Solvent Screening, Optimization and Kinetic Parameters of The Biocatalytic Epoxidation Reaction of Beta-Pinene Mediated by Novozym®435

Gustavo Dos Santos Martins
Federal University of Rio de Janeiro: Universidade Federal do Rio de Janeiro
Amanda Staudt
Federal University of Rio de Janeiro: Universidade Federal do Rio de Janeiro
Felipe Korbus Sutilli
Universidade Estadual Paulista Julio de Mesquita Filho - Campus de Botucatu
Camila Adão Rodrigues Malafaia
Federal University of Rio de Janeiro: Universidade Federal do Rio de Janeiro
Ivana Correa Ramos Leal (ivanafarma@yahoo.com.br)
Universidade Federal do Rio de Janeiro https://orcid.org/0000-0002-6917-9323

Research Article

Keywords: biocatalysis, beta-pinene, epoxidation, experimental design, lipase, Novozym 435.

Posted Date: January 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1218030/v1

Abstract

Monoterpenes are secondary metabolites widely used in the flavors and fragrance industries and can have their structure altered to enhance their applicability, such as producing epoxides, which are used as synthetic blocks for pharmaceuticals. Epoxides are commonly synthesized by the use of inorganic acids as catalysts, although the acid medium induces epoxide degradation. To overcome these limitations biocatalysis is shown as an alternative, in view that lipases can perform the reaction in a non-acidic medium. Related to, this work aimed to perform the synthesis of beta-Pinene epoxide using *Pseudozyma antarctica* lipase B (Novozym®435) as biocatalyst and to determine the independent variables that influence the reaction using experimental design tools. Different solvent systems were evaluated for until 72 h, in reactions with molar ratio of 2:2:1 (beta-Pinene, octanoic acid, and urea-hydrogen peroxide - UHP) at 40°C, 250 rpm, and 10%(w/v) of the biocatalyst. Ethyl acetate showed higher conversion (40% in 24 h) into the product without the formation of by-products. The atom economy (AE) was determined using metrics of green chemistry and ethyl acetate proved to have a higher atom economy (67.8%), while the other solvents that used octanoic acid as an acyl donor had 41.3%. In the following reactions, ethyl acetate was maintained as the solvent, while the temperature, molar ratio, and the percentage of the biocatalyst were varied. The increase in the molar ratio (beta-Pinene:UHP, 1:1) and percentage of biocatalyst (20%w/v) resulted in 80% of the product after 3 hof reaction at 40°C. To evaluate the impact of each independent variable, an FFD was performed by varying temperature, molar ratio, stirring, and percentage of enzyme, in one level. All variables were statistically significant, with different rates of impact. Due to this, the same variables were maintained on the CCRD, varying in two levels. The conversion ranged from good to excellent (32 - 93%). The independent variables that influenced the direction were temperature > stirring > molar ratio. In conclusion, the combination of two different tools of experimental design provided the development of an optimized model for beta-Pinene epoxidation, achieving high yields within 3 h.

1. Introduction

Monoterpenes belong to a class of natural products chemically generated by the coupling of two structural units containing five carbons atoms called isoprenes (Guimarães et al., 2013; Ruzicka, 1953). These molecules, originated from the plant secondary metabolism, have an important role due to their functions of defense, insecticidal and antimicrobial activity, communication, and production of hormones (Dambolena et al., 2016; Brahmkshatriya and Brahmkshatriya, 2013). In addition, recent studies have demonstrated the therapeutic activity (Mohamed et al., 2019; Dammak et al., 2019; Kang et al., 2016) and industrial applications of several monoterpenes as substrates for the synthesis of fragrances, flavors, and medicines (Juergens, 2014; Koziol et al., 2014). A strategy that has been used to increase the reactivity of these compounds is conducting the synthesis of their epoxides derivatives, which are highly reactive due to the oxirane ring inserted in the structure. The high reactivity of epoxides makes them susceptible to functionalization in reactions with several nucleophiles or undergoing oxidation, being able

to synthesize compounds as amino alcohols, diols, esters, and carbonyl compounds (Da Silva and Nascimento, 2012).

Epoxides are commonly chemically produced using strong oxidizing agents, such as *meta*-chloroperbenzoic acid (m-CPBA), or from the production of peracid *in situ*, from the reaction of hydrogen peroxide with organic acids, catalyzed by inorganic acids (Goud et al., 2006). Chemical epoxidation, in comparison to the enzymatic, has the limitation of leading to an epoxide degradation caused by the excessively acidic reaction media, favoring the formation of by-products (Sánchez-Velandia et al., 2019). To overcome these limitations the use of enzymes as catalysts is shown as a successful alternative.

It is known that several classes of enzymes are able to catalyze epoxidation reactions, such as monooxygenases of the P-450 family, and chloroperoxidase (Colonna, 1993). However, these enzymes have limitations in terms of industrial application, as they require specific cofactors and/or cannot maintain catalytic activity in organic solvents. One way to subdue these conditions is to apply catalysts such as the lipases that are included in the group of triacylglycerol hydrolases enzymes (EC 3.1.1.3). Lipases present a wide range of advantages, including: the use of hydrogen peroxide as precursor for the epoxide production; do not request cofactor; present high catalytic activity at mild temperatures (Bornscheuer, 2006); are stable in organic solvents (Tang et al., 2016); and, when immobilized, they have the possibility to be reused for several cycles (Moreira and Nascimento, 2007). In addition, they present high stability and are able to recognize a high broad of substrates, being used for different reaction process, including the epoxidation of different chemical class molecules.

