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Epoxidation of oleic acid catalyzed by PSCI-Amano lipase optimized by experimental design

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ABSTRACT

The present work focuses on the oleic acid epoxide production by using PSCI Amano Lipase as biocatalyst in the reaction. An experimental design (central composite design – CCD) adopting surface response was applied to this purpose. Reactions were performed in a shaker equipment and different variables were investigated, such as temperature (25–55 °C), enzyme load (10–20 wt% of oleic acid mass), hydrogen peroxide load (0.1–0.2%) and reaction time. PSCI-Amano enzyme showed its best behavior as biocatalyst after 3 h of reaction at 55 °C, 10% enzyme load, 0.2% hydrogen peroxide and, applying 150 rpm as stirring. On these conditions, the epoxide yield was around 88%.

1. Introduction

Oleochemicals are hydrocarbons derived from vegetable oils closely related to petrochemicals and well suited for transformations by the chemical industry [1]. Vegetable oils and fatty acids can be used in cosmetics, lubricants, chemical additives, detergents, pharmaceuticals, polymers, and other products [2].

Vegetable oils and their unsaturated fatty acids can be converted into epoxies which are useful intermediates in organic synthesis by participating in many reactions due to the high oxirane ring reactivity [3]. Among their important applications there is the function as plasticizer for polyvinyl chloride (PVC). Plasticizers increase flexibility, workability or distensibility of plastics, hence rendering them suitable for diverse applications [4]. One of the most important plastics additives currently adopted is the epoxidized soybean oil (ESBO) which has a stable market of approximately 100,000 tons/year [5]. Global demand for plasticizers is projected to grow to 7.6 million tons per year until 2018. The main market is the Asia-Pacific region, with China holding on to its dominating position with 65% share [6].

The most used procedure to produce epoxidized fatty acid esters currently consists of two steps involving alcoholysis of triglycerides/fatty acids using KOH as catalyst followed by epoxidation of peroxyacetic acid esters or peroxyformic (Scheme 1) [7].

The formation of byproducts occurs due to the high medium acidity. In addition, corrosion and production of large amounts of salts when acids are neutralized are one of the problems associated with this type of reaction [4].

Chemo-enzymatic epoxidation reaction often provides a more selective and environment-friendly alternative to the Prilezhaev epoxidation process [8,9]. In the chemo-enzymatic epoxidation reaction, the enzyme normally catalyzes the peracid formation from the corresponding fatty acid and hydrogen peroxide [10]. Then the peracid spontaneously transfers oxygen to the double bond forming the epoxide (Scheme 2) [11].

Lipases or triacylglycerol hydrolases are an important group of biotechnologically relevant enzymes with immense applications in food, dairy, detergent and pharmaceutical industries. Lipases are also defined as glycerol ester hydrolases that catalyze the hydrolysis of triglycerides into free fatty acids and glycerol. They can also catalyze esterification, acidolysis, interesterification, alcoholysis and aminolysis in addition to the hydrolytic activity on triglycerides. Lipases are produced from microbes, specifically bacteria and, they play a vital role in commercial ventures [12,13]. They represent a broadly employed renewable biocatalyst in lipids transformation.Therefore, this work aims at performing the epoxidation of oleic acid using lipase as biocatalyst and the Central composite

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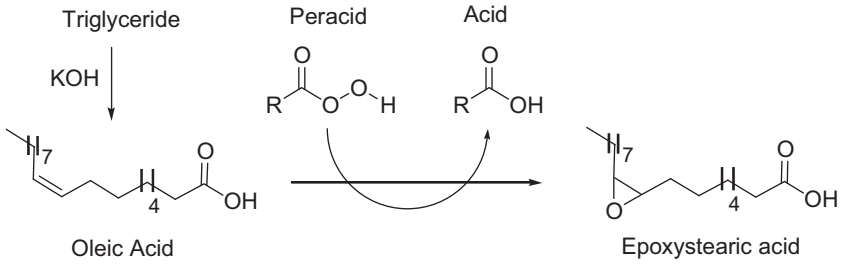


Scheme 1. Schematic presentation of alcoholysis of triglyceride using KOH as catalyst followed by epoxidation of oleic acid by peracid.

design (CCD) in conjunction with Response Surface Methodology to identify important variables to the process. The Amano PS-CI was for the first time used to catalyze the epoxidation reaction by using aqueous solution of hydrogen peroxide as oxidant.

