Enzymatic Synthesis of Eugenyl Acetate from Essential Oil of Clove Using Lipases in Liquid Formulation as Biocatalyst

Leandro Santolin
Federal Technological University of Paraná: Universidade Tecnologica Federal do Parana

Karina G. Fiametti
Federal Technological University of Paraná: Universidade Tecnologica Federal do Parana

Viviane da Silva Lobo
Federal Technological University of Paraná: Universidade Tecnologica Federal do Parana

João H. C. Wancura
Federal University of Santa Maria: Universidade Federal de Santa Maria

José Vladimir Oliveira (jose.vladimir@ufsc.br)
Universidade Federal de Santa Catarina  https://orcid.org/0000-0002-6196-3498

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Abstract

In this research, eugenyl acetate, a compound with flavoring, antioxidant and antimicrobial properties, was obtained from essential oil of clove (*Syzygium aromaticum*) via liquid lipase-mediated acetylation. Clove essential oil was extracted by drag water vapor from dry flower buds and its physic-chemical characteristics were analyzed. For the enzymatic synthesis, an extensive evaluation of reaction parameters was accomplished through employment of distinct reaction temperatures, acetic anhydride to eugenol molar ratios, enzyme loads and three different lipases (a lyophilized enzyme produced by solid-state fermentation of sunflower seed with *Penicillium sumatrense* microorganism and others two commercial lipases – Lipozyme TL 100L and CALB L). Characterization by Infrared Spectroscopy and Nuclear Magnetic Resonance (1H NMR and 13C) was used to confirm the presence of eugenyl acetate in the samples. Through optimized conditions (55 °C, acetic anhydride to eugenol molar ratio of 1:1, 10 wt% of Lipozyme TL 100L), 91.80 % of conversion after 2 h was achieved to the eugenyl acetate production. With the results obtained, it was possible to conclude that the use of lipases in liquid formulation is a promising alternative for the synthesis of essential esters largely applied on food, cosmetic and pharmaceutical industries.

Introduction

Esters with low molecular weight represent an important chemical class of compounds derived from short-chain acids such as acetates, propionates and butyrates [1, 2]. Such importance is due to the fact of numerous characteristics that allow its application in a wide area of industrial sectors that include food, pharmaceutical, energy, among others [3].

An example of component with these characteristics is the eugenyl acetate (EA). Eugenyl acetate (4-allyl-2-methoxifenol acetate) is a phenylpropanoid with pale yellow aspect belonging to class of compounds named vanilloids [4]. It is derivate from eugenol (2-allyl-4-methoxyphenol), which can be obtained by extraction from essential oil of clove (*Syzygium aromaticum*) [5]. Eugenol is the major component of the essential oil of clove with composition that range between 76 and 95 wt% [6–8].

Applications of EA are numerous. Researches published recently reports results of employments as flavoring agent in cosmetics and foods as well as antioxidant, anticarminatire, antispasmodic and antiseptic agent in pharmacology, bio-additive for fuels and still as antimicrobial and larvicidal agent [9–14]. Musthafa et al. [3] investigated the EA efficiency against clinical isolates of *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata*. Measurements of growth profile in time-kill study evidenced that minimum inhibitory concentrations of EA retarded the growth of *Candida* cells. Also, additional tests demonstrated that, upon treated with EA, cell morphology, cell damage and fragmented patterns were observed in *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata*, validating the hypothesis that the ester could cause cell death of *Candida* clinical isolates. Carrasco et al. [14] investigated the antioxidant and anticancer properties of the eugenol and its derivatives (among them the EA). The compounds were examined by in vitro model of cancer using androgen-insensitive prostate
cancer cells and oral squamous carcinoma cells. In the examined cancer cells, all compounds presented inhibition of the cellular activity, where the results obtained demonstrated that EA were significantly more active than eugenol.