A breakthrough in the biocatalytic synthesis of epoxides was reported by Björkling et al. (1990), in which an immobilized lipase was applied to catalyze perhydrolysis, originating the peracid *in situ* from the reaction of carboxylic acid and hydrogen peroxide. The peroxycarboxylic acid produced reacts with an alkene, leading the corresponding epoxide. An example of a perhydrolysis reaction involving beta-pinene as substrate is shown in Figure 1. In this biocatalytic type-process the peracid produced instantly reacts with the alkene, avoiding the excessively acidic environment (Goud et al., 2006).

Based on this, this work had the aim to perform the epoxidation of beta-pinene using the immobilized *Pseudozyma (Candida) antarctica* lipase B (Novozym®435 - N435) as biocatalyst, as well as to determine the ideal solvent and the independent variables that cause influence in the reaction model, employing tools of experimental design, such as temperature, stirring, molar ratio between the substrates (monoterpene: octanoic acid: urea hydrogen peroxide), and the percentage of the biocatalyst. Also, it was determined the kinetic parameters of the epoxidation reaction, such as Km and Vmax.

2. Methodology

2.1 Biocatalytic epoxidation of beta-Pinene

2.1.1. *Solvent screening*

Epoxidation reactions were set at at 40°C and 250 rpm, in 4 mL glass vials from a mixture of 2 mmol of beta-Pinene, 2 mmol of octanoic acid acyl donnor, 1 mmol of urea-hydrogen peroxide complex (UHP), 4Å molecular sieves, and 10% (w/v) of the enzyme Novozym®435 (activity of 1422 U/mL) in relation to the mass of monoterpene and octanoic acid, in 3 mL of solvent (cyclohexane, dichloromethane, acetonitrile or ethyl acetate). In the reactions conducted in ethyl acetate, octanoic acid was not added, in view that this solvent acts as an acyl donor, as described by Ankudey et al. (2006). The reactions were performed up to 72 h and aliquots (50 µL) were collected in duplicate.

2.1.2. *Variation of temperature, substrate molar ratio, and enzyme amount*

Sequential reactions with the selected solvent were performed in order to evaluate the influence of other variables into the conversion for the epoxide product. Reactions were conducted in a 4 mL glass vial using 3 mL of ethyl acetate as solvent/acyl donor. The molar ratio of beta-Pinene: UHP was changed to 1:1 (2 mmol of each reactant); the amount of N435 shifted in the range of 10-20% (w/v), and the temperature was evaluated in the range of 40-60°C. The other parameters were kept constant and the reactions were performed until 6 h.

2.2. The atom economy (AE) calculation

The atom economy (AE) was determined using metrics of green chemistry to define the conversion efficiency of the epoxidation process concerning the atoms involved and the epoxide produced using different solvents. Atom economy is calculated by dividing the product's molecular weight by the sum of the molecular weights of starting materials (Equation S1) (Sheldon, 2018). Nevertheless, this metric does not consider the solvent in the equation; for this, the E factor should be used since it considers all the reactants used in the process, including the solvents and co-reactants (Equation S2).

2.3 Fractional Factorial Design (FFD)

To evaluate the impact of the variables in the epoxidation reaction, it was performed a fractional factorial experimental design. There were evaluated four variables (molar ratio β-pinene: UHP, temperature, percentage of biocatalyst, and stirring) in two levels, as shown in Table 1. From the FFD, 11 experiments were perfo[rmed (Table S1) setting 3 h of reaction, including the triplicate of the central point.

Table 1
FFD experimental factors (+1: high level, 0: intermediate, −1: lower level) of the independent variables (2^{4-1}).

Independent variables	-1	0	+1
Stirring (rpm)	150	200	250
Temperature (°C)	40	50	60
Molar ratio (beta-Pinene: UHP)	1:1	1:2	1:3
Enzyme (%)	10	20	30

2.4 Central Composite Rotatable Design (CCRD)

The results obtained by FFD indicated the impact of each independent variable, serving as background to perform a further Central Composite Rotatable Design (CCRD). In this step, the four independent variables were maintained and varied in two levels (-2, -1, 0, +1, +2), as shown in Table 2. From CCRD, there were performed 27 experiments (Table S2) with triplicate of the central point. All reactions were conducted by 3 hours.

Table 2
CCRD experimental factors (+: higher level, 0: intermediate, −: lower level) of the independent variables (2^4).

Independent variables	-2	-1	0	+1	+2
Stirring (rpm)	200	225	250	275	300
Temperature (°C)	40	45	50	55	60
Molar ratio (beta-Pinene: UHP)	1:2	1:2,5	1:3	1:3,5	1:4
Enzyme (%)	5	10	15	20	25

2.5 Kinetic parameters

kinetic parameters (Km and Vmax) of the epoxidation reaction were established by setting the following conditions: 20% (w/v) of N435 in relation to the mass of beta-Pinene, 2 mmol of beta-Pinene, 3 mL of ethyl acetate, while the UHP concentration was evaluated in the range of 0.25 to 4 mmol. The reactions were maintained for 3 hours at 40°C and 250 rpm.