2. Experimental

2.1. Lipase and materials

The enzyme used in the present study was the PSCI-Amano Lipase from *Burkholderia cepacia* immobilized on ceramic and purchased from Sigma–Aldrich. Hydrogen peroxide (percentage given as 30% H_2O_2 (w/w) in water) and all chemicals (ethyl acetate, oleic acid, and N-methyl-N-(trimethylsilyl)trifluoroacetamide) were of analytical grade.

2.2. Enzymatic epoxidation reaction

Oleic acid (2 mmol) was dissolved in ethyl acetate (5 ml) and selected quantities of H_2O_2 were further added. The reaction was initiated with the addition of immobilized PSCI-Amano lipase from *B. cepacia*. Reactions were performed on orbital shaker (MARCONI) at 150 rpm with controlled internal temperatures for 3 h. Subsequently, the immobilized biocatalysts were removed by filtration and the solvent was distilled using a rotary evaporator. The reaction products were analyzed by gas chromatography coupled to mass spectroscopic detector (GC–MS) and calculated for its iodine value (item 2.4). We have also performed the experiment by using the ethyl oleate (~85% purity) as substrate in both, the best and worst condition found for oleic acid, in order to corroborate the mechanism proposed (Scheme 1).

2.3. Analytical procedure

2.3.1. GC–MS analysis

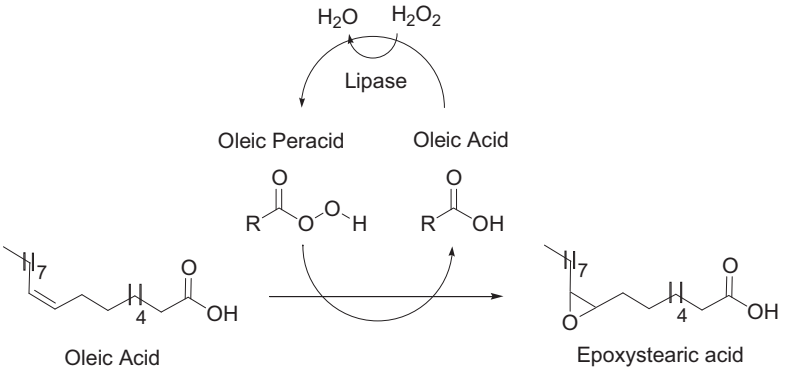
The GC–MS analyses were carried out on a Shimadzu CG-MS2010. Chromatographic separation was carried out on fused-silica capillary columns RTx-5MS (30 m × 0.25 mm × 0.25 μm) from Restek Corporation (USA). The column temperature was 60 °C for 1 min and then increased to 280 °C by 15 °C/min, and maintained for 10 min. The carrier gas used was helium (He) and the flow was 1.1 ml/min. The injection temperature was set to 250 °C and the split ratio was 20. The ion source and interface temperature were 250 and 300 °C, respectively. Free fatty acids were transformed into more volatile silylated derivatives in the presence of pyridine and N-methyl-N-(trimethysilyl)trifluoroacetamide (MSTFA). GC–MS samples were prepared by addition of 100 μL MSTFA at 100 μL of reaction. After 15 min, these reactants were dissolved on 2 ml ethyl acetate. 1 μL of this sample was then injected into a GC–MS equipment.