Conventionally, homogeneous acid-catalyzed esterification is applied industrially to esters production. However, operational problems arising from handling acids, generation of hazardous wastes as well as projects costs with equipment that must be resistant to corrosion are some drawbacks of the route [4]. Furthermore, the chemical synthesis leads to the formation of undesirable products to the food and pharmaceutical industries [1]. To circumvent such drawbacks, enzymatic synthesis of aromatic esters via acetylation reaction, catalyzed by lipases, had been proposed. Lipases (triacylglycerol ester hydrolases – E.C.3.1.1.3) are enzymes that act on organic-aqueous interface catalyzing hydrolysis of carboxylic triacylglycerol ester bonds with the formation of free fatty acids, diacylglycerols, monoacylglycerols and glycerol [15]. Lipases are also able to catalyze reactions of esterification, interesterification, hydroesterification and transesterification [16, 17]. Lipases have high specificity and selectivity, preventing that side reactions occurring in the system. Another interesting characteristic of enzymatic processes is the operation at mild reaction conditions (temperature and pressure), representing low energy costs with the process [18].

EA production using enzymatic catalysis is a topic that has been little explored until now, and the majority of published researches applied commercial immobilized lipases as reaction catalyst [4, 5, 10, 19, 20]. However, immobilized commercial lipases are expensive, affecting drastically the process feasibility [21, 22]. In this scenario, the use of lipases in liquid formulation, such as Lipozyme® TL 100L and Lipozyme® CALB L, both recently launched in the market by Novozymes A/K, arises as an interesting alternative to the EA synthesis. The use of liquid lipases for esters production can make the whole process cost-efficient, more competitive and sustainable since liquid lipases can be sold at prices 30 to 50 times lower than the immobilized ones and also can be reused after recovery from the system [23, 24].

The objective of this work is to investigate a EA synthesis by liquid lipase-mediated acetylation from eugenol (extracted from essential oil of clove), using different lipases: a lyophilized enzyme produced by solid-state fermentation of sunflower seed with Penicillium sumatrense microorganism and the commercial enzymes in liquid formulation Lipozyme® TL 100L and Lipozyme® CALB L. For this proposal, statistical design was utilized to evaluate the reaction conditions that maximize the ester conversion to the process.

**Materials And Methods**

**Materials**

Dry flower buds of clove (Iremar Alimentos, Toledo, Brazil) was purchased from a local market and use as feedstock to obtain the oil employed in the assays. Regarding the biocatalysts, three enzymes were used in the acetylation reactions; a lyophilized enzyme produced by solid-state fermentation of sunflower seed...
with *Penicillium sumatrense* microorganism (enzymatic activity of 31.83 U by gram of fermented solid). The others two are commercial enzymes in liquid formulation recently launched and kindly provided by Novozymes Latin America LTDA (Araucária, Brazil): Lipozyme® TL 100L (obtained from *Thermomyces lanuginosus* microorganism and nominal enzymatic activity of 100 kLU·g⁻¹) and Lipozyme® CALB L (obtained from *Candida antarctica* B microorganism and nominal enzymatic activity of 5,000 LU·g⁻¹). The chemicals used in the tests and analysis are acetic anhydride, anhydrous sodium sulfate and sodium hydroxide (all analytical grade, Synth, Diadema, Brazil); methanol, ethanol and dichloromethane (all 99.5 % purity, Merck, Rio de Janeiro, Brazil); cyclohexane, acetic acid and dimethylsulfoxide – DMSO-d₆ (all purity ≥ 99.5 % - chromatographic grade, Sigma-Aldrich, São Paulo, Brazil). Standards used in the analysis, with chromatographic grade, included eugenol (99 % purity), β-caryophyllene (purity ≥ 80%), humulene (purity ≥ 96%) and eugenyl acetate (purity ≥ 98%), all purchased from Sigma-Aldrich (São Paulo, Brazil).