2.6 Analytical procedure by gas-chromatography coupled to mass spectrometry detector (GC–MS)

The epoxide conversion was determined by GC-MS (GCMS-QP2010 SE, Shimadzu) equipped with a Quadrex capillary column (DB-5MS) (5% diphenyl dimethylsiloxane) capillary column (30 m x 0.25 mm, 0.25 µm film thickness, Agilent Technologies). Aliquots (50 µL) collected from the reaction media were

diluted in ethyl acetate HPLC grade (450 µL) (1: 9) previous to analysis. The injector temperature was set at 250°C, with helium (99.999%) as carrier gas at flow rate of 1 $mL.min^{-1}$, and injection of 1 µL of sample, at a split ratio of 30. The column oven temperature program was set at 60°C and increased up to 260°C at a ratio of $10°C.min^{-1}$, being held constant for additional 1 min, resulting in a total 21 min method. The equipment was operated by electron impact ionization mode at 70 eV and scanned at the range of 40 to 600 m/z. The products were identified comparing the obtained mass spectrum and the *National Institute of Standard and Technology (NIST)* - version 05 - library.

2.7 Statistical analysis

The experimental designs and data processing were carried out using the software Statistica 10.0 (Statsoft, Inc., USA) according to the significance level established to obtain the mathematical model. The significance of the regression coefficients and the associated probabilities, p(t), were determined by Student's t test; the model equation significance was determined by Fisher's F test. The variance explained by the model is given by the multiple determination coefficients, R2.

3. Results And Discussion

Four solvents with different polarities were tested to determine the ideal system to conduct to beta-Pinene epoxidation (item 2.1.1.), that were: ethyl acetate, cyclohexane, toluene, and acetonitrile. Reactions conducted in acetonitrile, dichloromethane, and hexane, did not show the conversion to the desired epoxide product, under the conditions studied (*data not shown*). This can be explained, among other, by the need for long-chain fatty acids (C_8-C_{12}) as perhydrolysis substrate or, grater amount of reagents and possibly more time-consuming reaction. Anyway, it is important to highlight that large quantity of acids in the reaction medium can generate a biphasic system, limiting the access of the substrate to the enzyme active site (Daniel, 2014).

Interestingly, within 24 hours of reaction using ethyl acetate as solvent, it was observed a conversion of 40% to the epoxide product (Spiro[biciclo[3.1.1]heptano-2,2'-oxirano). In grater reaction times, it was observed the degradation of the epoxide and the formation of several by-products, such as ketone, enol, and diol. It is also worth mentioning that no product formation was detected in the absence of the biocatalyst regardless of the solvent system employed, ruling out the possibility of spontaneous reaction.

An advantage of using ethyl acetate as solvent is that it acts as an acyl donor to the perhydrolysis, being unnecessary the use of other acids and avoiding the high acidity of the reaction medium (Ankudey et al., 2006). In addition, it avoids the degradation of the epoxide caused by the acidic medium (MEYER-WAßEWITZ et al., 2017). Besides, this capacity makes the method more environmentally friendly and economically viable, in view that it is a low-cost reagent and easy to remove by evaporation in further purification steps.

The atom economy (AE) was determined to define the efficiency of the epoxidation process concerning the atoms involved and the epoxide produced using different solvents (reactions with or without octanoic

acid). From this, the reaction using ethyl acetate proved to furnish a higher atom economy (67.8%) compared to the other that used solvents and octanoic acid as acyl donor (41.3%) (Table S3). In an eco-friendly reaction, the E factor must be close to zero or as close as possible, which means no negative environmental impact. On the other hand, the use of solvents increases the E-factor, whereas this represents part of the amount of waste and contamination generated in synthetic reactions. For this reason, the use of ethyl acetate as solvent and acyl donor reduces the use of reactants, decreasing the environmental impact as shown by the reduced E-factor.

As ethyl acetate proved to be the most promising solvent, this solvent/acyl donor was employed in the subsequent steps. To investigate the influence of the reaction variables on the conversion to the epoxide, a new reaction was performed with an increase on the molar ratio from 1 mmol to 2 mmol (beta-Pinene: UHP) (1:1) and temperature ranging from 40 to 60°C. In addition, the reaction time has been reduced to up 6 h in order to avoid product degradation, since no higher conversions were observed after this time.

Among the temperatures investigated, 40°C showed the highest conversion (Figure 2). The increase in the molar ratio resulted in a conversion of 66% to the product of interest within 3 hours of reaction. At 50 and 60°C, there were obtained 63 and 51% product conversions, respectively, which indicates that the increase in temperature led to lower conversion to the beta-Pinene epoxide. However, it was discovered that there is no significant statistical difference among the temperatures tested (Tables S4 and S5). In order to promote energy economy and avoid product and/or substrate degradation, further experiments were performed at the reduced temperature employed, 40°C.

It is known that N435 presents high activity in the range of 30 to 80°C and higher temperatures are generally related to an increase in catalytic activity, but, for sure, it strictly depends on the reaction model (Bornscheuer, 2006). In this study, the increase in temperature resulted in less conversion to the product of interest within the conditions employed probably due to the degradation of the main product. It was realized that temperatures above or equal to 50°C seems to accelerate the degradation of hydrogen peroxide (Aouf et al., 2014), leading to water formation, which acts as a nucleophile for the β-pinene epoxide hydrolysis, and hence, for by-products formation (Skuridou *et al.*, 2003).

Several studies have already demonstrated that, within certain concentration limits, the increase in the hydrogen peroxide percentage the conversion to the product raises, such as observed for alpha-pinene epoxide, as an example. Otherwise, high UHP concentrations can inhibit the enzyme and cause oxidation of amino acids, causing enzyme inactivation (Abreu et al., 2012). Therefore, the sum of mild temperature conditions and a low molar ratio of beta-Pinene: UHP are ideal conditions for conducting the reaction, in agreement with the model proposed by Abreu et al. (2012).