2.4. Iodine value (IV)

Iodine value (IV) was obtained using the Wij's method [14]. Aliquots of the samples (1.0 g) were weighed directly into the Erlenmeyer and dissolved in solvent mixture (cyclohexane and glacial acetic acid 1:1). Wij's reagent (25 mL) was added and the flask was capped, mixed thoroughly and kept in the dark for 1 h. At the end of reaction time, 20 ml of 10% KI and 150 ml of distilled water were added. The blank was prepared simultaneously on the analysis. The mixture was titrated with 0.1 M sodium thiosulfate solution until the yellow color almost disappears. A few drops of 0.5% starch solution were added and titration continued until the blue color disappears after vigorous shaking. The volume of sodium thiosulfate solution spent in the titration was used to calculate the iodine value of samples. The result was calculated using the following expression:

$$\text{Iodine value (IV)} = \frac{12.69 \times C \times (V_1 - V_2)}{m}$$

where C is the concentration, in mol/l, of sodium thiosulfate solution standard; V_1 is the volume, in mL, of sodium thiosulphate used in titration of blank; V_2 is the volume, in mL, solution of sodium



Scheme 2. Schematic presentation of the chemo-enzymatic epoxidation of oleic acid.

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)	25	40	55
Amount of enzyme (%)[a]	10	15	20
Peroxide concentration (%)	0.1	0.15	0.2

[a] By weight of oleic acid.

thiosulfate titration of the sample spent; *m* is the mass, in g, of the sample aliquot.

3. Results and discussion

The reaction time is not generally considered as a variable in the case of experimental designs since it causes the greatest influence on the system and on other variables, mistaking eventual responses. However, we recognize that it is very significant for final conversion rates. Based on that, we followed the literature data to select the reaction time and fixed that on 3 h [15,16].

The central composite design (CCD) consisting of three variables and varying in two levels was used to identify the important variables for the epoxidation reaction of oleic acid catalyzed by immobilized PSCI-Amano lipase from *B. cepacia*. Temperature, amount of enzyme, and peroxide concentration were considered critical variables (independent) and therefore assessed in planning [17,18]. The CCD 2^3 was applied with triplicate central points for calculating the experimental error. The three variables and their real and coded levels for the enzyme evaluated are shown in Table 1.

3.1. Central composite design

The experimental designs and data analysis were carried out using the software Statistica 6.0 (Statsoft Inc., USA), according to the significance level established for obtaining the mathematical model. The variance explained by the model is given by the multiple determination coefficients, R^2. The significance of regression coefficients and associated probabilities, $p(t)$, were determined by the Student's *t*-test; the model equation significance was determined by the Fisher's *F*-test.

Table 2 shows the 11 treatments considering the three variables and the percentage yield conversion for each experiment. The first eight treatments were used to determine the mathematical model and refer to statistical design. Treatments 9–11 represent the triplicates of the central points for obtaining the experimental error.

Table 2
Experimental design and oleic acid epoxidation conversion rates in different temperature, enzyme load and hydrogen peroxide load.

Entries[a]	Reaction temperature (°C)	Enzyme load (%)[b]	H_2O_2 (%)[c]	Conversion (%)[d]
1	−1	−1	−1	37
2	+1	−1	−1	34
3	−1	+1	−1	34
4	+1	+1	−1	50
5	−1	−1	+1	72
6	+1	−1	+1	88
7	−1	+1	+1	61
8	+1	+1	+1	83
9	0	0	0	59
10	0	0	0	59
11	0	0	0	61

[a] Numbers were run in random order.
[b] Enzyme load (%, relative to the weight of oleic acid).
[c] Hydrogen peroxide load (%, relative to the mol).
[d] Analyzed by GC–MS.

Table 3
Effect of parameters estimates of CCD 2^3 for enzyme studied.

