraction unit available at the Faculty of Chemical Engineering at UFPA is depicted in Figure 3. The apparatus has been modified in order to carry out the reaction experiments. The experimental apparatus consists basically of a thermal stabilized extractor with 3500 cm³, a diaphragm-type compressor (Andreas Hofer, Germany), a sampling system of 20 cm³, a thermostatic bath (Haake Messtechnik GmbH, Germany), a CO_2 reservoir, and a control unit that displays the temperature and pressure inside of the extractor. For more details a complete description is found elsewhere in the literature[5].

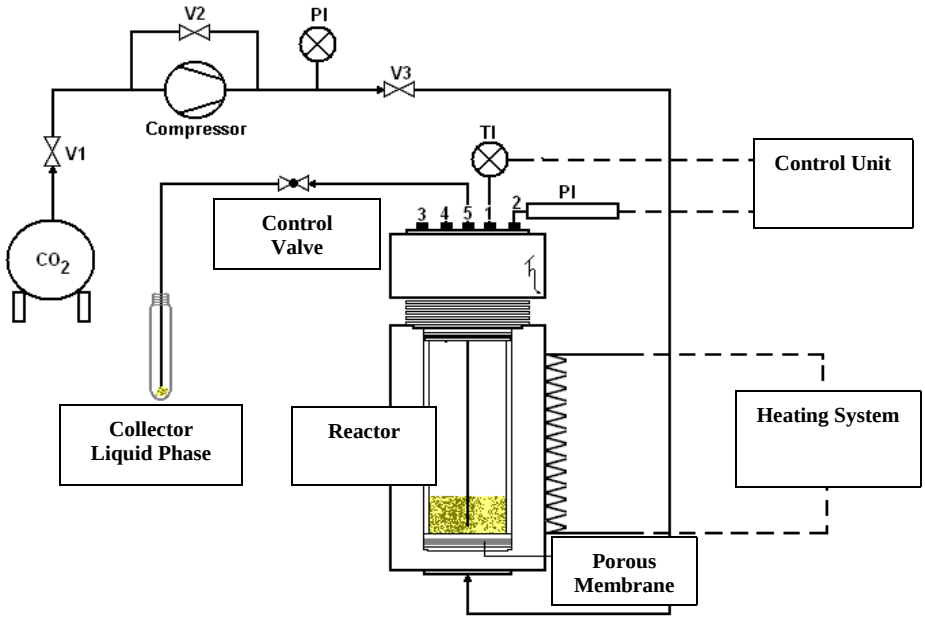

Figure 3: A schematic diagram of the experimental apparatus (reactor).

2.3.2 Experimental Procedures

The reagents (Oleic Acid and Ethanol) were introduced into the high pressure vessel (Reactor) in different fatty acid to alcohol molar ratios, as well as the immobilized lipase Lipozyme® RM IM (*Rhizomucor miehei*). Afterwards, the thermostatic bath was set-up to reach the desired temperature. Then, the reservoir valves were opened and the compressor started. CO_2 inside the reservoir at 6 MPa was compressed until the desired system pressure could be achieved inside the high pressure vessel. The supercritical CO_2 flows upwards through the fixed bed porous membrane and mixers the reagents inside the reactor. After the operating pressure and temperature were stabilized, the reaction process initializes. The samples from the liquid phase were collected by means of a capillary tube and a micro-metering valve in small glass tubes, as shown in Figure 4, in intervals of one hour for a total reaction time of five hours. For a gravimetric determination of the amount of collected liquid phase, the glass tubes were weighed after each time interval of one hour, and the mass of collected material was determined.

2.4 Chemical Analysis

The chemical analysis of fatty acid esters was determined by computing the Fatty Acid Index using the official method of AOCS[6] (Ca 5a-40, 1990) as follows, defined by equation (1):

$$I.A = \frac{(V_{KOH} * C_{KOH} * 56.1094)}{m_A} \quad (1)$$

Where V_{KOH} is the KOH volume in mL necessary to neutralize the fatty acids present in the liquid phase samples, C_{KOH} the molar concentration of KOH solution and m_A is the amount of weighted liquid phase samples in g. Using the chemical equation described in Figure 2, and knowing that 01 (one) mole of oleic acid is consumed to produce 01 (one) mole of ethyl ester of oleic acid, it is ease to compute the conversion rate of ethyloleate, given by equation (6), by integrating equation (3) from $\tau = 0$ to $\tau = \tau$, taking the reaction volume as constant, as follows:

$$-\frac{dC_{OleicAcid}(\tau)}{d\tau} = \frac{dC_{Ethyloleate}(\tau)}{d\tau} \quad (2)$$

$$-\frac{1}{V_{Reaction}}\frac{dn_{OleicAcid}(\tau)}{d\tau} = \frac{1}{V_{Reaction}}\frac{dn_{Ethyloleate}(\tau)}{d\tau} \quad (3)$$

$$n_{OleicAcid}(\tau = 0) - n_{OleicAcid}(\tau) = n_{Ethyloleate}(\tau) - n_{Ethyloleate}(\tau = 0) \quad (4)$$

$$n_{OleicAcid}(\tau = 0) * \chi_{OleicAcid} = n_{Ethyloleate}(\tau) \quad (5)$$

$$\chi_{OleicAcid} = \frac{I.A(\tau = 0) - I.A(\tau)}{I.A(\tau = 0)} = (1 - \frac{I.A(\tau)}{I.A\tau(\tau = 0)}) \quad (6)$$

Where $n_{Oleic\ Acid}$ and $n_{Ethyloleate}$ are the number of moles of oleic acid and ethyloleate, and $\chi_{Oleic\ Acid}$ is the conversion of oleic acid, and $\chi_{Ethyloleate}(\tau=0)$ is zero.

3. Results and Conclusions

The reaction experiments have been carried out in a high pressure reactor of 3.5 L, at pressures of 12 and 21.4 MPa, temperatures of 313 and 323 K, enzyme concentration of C_{Enzyme} = 0.0132 g.mL^{-1} and 0.0228 g.mL^{-1}, and substrate concentrations of $C_{Substrate}$ = 0.1325 g.mL^{-1} and 0.2282 g.mL^{-1}, as described in Table 1. The process feasibility has been investigated by analyzing the conversion of oleic acid into ethyloleate and the quality of the desired product (fatty acid esters). The operating conditions for all the reaction experiments carried out in the high pressure reactor are given in Table 1.

Table 1: Operating conditions for all the reaction experiments, reagents and enzyme quantities.

Experimental Conditions	Molar Ratios 1 : 5	Molar Ratios 1 : 2.5
T [K]	313, 323	313, 323
P [MPa]	12, 21.4	12 e 21.4
Oleic Acid [g]	43	43
Ethanol [mL]	272.2	136.1
Enzyme [g]	4.3	4.3
$C_{Oleic\ Acid}$ [g.mL^{-1}]	0.1325	0.2281
C_{Enzyme} [g.mL^{-1}]	0.0132	0.0228
C_{EtOH} [g.mL^{-1}]	0.6624	0.5702

* The concentrations of fatty acid, ethanol and enzyme in relation to reaction volume.

The experimental results obtained by computing the Fatty Acid Index (I.A) during the course of reaction were used to compute the rate of conversion of ethyloleate, and to analyze the influence of state conditions and fatty acid/alcohol molar ratios on the esterification reaction.

3.1 Influence of Pressure

The influence of system pressure on the esterification reaction at 313 K, and oleic acid/alcohol molar ratio of 1: 2.5 is shown in Figure 4.