Taking into account the data observed, the molar ratio and temperature parameters were maintained, while the influence of the amount of biocatalyst was evaluated. Comparing to the previous experiment with 10% (w/v) of N435, a reaction with 20% (w/v) was now performed. The reaction time was conducted in up to 3 h as the enzyme can be inactivated at a faster rate when exposed to peracid and hydrogen

peroxide for longer times and therefore the reduction of reaction time can better preserves it (MEYER-WAßEWITZ et al., 2017).

The results (Figure 3) show that the increases in the percentage of biocatalysts resulted in enhanced conversion, obtaining nearly 80% to the epoxide product in 3 hours reaction. This result agrees with the observed by Skouridou *et al*. (2003) to the alpha-pinene epoxidation, in which the increase in the percentage of biocatalyst provided the enhancement in the conversion to the product in the first 3 hours. This effect is due to the increase of peracid production, the limiting agent of the reaction, which shifts the reaction equilibrium in direction to the product formation (Goud et al., 2006). Although, it is relevant to highlight that excessive peracid production might lead to a more acidic medium, able to degrade the epoxide and inactivate the enzyme (Aouf et al., 2014).

A Fractional Factorial Design (FFD) was designed to investigate the correlation of each independent variable and their impact on the conversion to the desired product. For this, the independent variables: molar ratio beta-Pinene:UHP, temperature, percentage of biocatalyst, and stirring were considered. In order to calculate the experimental error, each independent variable was varied in two levels (Table 2), with triplicate at the central point.

Interesting results and conversions were obtained from the FFD **(Table S1)**, demonstrating that all independent variables investigated had statistical significance ($p < 0.05$), as shown in Table 3. The model presented an error (R^2) of 0.92, indicating that 92% of the reaction model can be explained by the variables considered. The model also presented a low error (0.25), indicating high reproducibility of the data obtained.

Table 3
Effect of the independent variables by Fractional Factorial Design statistical analysis

Factor	Effect	Error	P
Mean/Interaction	81.30	0.13	0.000002*
Curvature	18.46	0.48	0.000687*
Stirring (rpm)	8.11	0.25	0.000970*
Temperature (°C)	8.21	0.25	0.000946*
Molar ratio (beta-pinene: UHP)	17.39	0.25	0.000211*
Enzyme (%)	-1.40	0.25	0.030986*
* Statistically significant at 95% of confidence level.			

As shown in Table 4, the variable with the highest impact on the epoxidation of β-pinene was the molar ratio between beta-Pinene and UHP, followed by temperature and stirring. Although the percentage of the enzyme presented a negative value (-1,40), it was quite minor, suggesting that the range considered for this variable was already near the ideal. The same effect was observed by Ranganathan et al. (2016),

which used the Taguchi's method of Experiments Design, and demonstrated that hydrogen concentration and substrate concentration were the variables with the higher impact in the biocatalytic epoxidation of limonene.

In general, it was expected that increasing stirring speed favors mass transfer in biocatalytic reactions, so enhancing the conversion to the product of interest. However, high agitation might generate a fluid shear process, causing degradation of the biocatalyst solid support (Bhalerao et al., 2018). The range applied (150-250 rpm) to this FFD showed a low effect, probably because it is already in the optimal stirring speed range, favoring mass transfer without causing degradation.

The Table S1 summirizes the real and coded values of the independent variables (2^{4-1}) applied for the Factorial Fractional Design (FFD). In entry 8 **(Table S1)**, for example, when there were used the highest temperature (60°C), the molar ratio 1:3, stirring speed 250 rpm, and percentage of the enzyme 30%, 95% of conversion to the epoxide was obtained. Entries 9 (200 rpm, 50°C, molar ratio 1:2, and 20% (w/v) of enzyme) and 6 (250 rpm, 40°C, molar ratio 1:3, and 10% (w/v) of enzyme) have shown similar conversions: 93% and 90%, respectively. Even though entry 8 had the highest conversion, entries 9 and 6 have similar results using less biocatalyst, making the epoxidation reaction more economically feasible.

The application of the Fractional Factorial Design provided the background to the development of a Central Composite Rotatable Design (CCRD), aiming to deeply understand the effect of the independent variables and their influence on the β-pinene epoxidation. The variables with positive impact (molar ratio, stirring) had the values increased in the CCDR, while the percentage of the enzyme, that showed a negative impact, the new values attributed were less than in DFF. Despite the positive impact of temperature, the interval was not modified due to technical limitations of the equipment used. The CCRD was designed with 16 factorial points, 8 axial points, and 3 center points.

CCRD results have shown that solely the independent variable *enzyme amount* does not exhibit a statistical significance in the biocatalytic synthesis of the epoxide of interest, in agreement with the previously visualized in the FFD. It corroborates that the model is already being conducted with an ideal mass of biocatalyst. Also, regarding the impact of the other three independent variables, it was observed, by the Pareto's Graphic **(Figure S2)**, that they significantly influence the process ($p<0.05$), in this order and magnitude: temperature (-24.0820) > stirring (+10.5425) > molar ratio (-7.8835). This result differed from the obtained by FFD, which might be caused by the higher range applied, showing the relevance of combined experimental planning designs.

The analysis of variance (ANOVA) also shows the magnitude of the model, in which the F calculated (13.2) showed to be higher than F tabulated (2.6), supporting the experimental validity. The model also demonstrated higher conversions using less biocatalyst, as seen in entries 9 and 19, which achieved 92.7% of β-Pinene epoxide using 20% w/v and 15% w/v of biocatalyst, respectively. Also, it is important to highlight that both entries were conducted in mild temperatures (45°C and 40°C) and with a molar ratio of 2,5 and 3.0, respectively.