Variables	Effect	*p*-Value
Mean	58.00	<0.0001*
Temperature	12.75	0.0004*
Amount of enzyme	−0.75	0.4552
Peroxide concentration	37.25	0.0004*
Temperature × Amount of enzyme	6.25	0.0166*
Temperature × Peroxide concentration	6.25	0.0166*
Amount of enzyme × Peroxide concentration	−7.25	0.0124*

* Statistically significant at 95% of confidence level.

It can be checked in Table 2 that the best results are among entries 5 and 8, turning evident the positive effect of the hydrogen peroxide concentration variable in the epoxide conversion. This positive effect occurs within the analyzed range 0.1–0.2%. It is important to highlight that previous tests performed in our laboratory showed that peroxide concentrations upper to that inhibited the activity of the immobilized *B. cepacia* lipase. Several studies have already reported the occurrence of this increased effect on epoxide conversion with increasing concentration of hydrogen peroxide in the reaction medium [19,20]. H_2O_2 has been reported to oxidize methionine residues of various enzymes and, residues at the surface of the protein seem to be most susceptible. The oxidation of surface residues may not affect the enzyme activity but side-chain to side-chain transfer reactions were already described, transferring the initial site of oxidation to readily oxidized amino acids [21].

Table 3 shows the estimative of the effects for the experimental design 2^3 and *p* values. Variables with $p < 0.05$ were significant in the process. Only the variable concerned to the amount of enzyme was not significant in the reaction within the range studied (10–20%), once it has presented a $p > 0.05$ (0.4552). Added to that, the lowest lipase concentration (10%) did not harm the reaction rate once the best result could be obtained at this percentage (entry 6, Table 2). Focusing on the industrial applicability, this result is considerable relevant once it implies directly in operating costs due to the smaller amount of biocatalyst required.

The variable temperature promoted positive effect (12.75, Table 3). By increasing the temperature the product formation rate is also enhanced within the range studied. It is corroborated by entries 6 and 8 (Table 2) which achieved the highest conversions (88 and 83, respectively). However, the continuous increase in temperature, there may be a gradual inactivation of the enzyme to total inactivation, caused by protein denaturation by heat [22].

The experimental data have been adjusted to the proposed model and its adequacy was performed by the analysis of variance and parameter R^2. Eq. (1) represents the mathematical model of epoxystearic acid conversion depending on the variables.

$$Y = 58.00 + 18.62H_2O_2 + 6.37T - 3.62E \times H_2O_2 + 3.12T \times E + 3.12T \times H_2O_2 \quad (1)$$

where *Y* is the percentage yield conversion, *T*, *E* and H_2O_2 are the coded values of temperature, amount of enzyme and peroxide concentration, respectively. Statistical testing of the model was performed by the Fisher's statistical test for ANOVA (Table 4).

Table 4 of analysis of variance (ANOVA) shows the model validity by *F* test and the residue shows the magnitude of experimental error. The *F* calculated (92.41) was higher than the *F* tabulated ($F_{5,5} = 5.05$), showing the experimental model validity. The goodness of a model can be checked by the determination coefficient (R^2). The determination coefficient ($R^2 = 0.98$) implies that the 98% sample variation for epoxystearic acid 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).*

Factor	Sum of squares	Degrees of freedom	Mean square	*F* calculated	*F* tabulated	*p*-Value
Regression	3361.63	5	672.32	92.41	5.05	$6.37E^{-5}$
Residuals	36.38	5	7.27	–	–	–
Lack of fit	33.70	3	–	–	–	–
Pure error	2.66	2	–	–	–	–
Total	3398.0	10	–	–	–	–

* Confidence level 95%.