**Essential Oil of Clove (*Syzygium aromaticum*): Obtaining and Characterization**

Previously grounded dry flower buds were used to obtain essential oil of clove via drag water vapor distillation in a Clevenger-type distiller (Solab, model SL-76, Piracicaba, Brazil) coupled to a thermostated bath (Tecnal, model TE-2005, Piracicaba, Brazil). In all, five batches of 60 min were performed using 200 g of ground material in each operation. All the extracted oil was dried by percolating on anhydrous sodium sulfate. For this step, the mass yield of the extraction process was calculated.

For the clove oil obtained, was determined physical-chemical characteristics (color, appearance, relative density, oil refraction index, moisture by Karl Fischer and oil solubility in ethanol 70 vol%), according methodology proposed by The United States Pharmacopeia – USP 41 [25].

The quantification and identification of the main constituents of the essential oil of clove was determined by gas chromatograph (7890B from Agilent® equipped with flame ionization detector and an Agilent DB-1 capillary column, 100 % dimethylpolysiloxane with 30 m x 0.25 mm x 0.25 μm of coating film). Temperature program: 280 °C in isotherm for 15 minutes, injector temperature of 290 °C and detector temperature of 290 °C. Helium (99.999 % purity) with a column flow rate of 1 mL·min⁻¹ and split ratio 100:1 was used as carrier gas. Nitrogen with a flow rate of 35 mL·min⁻¹ was used as make up gas. Hydrogen and synthetic air with flow of 30 and 300 mL·min⁻¹, respectively, was used as flame gas. To identify the compounds, standard solutions of eugenol, β-caryophyllene, humulene and eugenyl acetate (diluted in cyclohexane) were prepared.

**Synthesis of Eugenyl Acetate**

EA synthesis was performed in an orbital shaker (operating at 150 rpm) with the addition of the clove oil, lipase and acetic anhydride until complete dissolution. Molar proportion of substrates was established
considering the molar mass of acetic anhydride (102 g·mol⁻¹) and the mass concentration of eugenol (molar mass of 164 g·mol⁻¹) present in the clove oil previously determined (86.87 wt%). Thus, 187 g of oil contain the equivalent to 1 mol of eugenol, representing the mass of raw material applied in each assay.

After reaction finishing, the catalyst was separated from the reaction medium by filtration and the product of the enzymatic reaction was purified by washing with solution of NaOH 0.1 N and cyclohexane (25 vol%). After washing, the organic phase (rich in EA and cyclohexane) was separated from the aqueous phase (NaOH solution plus acetic acid formed in the reaction). The solvent was evaporated from the organic phase and the purified EA was stored under refrigeration for analysis.

## Selection of the Ideal Biocatalyst

Preliminary tests (in triplicate) were performed with each enzyme (lipase obtained from *Penicillium sumatrense* microorganism, Lipozyme® TL 100L and Lipozyme® CALB L) in order to determine which biocatalyst would return the best performance for the EA synthesis. For these tests, experimental conditions obtained by Radünz et al. [26] was used: 50 °C, eugenol to acetic anhydride molar ratio of 1:3, enzyme load of 5.5 wt.%, 150 rpm and 2 hours of reaction.

After catalyst selection, a statistical design was carried out to determine the reaction conditions that optimize the EA synthesis for the enzyme chosen. The reaction conditions that was evaluated were eugenol to acetic anhydride molar ratio (mol·mol⁻¹), temperature (°C) and enzyme load (wt%). A complete factorial design $2^3$ with triplicate in the center point (resulting in 11 trials) was applied. Table 1 presents the real and coded variables that were tested for the enzyme selection among the three biocatalysts considered. StatSoft Statistica (version 11.0) was used to data processing using a reliability level of 95 %. After determining the catalyst with better performance to the enzymatic process and the appropriate experimental conditions, a time course of the EA synthesis was accomplished.

