EPOXIDATION OF OLEIC ACID CATALYZED BY CANDIDA ANTARCTIC LIPASE IMMOBILIZED IN MICROEMULSION BASED ORGANOGELS

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ABSTRACT – The use of *Candida antarctica* lipase B immobilized in hydroxyl-propyl-methyl cellulose microemulsion-based organogels as media for the catalysis of oleic acid epoxidation with H_2O_2, has been tested. The optimization of several reaction conditions such as temperature, concentration of substrates and the enzyme content of the organogel have been examined. For this purpose an experimental design (central composite rotatable design) was applied. The results showed that the highest yields are observed for the lower temperatures, while the enzyme and oleic acid concentrations do not affect significantly the reaction yield. A yield of 84% was obtained within 48 hours of reaction, 35° C, 0.8 mg of enzyme/ml of microemulsion and 1.2 mL of H_2O_2. Moreover, the reusability of the catalyst has been verified.

1. INTRODUÇÃO

Epoxidation of olefinic compounds leads to the formation of commercially important precursors that are useful intermediates for various syntheses of high added value products *(*Lutz, 1980*)*. Epoxides are also used directly as plasticizers and plastic stabilizers. More specifically, epoxides of vegetable oils have been employed to manufacture adhesives, polymers and plastics such as polyvinyl chloride (PVC) and other related resins to improve flexibility, elasticity, toughness of the plastics and impart thermal and photo stability.

In laboratory scale, epoxidation is still usually achieved using organic peroxides and metal catalysts *(*Sharpless & Verhoeven, 1979*)* or peracids such as m-chloroperbenzoic acid *(*Vogel *et al.*, 1989*)*. In industrial scale, many of the epoxides produced are synthesized by using the chlorohydrin method or via in situ generated peracids (a method which demands the presence of a strong mineral acid) *(*Slayden *et al.*, 1993*)* or organic hydroperoxides and molybdenum or tungsten salts as catalytic additives *(*Pai *et al.*, 2009*)*. Besides the numerous safety issues that these methods present, the generation of large quantities of by-products and acidic waste make these approaches undesirable.

To overcome these problems, over the last two decades several authors have been oriented to

the use of a chemoenzymatic method for the oxidation of olefins, which is a more environmentally friendly alternative to the traditional processes. This method is based on the use of enzymes, and particularly lipases, for the formation of peracids (Piazza *et al.*, 2000; Carboni-Oerlemans *et al.*, 2006). The lipase catalyzes the formation of a peroxy acid from a corboxylyc acid and an oxidant. The formed peracid donates oxygen to the double bond producing an epoxide or oxirane and regenerating the carboxylic acid (Bjorkling *et al.*, 1990). Most of the chemoenzymatic epoxidation studies that have been published, report on the use of a commercially available preparation of *Candida antarctica* lipase (Novozym 435®) for the catalysis (Warwel & ger Klaas, 1995; Yadav & Devi, 2001; Kudanga *et al.*, 2010; Gitin *et al.* 2006; Skouridou *et al.*, 2003; Wiles *et al.*, 2009; Moreira *et al.*, 2005; Schneider *et al.*, 2009). The present study reports on the use of free *C. antarctica* lipase, that is immobilized on a matrix consisted of an organogel based on a natural polymer, namely hydroxyl-propyl-methyl cellulose (HPMC), and a water-in-oil (w/o) lecithin microemulsion containing the lipase and is being used for the first time as a catalyst of the epoxidation reaction.

Among low water media, water-in-oil (w/o) microemulsions have been widely reviewed as tool for achieving various enzymatic reactions in hydrophobic solvents (Stamatis *et al.*, 1999; Biasutti *et al.*, 2008; Pavlidis *et al.*, 2009). The medium consists of small aqueous nanodroplets dispersed in a non-polar organic phase stabilized by surfactants and short carbon chain alcohols. These thermodynamically stable liquid media with a large interfacial area provide an aqueous phase where hydrophilic enzymes can be hosted, an interface where surface-active enzymes can be anchored, and a non-polar organic phase where hydrophobic substrates or products are solubilized. (Stamatis *et al.*, 1999).

Interestingly, many w/o microemulsions can be "gelled" by the addition of a gelling agent such as cellulose derivatives, yielding a matrix suitable for enzyme immobilization. These organogels (MBGs) offer many of the advantages of the enzyme-containing microemulsions such as the ability to disperse the biocatalyst at a molecular level, enormous interface area and the ability to incorporate enzyme cofactor. Moreover, these organogels, to a great extent overcome the major problems that prevent the use of microemulsions in industrial processes as they offer easy catalyst separation, good storage stability and reusability as well as easy operation and effective use under mild conditions (Delimitsou *et al.*, 2002; Zoumpanioti *et al.*, 2006; Zoumpanioti *et al.*, 2010a; Zoumpanioti *et al.* 2010b).