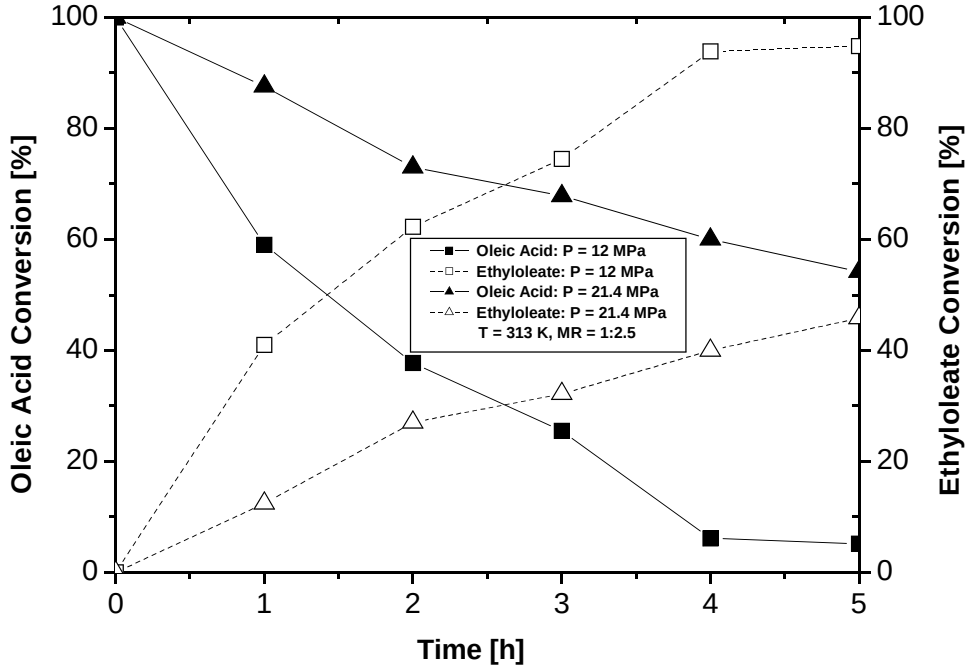

Figure 4: Course of esterification reaction of oleic acid in supercritical CO_2 [T = 313 K, MR = 1:2.5].

The experimental results show that the fatty acid ester conversion decreases as pressure increases. By increasing the system pressure maintaining all others variables unchanged, increases the mutual solubility's of carbon dioxide in the coexisting liquid and gaseous phases, thus providing theoretically a higher degree of mixing and hence a better contact between the reagents, at the same time an increase on system pressure causes a higher distribution of ethanol in the gaseous phase, thus decreasing the excess of ethanol to oleic acid in the liquid phase, witch is a process parameter of fundamental importance on the reaction rate, so this effect may prevail in comparison to those associated to the increase of system pressure described above. In this case conversions up to 94.83 % have been achieved by T = 313 K and P = 12 MPa, compared to a maximum conversion of 45.84 % achieved T = 313 K and P = 21.4 MPa, keeping the oleic acid/alcohol molar ratio of 1: 2.5 constant.

3.2 Influence of Temperature

The influence of temperature on the esterification reaction at 313 K, and oleic acid/alcohol molar ratio of 1: 2.5 is shown in Figure 5. The experimental results show that the fatty acid ester conversion decreases as temperature increases. By increasing the temperature keeping the pressure and oleic acid/alcohol molar ratios constant, decreases the carbon dioxide density, as a consequence, the mutual solubility's of carbon dioxide in the coexisting liquid and gaseous phases decreases, thus providing a worse degree of mixing and contact between the reagents, and hence a decreasing on the reaction rate. In this case conversions up to 94.83 %

have been achieved by T = 313 K and P = 12 MPa, compared to a maximum conversion of 61.56 % achieved T = 323 K and P = 12 MPa, keeping the oleic acid/alcohol molar ratio of 1: 2.5 constant.