It was observed that the temperature and molar ratio had a negative impact on the model. In this context, it's important to highlight that higher conversions (>90%) were obtained at mild temperatures (45°C) and using a molar ratio in the range of 1:2.5-1:3.5. These results are demonstrated by the Surface response graphic (Figure 4). The impact of the molar ratio was close to zero, indicating that the values are already near the ideal, which means that the reaction can be conducted using these conditions and resulting in high conversions. The reduction in the use of UHP is also important to maintain the viability of the biocatalyst since a high concentration of this oxidant agent can induce degradation.

The only independent variable with a positive impact was the stirring (+10.54). The agitation speed is important in heterogeneous catalysis since it favors the mass transfers and the progression of the reactions to form the product of interest. As observed by Sun *et al.*(2011), the increase of stirring speed from 150 rpm to 300 rpm increased oxirane percentage formation. The same can be seen for the proposed model, in which the increase of stirring speed results in the improvement of conversion, in the range applied in the study. The response surface graphic (Figure 5) demonstrates that the increase of stirring and the reaction in mild temperatures result in conversions around 100%.

In order to evaluate the kinetics parameters of the reaction, the UHP concentration was ranged between 0.25 to 4 mmol, while the temperature (40°C), percentage of biocatalyst (20%w/v), and stirring (250 rpm) were kept constant. From these reactions there were determined the initial rate (Table 3), the maximum speed (Vmax of 10.7 mmol.min^{-1}, **Table S7 and Figure S1**), and the Michaelis-Menten constant (Km of 30.1 mmol, **Figure S1**).

Table 4
Kinetics parameters of beta-Pinene epoxidation reaction with different UHP concentrations

UHP concentration (mmol)	Initial rate, V_0 (mmol. min^{-1})	Conversion (%)
0.25	$8.88.10^{-2}$	22
0.5	0.103	35
1	0.321	52
2	0.642	77
3	0.752	88
4	3.73	78
Epoxidation reaction conditions: 2 mmol of β-Pinene, 0.25 to 4 mmol of UHP, 20% (w/v) of Novozym®435, in 3 mL of ethyl acetate, 250 rpm and 40°C for 3 h. Reactions were conducted in duplicate.		

The results (Table 4) show that the highest conversion was obtained when 3 mmol of UHP was applied, being possible to reach 88.3% of the epoxidated product. Although, its initial rate (V_0) was lower than when employing 4 mmol of UHP. Despite this condition (4 mmol of UHP) presented a higher initial rate

(V_0), the speed was not conserved throughout the process. The same effect was also described by Salvi and Yadav (2020) on the kinetics of limonene epoxidation using hydrogen peroxide and octanoic acid as acyl donor. With the increase of the concentration of oxidizing agent, the reaction rate decreased over time. The UHP concentration of 4 mmol exceeds the limit proposed by Mashhadi *et al*. (2018), which demonstrated that for each 1 mmol of fatty acid, 1.61 mmol of an oxidizing agent should be used, which may have caused enzymatic inactivation. Also, these results can be compared with Ranganathan and Sieber (2017), that also used ethyl acetate as an acyl donor in the epoxidation reaction of alpha-pinene, molar ratio of 0.2:7.5 (alpha-pinene:H_2O_2) and 40 mg of N435, resulting in a rate of 5.49×10^{-4} $mmol\cdot min^{-1}\cdot mg^{-1}$. Although alpha-pinene is more reactive than beta-pinene, because of the higher degree of double bond substitution (Swern, 1947), the reaction presented a slower reaction rate, indicating that the excess of hydrogen peroxide is, probably, reducing the activity of the enzyme.

4. Conclusion

The work demonstrated that ethyl acetate was the ideal solvent to conduct the reaction, among the tested solvents, due to the ability to act as a solvent and an acyl donor. Furthermore, when applied metrics of green chemistry, the dual activity of ethyl acetate makes this solvent more environmentally friendly, as can be seen by the high Atomic Economy and low E-value. After this, it was evaluated the unifactorial impact of independent variables, by increasing the molar ratio, the percentage of biocatalyst, and the temperature. So, by the increase of biocatalyst and the molar ratio, the conversion increased to 80% of the product after 3 hours of reaction. The kinetics parameters also demonstrated that the enzyme has a high affinity to the substrate (hydrogen peroxide) and can reach high conversion rates in only a few minutes of reaction, as can be seen by the V0, Km and Vmax parameters. To better understand the correlation between these variables, an FFD was proposed to evaluate four independent variables (stirring, temperature, molar ratio, and percentual of biocatalyst), which demonstrated that all were statistically and with different rates of impact. Then the model continued to be optimized using a CCRD that considered these independent variables, which resulted in an optimized model with entries that achieved conversions in the range of good to excellent (30-92.7%).

Declarations

Funding

This study was financed by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro - FAPERJ (grant numbers 201.511/2018 and E-26/202.728/2018) and PNPD-CAPES.

Data Availability Statement

Data is contained within the article and the supplementary material.