In the study presented here, the use of *C. antarctica* lipase immobilized in HPMC-lecithin MBGs have been tested for the epoxidation of oleic acid and the reaction parameters have been optimized via experimental design.

2. MATERIALS AND METHODS

2.1. Materials

Candida antarctica lipase B (CaL) recombinant from *Aspergillus oryzae* was purchased from Sigma Aldrich, Germany. Lecithin containing 36% phosphatidylcholine and hydroxypropyl methyl

cellulose (HPMC) were purchased from Sigma Aldrich, Germany. Oleic acid, analytical grade, was acquired from Vetec, Brazil. Hydrogen peroxide was 35% w/w. Ethyl acetate, isooctane, n-dodecane, 2-propanol were of analytical grade and were obtained from Merck, Germany. HPLC-grade methanol was acquired from Lab-Chem, UK.

2.2. Preparation of microemulsion-based organogels (MBGs)

Lecithin microemulsions and HPMC organogels are prepared as described elsewhere (Delimitsou *et al.*, 2002). In a typical experiment, 1 mL of microemulsion containing 0.6 mg of enzyme was mixed with 1 g HPMC and 2 mL of distilled water at room temperature. The mixture was stirred until homogenous.

2.3. Epoxidation reactions

The reaction medium consisted of 5 mL of ethyl acetate containing predetermined amount of oleic acid and MBGs in a flash incubated using a incubator at the desired temperature and speed agitation 150 rpm. The reaction was initiated by adding 1.2 mL H_2O_2 (35% solution). At fixed time intervals aliquots were taken and analyzed by GC.

2.4. Reuse of the biocatalyst

In order to determine the stability of the lipase immobilized in HPMC organogels, the biocatalyst was reused in consecutive independent batches. Each batch reaction was continued for 80 min at 35°C. The total volume of the batch was 6.2 mL in each case. After each run, the organogels containing lipase were washed three times with 5 mL ethyl acetate and a new epoxidation reaction was started as described before.

2.5. Analytical methods

Conversions yield for the epoxidation reactions were monitored via GC analytical method. At fixed time intervals samples of 30 µL were withdrawn from the reaction solution and analyzed using HP-INNOWax capillary column (30 m x 0.32 mm i.d., 0.25 µm film thickness) mounted on a Hewlett Packard (HP) Model GC-6890C. Injector temperature, 270°C; FID temperature, 290°C; oven temperature increased from 150 to 260°C with a rate of 30°/min and then maintained constant for 12 min. N-dodecane was used as an internal standard.

3. RESULTS AND DISCUSSION

3.1. Reaction development

In order to assess the most important parameters that affect the yield of oleic acid epoxidation catalyzed by CaL B in lecithin-HPMC MBGs, the use of factorial design is proposed. It takes into account the three most important parameters which are temperature (T), enzyme quantity (E) and oleic acid (OA) concentration and vary in two levels. The advantage using central composite design

(CCD) is that it offers a central point, where three experiments are conducted in order to assess the experimental error *(*Jiju, 2003*)*. The three variables and their real and coded levels for the system evaluated are shown in Table 1.

Table 1 – Real and coded (+ superior level, 0 intermediate, - inferior level) values for the variables evaluated in the epoxidation reactions

Factors	-1	0	+1
Temperature (°C)	35	45	55
Amount of enzyme (mg)	0.4	0.6	0.8
Oleic acid concentration (mM)	50	75	100

Table 2 shows the 11 treatments considering the three variables and the conversion yield for each experiment. The conversion yield taken into consideration corresponds to 10 h of reaction as the conducted preliminary experiments showed that the conversion reaches a plateau at 10 h. In table 2, the first eight entries were used to determine the mathematical model and refer to statistical design. Entries 9-11 represent the triplicates of the central points for obtaining the experimental error. As shown, best results are obtained among entries 1-4 and entries 9-11.

Table 2 – Experimental design and oleic acid epoxidation conversion rates in different temperature (T), enzyme load (E) and oleic acid concentration (OA) for 10 h of reaction.

Entries	T	E	OA	Conversion (%)
1	-1	-1	-1	47
2	-1	+1	-1	53
3	-1	-1	+1	43
4	-1	+1	+1	53
5	+1	+1	+1	39
6	+1	-1	+1	35
7	+1	+1	-1	34
8	+1	-1	-1	33
9	0	0	0	48
10	0	0	0	46
11	0	0	0	51

The estimated effects and the p values are shown in Table 3. Variables with p-value < 0.05 are significant in the process. Enzyme load and oleic acid concentration present p values > 0.05 which means they do not affect significantly the conversion. It should be noted here that the enzyme load values of 0.4 - 0.8 mg used in the present study correspond to only 0.29 - 0.58% by weight of H_2O_2 and 0.2 - 0.9% by weight of oleic acid, which is considered to be low. Consequently, the lecithin-based HPMC organogel containing the lipase offers an advantage in industrial applications as it is a more economic procedure in terms of environmental effect and cost of the biocatalyst. More

specifically, the biocatalyst studied in the present work presents better results compared to the epoxy stearic acid production catalyzed by PSCI Amano Lipase. In that study, the enzyme load range by weight of oleic acid is 10-20% which is 100 times more than the enzyme load in the present study (Correa *et al.*, 2012).