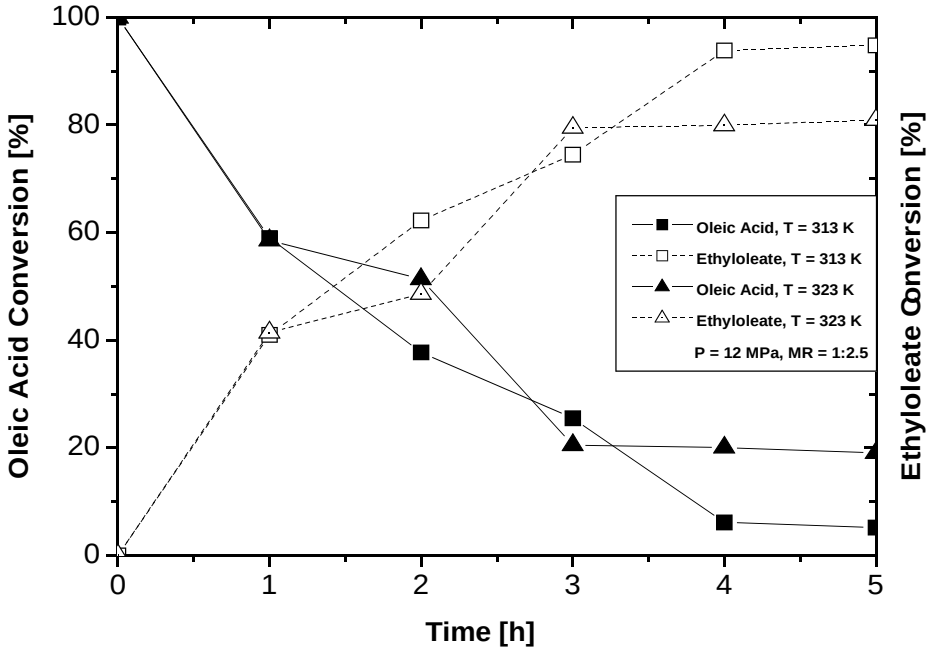

Figure 5: Course of esterification reaction of oleic acid in supercritical CO_2 [P = 12 MPa, MR = 1:2.5].

3.3 Influence of Fatty Acid/Alcohol Molar Ratios

The influence of oleic acid/alcohol molar ratios on the esterification reaction at 313 K and 12.0 MPa is shown in Figure 6.

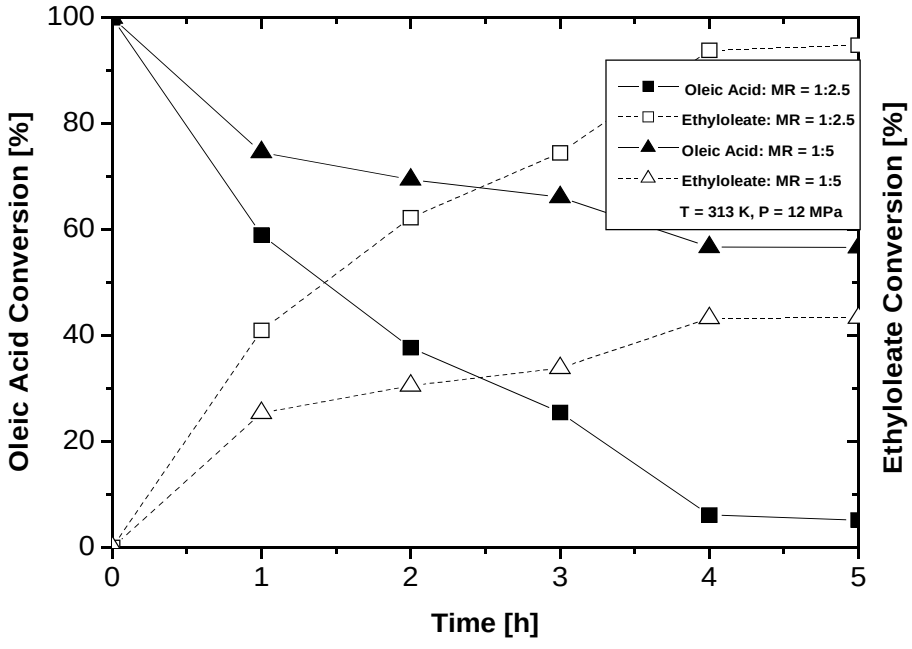

Figure 6: Course of esterification reaction of oleic acid in supercritical CO_2 [P = 12 MPa, T = 313 K].

The experimental results show that increasing the oleic acid/alcohol molar ratios decreases the ethyl ester conversion maintaining the system pressure and temperature constant. In principle, an increase of the ethanol excess in the liquid phase, witch is a process parameter of fundamental importance on the reaction rate, should increase the conversion of ethyl esters. On the other hand, an increase on oleic acid/alcohol molar ratios may cause a higher distribution of ethanol in the gaseous phase, thus decreasing the excess of ethanol to oleic acid in the liquid phase. In this case conversions up to 94.83 % have been achieved by an oleic acid/alcohol molar ratio of 1: 2.5, T = 313 K and P = 12 MPa, compared to maximum conversion of 45.34 % achieved by an oleic acid/alcohol molar ratio of 1: 5, T = 313 K and P = 12 MPa.

3.3 Conclusions

The use of immobilized lipases on the esterification reaction of oleic acid catalyzed by immobilized lipase Lipozyme® RM IM (*Rhizomucor miehei*) using carbon dioxide as supercritical media has shown high conversion rates. The highest ethyloleate conversion rate of 94.38 % was achieved by T = 313 K, P = 12.0 MPa, and oleic acid/alcohol molar ratio of 1: 2.5 after five hours of reaction. The experimental results show that state conditions, initial concentration and oleic acid/alcohol molar ratios have great influence on the reaction rate and conversion as well.

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