Conflicts of Interest

The authors declare no conflict of interest. Also, the funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Authors contribution

Gustavo dos S. Martins and Ivana C.R. Leal conceived the project; Gustavo dos S. Martins performed conceptualization, investigation, methodology, formal analysis, and writing – original draft - of the paper that all authors contributed; Amanda Staudt performed conceptualization, formal analysis, and writing original draft; Felipe Korbus Sutilli performed conceptualization and formal analysis; Camila R.A. Malafaia performed conceptualization, investigation, and project administration; Ivana C.R. Leal performed conceptualization, resources, writing – review and editing, supervision, project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

References

1. Abreu F De, Sutili FK, Miranda LSM, et al (2012) Journal of Molecular Catalysis B : Enzymatic Epoxidation of oleic acid catalyzed by PSCI-Amano lipase optimized by experimental design. 81:7–11. https://doi.org/10.1016/j.molcatb.2012.03.011
2. Andrus MB, Poehlein BW (2000) Epoxidation of olefins with peracid at low temperature with copper catalysis. Tetrahedron Lett 41:1013–1014. https://doi.org/10.1016/S0040-4039(00)00070-8
3. Ankudey EG, Olivo HF, Peeples TL (2006) Lipase-mediated epoxidation utilizing urea-hydrogen peroxide in ethyl acetate. Green Chem 8:923–926. https://doi.org/10.1039/b604984b
4. Aouf C, Durand E, Lecomte J, et al (2014) The use of lipases as biocatalysts for the epoxidation of fatty acids and phenolic compounds. Green Chem 16:1740–1754. https://doi.org/10.1039/c3gc42143k
5. Bhalerao MS, Kulkarni VM, Patwardhan A V. (2018) Ultrasound-assisted chemoenzymatic epoxidation of soybean oil by using lipase as biocatalyst. Ultrason Sonochem 40:912–920. https://doi.org/10.1016/j.ultsonch.2017.08.042
6. Björkling F, Godtfredsen SE, Kirk O (1990) Lipase-mediated formation of peroxycarboxylic acids used in catalytic epoxidation of alkenes. J Chem Soc Chem Commun 1301–1303. https://doi.org/10.1039/C39900001301
7. Bornscheuer, U.T., Kazlauskas, R.J., 2006. Hydrolases in Organic Synthesis Regio- and Stereoselective Biotransformations. Wiley-VCH, Weinheim, Germany. https://doi.org/10.1021/op060064f
8. Brahmkshatriya, P.P., Brahmkshatriya, P.S., 2013. Terpenes: Chemistry, Biological Role, and Therapeutic Applications, in: Ramawat, K.G., Mérillon, J.-M. (Eds.), Natural Products. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 2665–2691. https://doi.org/10.1007/978-3-642-22144-6_120

9. Colonna S (1993) Enantiasel~ve Epoxidation dStyrene Derivativea by Cbloropeddase Cdalysi8. 4:1325–1330
10. Da Silva JMR, Nascimento MDG (2012) Chemoenzymatic epoxidation of citronellol catalyzed by lipases. Process Biochem 47:517–522. https://doi.org/10.1016/j.procbio.2011.12.019
11. Dambolena JS, Zunino MP, Herrera JM, et al (2016) Terpenes: Natural Products for Controlling Insects of Importance to Human Health - A Structure-Activity Relationship Study. Psyche (London) 2016:. https://doi.org/10.1155/2016/4595823
12. Dammak, I., Hamdi, Z., Kammoun El Euch, S., Zemni, H., Mliki, A., Hassouna, M., Lasram, S., 2019. Evaluation of antifungal and anti-ochratoxigenic activities of Salvia officinalis, Lavandula dentata and Laurus nobilis essential oils and a major monoterpene constituent 1,8-cineole against Aspergillus carbonarius. Industrial Crops and Products 128, 85–93. https://doi.org/10.1016/j.indcrop.2018.11.006
13. Goud V V., Patwardhan A V., Pradhan NC (2006) Studies on the epoxidation of mahua oil (Madhumica indica) by hydrogen peroxide. Bioresour Technol 97:1365–1371. https://doi.org/10.1016/j.biortech.2005.07.004
14. Guimarães AG, Quintans JSS, Quintans-Júnior LJ (2013) Monoterpenes with analgesic activity - A systematic review. Phyther Res 27:1–15. https://doi.org/10.1002/ptr.4686
15. Juergens, U., 2014. Anti-inflammatory Properties of the Monoterpene 1.8-cineole: Current Evidence for Co-medication in Inflammatory Airway Diseases. Drug Res (Stuttg) 64, 638–646. https://doi.org/10.1055/s-0034-1372609
16. Kang, S.-H., Kim, Y.-S., Kim, E.-K., Hwang, J.-W., Jeong, J.-H., Dong, X., Lee, J.-W., Moon, S.-H., Jeon, B.-T., Park, P.-J., 2016. Anticancer Effect of Thymol on AGS Human Gastric Carcinoma Cells. Journal of Microbiology and Biotechnology 26, 28–37. https://doi.org/10.4014/jmb.1506.06073
17. Koziol, A., Stryjewska, A., Librowski, T., Salat, K., Gawel, M., Moniczewski, A., Lochynski, S., 2014. An Overview of the Pharmacological Properties and Potential Applications of Natural Monoterpenes. MRMC 14, 1156–1168. https://doi.org/10.2174/1389557514666141127145820
18. MEYER-WAßEWITZ, Janine et al (2017). An organic-single-phase CSTR process for the chemo-enzymatic epoxidation of α-pinene enables high selectivity and productivity. Biochemical engineering journal, v. 126, p. 68-77, 2017.
19. Mohamed, A.A., Behiry, S.I., Younes, H.A., Ashmawy, N.A., Salem, M.Z.M., Márquez-Molina, O., Barbabosa-Pilego, A., 2019. Antibacterial activity of three essential oils and some monoterpenes against Ralstonia solanacearum phylotype II isolated from potato. Microbial Pathogenesis 135, 103604. https://doi.org/10.1016/j.micpath.2019.103604
20. Moreira MA, Nascimento MG (2007) Chemo-enzymatic epoxidation of (+)-3-carene. Catal Commun 8:2043–2047. https://doi.org/10.1016/j.catcom.2007.02.032
21. Ranganathan S, Tebbe J, Wiemann LO, Sieber V (2016) Optimization of the lipase mediated epoxidation of monoterpenes using the design of experiments—Taguchi method. Process Biochem 51:1479–1485. https://doi.org/10.1016/J.PROCBIO.2016.07.005