Table 3 – Effect of parameters estimates of CCD 2^3 for epoxidation of oleic acid catalyzed by CaL B immobilized in HPMC MBGs.

Variables	Effect	p-value
Mean	43.69	0.0002
Temperature	-13.55	0.0130
Enzyme	5.55	0.0708
OA concentration	0.55	0.7582
Temperature x Enzyme	-2.65	0.2316
Temperature x OA concentration	2.65	0.2316
Enzyme x OA concentration	1.85	0.3576

As shown in Table 3, temperature had a negative effect (-13.55) within the chosen range of 35 to 55°C. By choosing the lowest T within the range studied, higher conversion yields are achieved. Namely, when temperature increases from 35 to 55°C and the other two variables (enzyme and OA concentration) are kept constant, the conversion yield decreases from 53% to 39% for the lowest values (0.4 mg of enzyme, 50 mM OA) and from 47% to 33% for the highest values (0.8 mg of enzyme, 100 mM OA), thus indicating the system's preference to lower temperatures.

The experimental data have been adjusted to the proposed model and adequacy was performed by the analysis of variance and parameter R^2 and statistical testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA). Equation (1) represents the mathematical model of the conversion of oleic acid to epoxy stearic acid catalyzed by CaL B in lecithin-HPMC MBGs employing statistical significance variables only.

$$Y = 43.69 - 6.77 \bullet T \quad (1)$$

where Y is the conversion yield and T is the uncoded value of temperature. Table 4 shows the variance analysis which depicts the validity of the model by F test and residue that shows the magnitude of experimental error. The calculated F (16.59) was higher than the tabulated F (5.12), showing the validity of the experimental model. The determination coefficient (R^2 = 0.65) implies that the sample variation of 65% for epoxide production is attributed to the independent variables and can be accurately explained by the model.

Table 4 – Variance analysis for validation of mathematical models (ANOVA)[a]

Factor	Sum of squares	Degrees of freedom	Mean square	F calculated	F tabulated
Regression	367.20	1	367.20	16.59	5.12
Residuals	199.20	9	22.13		
Lack of fit	189.46	7	-		
Pure error	9.74	2	-		
Total	566.41	10	-		

[a]confidence level 95%

3.2. Reuse of the catalyst

During the catalysis of the epoxidation reaction the biocatalyst is in contact with hydrogen peroxide that has a negative effect on the enzyme. Therefore, the operational stability (i.e. the ability to reuse) of the biocatalyst should be tested this is an important parameter determining the economic viability of the biocatalytic process.

For this purpose, the ability of the biocatalyst to retain its activity was tested by catalyzing the epoxidation of oleic acid with H_2O_2 for 7 consecutive times. As can be seen from Figure 1 the lipase activity after 4 uses is well preserved. The conversion yield reduced after 4 runs by only about 10%. However, after the 5th and 6th run the conversion yield appeared to be about 40% reduced and after the 7th run the conversion yield was 25% with respect to the first use.

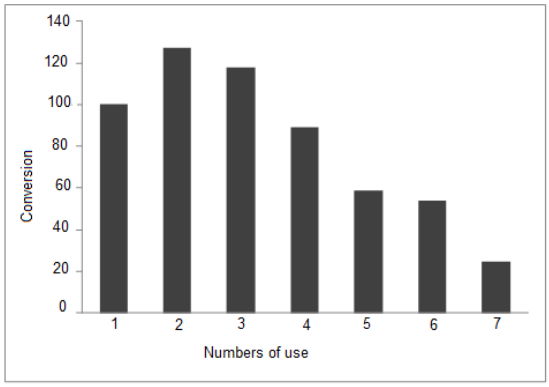

Figure 4 – Catalytic activity of CaL B immobilized in HPMC MBGs towards repeated synthesis of epoxy stearic acid.

It should be noted here that after the first use a hyperactivity of the enzyme can be observed. This was also observed for the same lipase immobilized in HPMC organogels used for the propyl laurate synthesis (Zoumpanioti *et al.*, 2006), but it was not observed for R. miehei lipase immobilized in HPMC organogels and used for the same esterification reaction (Delimitsou *et al.*, 2002).

4. CONCLUSIONS

The epoxidation of oleic acid with H_2O_2 has been achieved by chemoenzymatic catalysis using *C. antarctica* lipase immobilized in organogels based on a cellulose derivative (HPMC) and lecithin w/o microemulsion. The central composite design revealed that the enzyme load in the organogel and the oleic acid concentration do not affect significantly the reaction conversion. On the other hand, temperature strongly affects the conversion with the highest conversion yield appearing for the lowest temperature, namely 35°C. The biocatalyst was reused successfully for 6 runs.

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