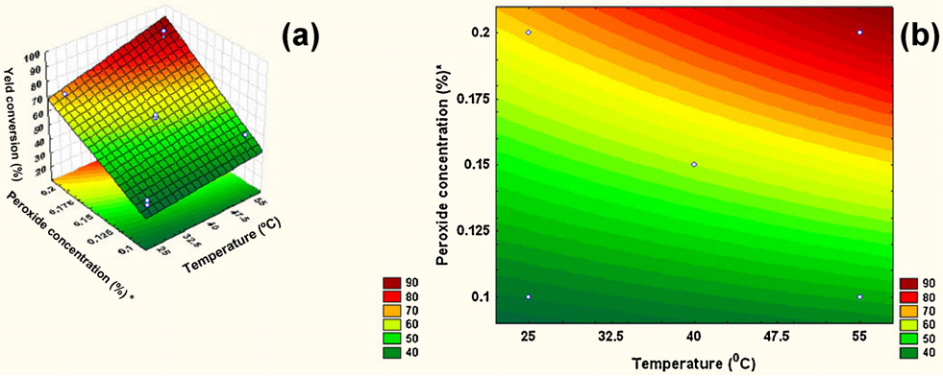


Fig. 1. (a and b) Response surface (a) and counter curve (b) for the epoxidation reaction catalyzed by the immobilized *Burkholderia cepacia* lipase. IM in function of the temperature and peroxide concentration.

Fig. 1a and b shows that increasing the concentration of hydrogen peroxide and increasing the temperature results in optimal response, around 90%. Above the rated temperature (55 °C) it is suggested that it can lead to enzyme denaturation. Likewise, increasing the peroxide concentration above 0.2% the lipase activity seems to be inhibited.

The interaction between reaction temperature and peroxide concentration was evaluated by Sun et al. [17]. It was observed that the combination of high substrate content and mild temperatures (35–50 °C) led to increased formation of epoxides (>4.5%). However, once the substrate ratio is decreased and temperature increased, the formation of epoxide does not increase. This is probably due to the lack of enough H_2O_2 to perform the epoxidation of C=C bond [17].

We finally analyzed the conversion of ethyl oleate epoxide considering the best (55 °C; 3 h; 10% *E*; 0.2 H_2O_2) and the worst variables (25 °C; 3 h; 10% *E*; 0.1 H_2O_2) proposed by the mathematic model for oleic acid peroxide. At these two different conditions the ethyl oleate epoxide was detected, but merely in minor amounts (≤10%), which can be justified by the impurity of the substrate.

Table 5
Calculated iodine values for the reaction products.

Entry	[a]IV	[b]IV(*x*)	Conversion (%)
1	3.52	0.30	37
2	3.03	0.40	34
3	2.90	0.42	34
4	2.43	0.52	50
5	1.50	0.70	72
6	0.62	0.88	88
7	1.85	0.63	61
8	0.74	0.85	83
9	2.39	0.53	59
10	2.10	0.58	59
11	2.00	0.60	61

[a] IV: Iodine value by the Wij's method (EN 14111:2003).
[b] $IV(x) = [(IV_o - IV)/IV_o]$; IV_o: initial iodine value.

3.2. Iodine values

The double bound conversion, as related to the iodine values, correspondent to the entries 1–11 are given in Table 5. As can be seen, the iodine value (IV) is in accordance with the conversion obtained, once it has shown smaller values in reactions which furnished higher product conversions (entries 6 and 8). Contrary, the entries 1–3 presented the minor conversion rates, and, hence higher iodine values (IV) were attributed. The iodine values as related to the initial IV (IV_o) are also listed below in Table 5.

4. Conclusion

We worked on different parameters which found to be important for the PSCI Amano lipase activity. The enzyme showed its best behavior as biocatalyst for epoxidation reaction after 3 h at 55 °C, 10% enzyme load, 0.2% hydrogen peroxide and, applying 150 rpm as stirring. On these conditions, the epoxide yield was around 88%. An experimental design (central composite design – CCD) adopting surface response was applied to measure the variables interference and, finally, the mathematical model was satisfactorily validated. We could also demonstrate by the ethyl oleate experiment that, on the conditions adopted, the carboxyl group seems to be exhibiting a very important role for the obtainment of high epoxide conversion rates.

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