22. Ranganathan, S; Sieber, V (2017). Development of semi-continuous chemo-enzymatic terpene epoxidation: combination of anthraquinone autoxidation and the lipase-mediated epoxidation process. Reaction Chemistry & Engineering, 2:885-895.
23. Ruzicka, L., 1953. The isoprene rule and the biogenesis of terpenic compounds. Experientia 9, 357–367. https://doi.org/10.1007/BF02167631
24. Sánchez-Velandia JE, Gelves JF, Dorkis L, et al (2019) Ring-opening of B-pinene epoxide into high-added value products over Colombian natural zeolite. Microporous Mesoporous Mater 287:114–123. https://doi.org/10.1016/j.micromeso.2019.05.053
25. SALVI, H. M.; YADAV, Ganapati D (2020). Chemoenzymatic epoxidation of limonene using a novel surface-functionalized silica catalyst derived from agricultural waste. ACS omega, 5:22940-22950
26. Sheldon RA (2018) Metrics of Green Chemistry and Sustainability: Past, Present, and Future. ACS Sustain Chem Eng 6:32–48. https://doi.org/10.1021/acssuschemeng.7b03505
27. Sun S, Ke X, Cui L, et al (2011) Enzymatic epoxidation of Sapindus mukorossi seed oil by perstearic acid optimized using response surface methodology. 33:676–682. https://doi.org/10.1016/j.indcrop.2011.01.002
28. Swern, D. (1947): Electronic Interpretation of the Reaction of Olefins with Organic Per-acids. Journal of the American Chemical Society 69 (7) 1692– 1698. 26
29. Tang Q, Popowicz GM, Wang X, et al (2016) Lipase-Driven Epoxidation Is A Two-Stage Synergistic Process. ChemistrySelect 1:836–839. https://doi.org/10.1002/slct.201600254

Figures

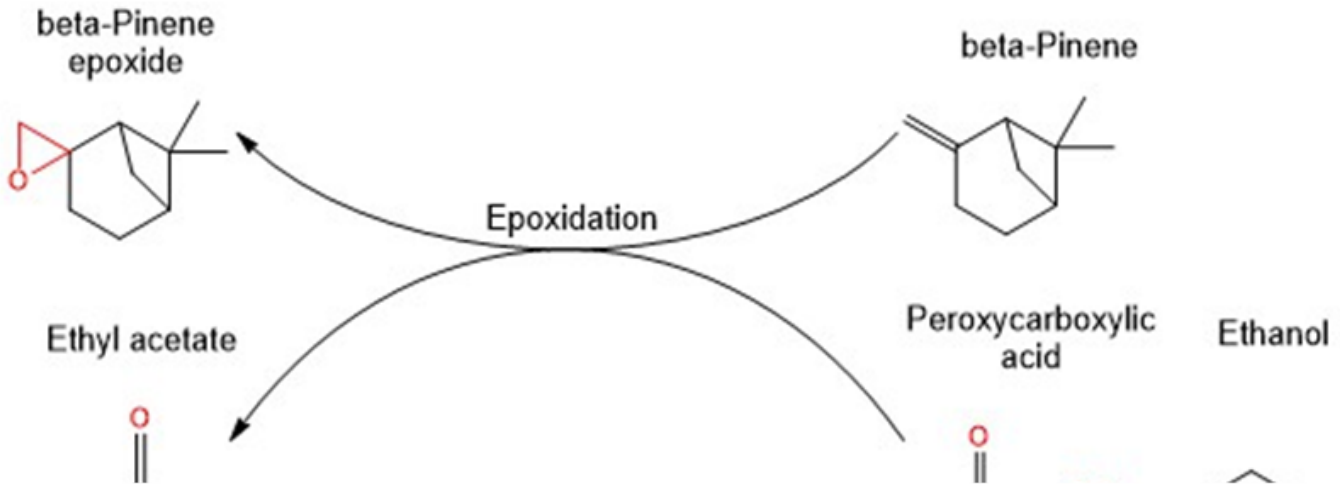

Figure 1

Biocatalytic epoxidation of beta-Pinene mediated by Novozym®435.

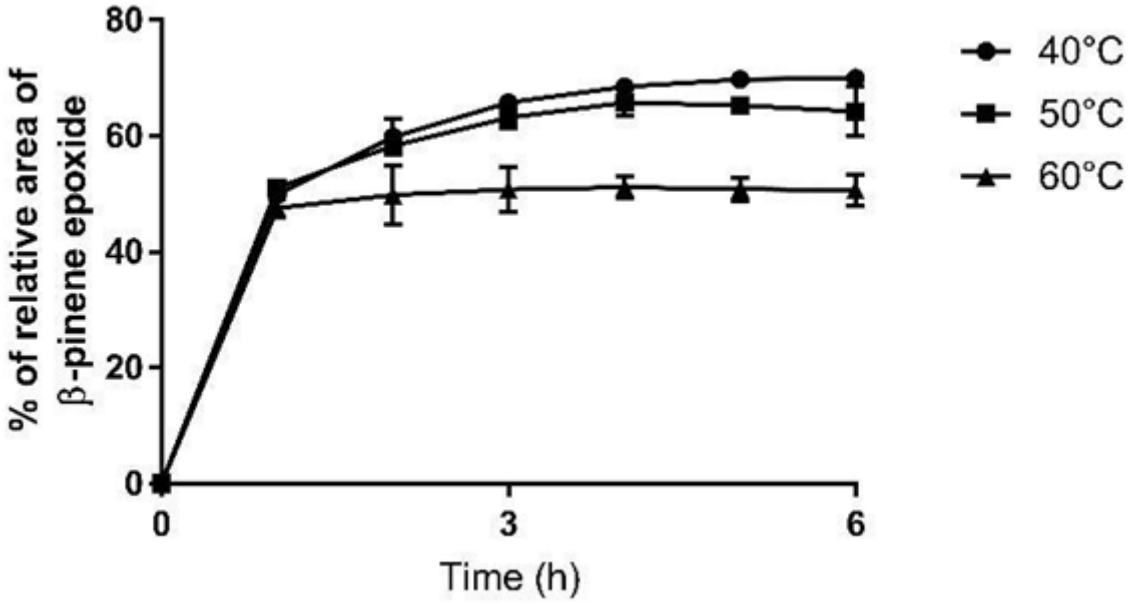

Figure 2

Biocatalytic reaction of beta-Pinene to produce β-pinene epoxide (%) at different temperatures over time. Epoxidation reaction conditions: molar ratio 1:1 (beta-Pinene: UHP), the concentration of 2 mmol of beta-pinene, 10% (w/v) of Novozym®435, in 3 mL of ethyl acetate, 250 rpm and 40, 50 or 60°C for 6 h. Reactions were conducted in duplicate.

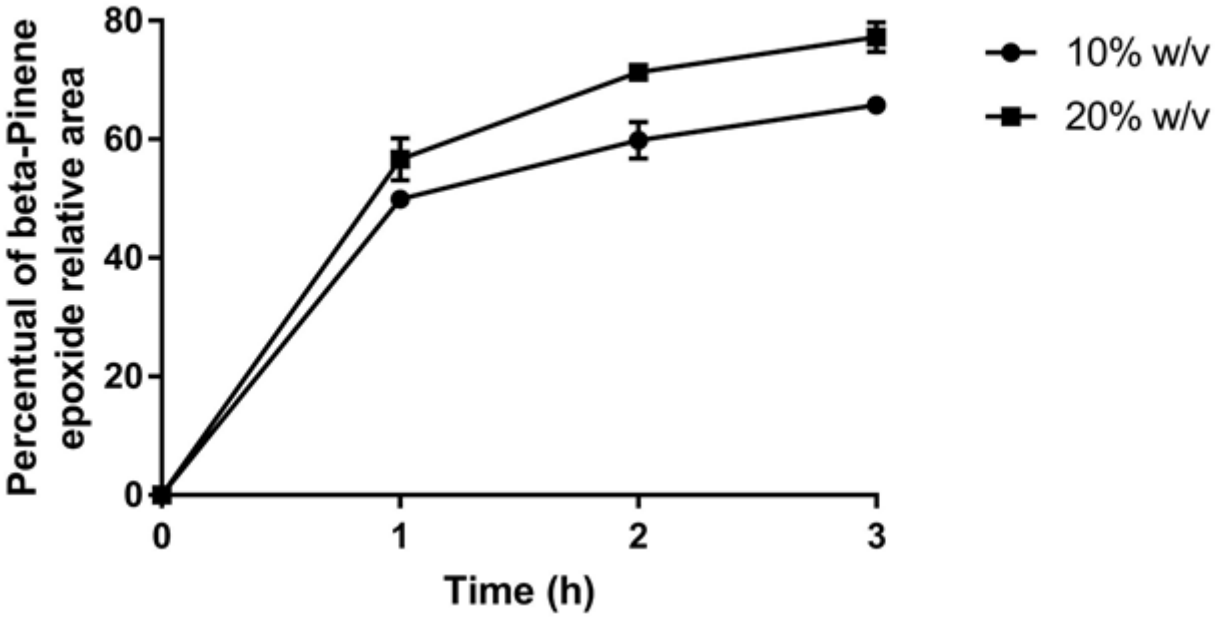

Figure 3

Biocatalytic reaction of beta-Pinene to produce β-pinene epoxide (%) with different biocatalyst amounts over time. Epoxidation reaction conditions: molar ratio 1:1 (beta-Pinene: UHP), the concentration of 2 mmol of beta-Pinene and 2 mmol of UHP, 10 or 20% (w/v) of Novozym®435, in 3 mL of ethyl acetate, 250 rpm and 40°C for 3 h. Reactions were conducted in duplicate. Control reactions without enzymes did not result in an epoxidated product.

Figure 4

Response surface for the epoxidation reaction catalyzed by N435 in function of the molar ratio and temperature.

Figure 5

Response surface for the epoxidation reaction catalyzed by the N435 in function of the temperature and stirring.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GRAPHICALABSTRACT.png
- SupplementaryInformationICRLBiotechLetter.doc