**Table 1** Real and coded parameters evaluated in the EA production and conversions obtained
| Assay | Molar Ratio (mol·mol⁻¹) | Temperature (°C) | Lipase Load (wt%) | Conversion (%) |
|-------|-------------------------|------------------|-------------------|---------------|
| 1     | 1:1 (-1)                | 45 (-1)          | 5 (-1)            | 38.12         |
| 2     | 1:5 (1)                 | 45(-1)           | 5 (-1)            | 47.10         |
| 3     | 1:1 (-1)                | 55 (1)           | 5 (-1)            | 82.15         |
| 4     | 1:5 (1)                 | 55 (1)           | 5 (-1)            | 68.23         |
| 5     | 1:1 (-1)                | 45 (-1)          | 10 (1)            | 58.50         |
| 6     | 1:5 (1)                 | 45 (-1)          | 10 (1)            | 77.18         |
| 7     | 1:1 (-1)                | 55 (1)           | 10 (1)            | 91.80         |
| 8     | 1:5 (1)                 | 55 (1)           | 10 (1)            | 88.85         |
| 9     | 1:3 (0)                 | 50 (0)           | 7.5 (0)           | 66.70         |
| 10    | 1:3 (0)                 | 50 (0)           | 7.5 (0)           | 67.50         |
| 11    | 1:3 (0)                 | 50 (0)           | 7.5 (0)           | 64.30         |

**Eugenyl Acetate Analysis**

The chromatographic methodology described on Section 2.2 were used to quantify the eugenol conversion in esters, following the reduction in the signal area of the limiting agent (eugenol) as well as the appearance of the peak product. The area of the reaction mixture without the catalyst (blank) and the area of the reaction were related by Equation 1.

\[
QC(\%) = \frac{100 - (100 \cdot A_c)}{A_{Blank}} \quad (1)
\]

where \( QC \) is the amount of eugenol converted in EA, \( A_c \) is the peak area of conversion and \( A_{Blank} \) is the area of the blank.

After purification process, the EA samples were analyzed in the GC (7890B from Agilent® equipped with flame ionization detector and an Phenomenex ZB-Wax G16 column, 100 % polyethylene glycol, with 30 m x 0.32 mm, 50 μm). Chromatographic system was configured using injection volume of 0.6 μL, injector temperature of 180 °C, detector temperature of 250 °C and helium as carrier gas with a column flow rate of 5 mL·min⁻¹ and split ratio 1:15. Column temperature was configured according description: initial temperature of 40 °C, 40 °C to 180 °C in 7 min at 20 °C·min⁻¹ and 180 °C to 250 °C in 12 min at 50 °C·min⁻¹ with a temperature holding time of 3.5 min. To identify the compound, standard solutions of EA (diluted in cyclohexane) were prepared.
Infrared spectroscopy (FT-IR, PerkinElmer Spectrophotometer® 400 equipped with Attenuated Total Reflectance – ATR device) was used to identify the main chemical functions of the synthesized molecule. The equipment was configured for 16 scans, resolution of 2.0 and scanned in the region from 4,000 to 650 cm$^{-1}$.

Nuclear Magnetic Resonance (NMR, $^1$H and $^{13}$C) for 1D and 2D NMR spectra were performed on a Bruker Avance III HD 600 Spectrometer with a magnetic field of 14.1 T and 5 mm of TCI (Triple Chanel Inverse) cryoprobe. The chemical displacements of $^1$H and $^{13}$C were referenced according to the peak of the solvent dimethylsulfoxide (DMSO-d$_6$) used to solubilize the samples ($\delta_C$ of 39.5).

**Results And Discussion**

**Physical-Chemical Characteristics and Chemical Composition of the Oil**

Based on the extractions process of clove oil from dry flower buds, 11.2 ± 0.9 mL of oil was obtained, representing a mass 11.7 ± 1.0 g. As the oil density was determined at 1.041 g·mL$^{-1}$, the mass yield was estimated at 5.83 ± 0.5 %. In a similar study, Radünz et al. [26] reported yields ranging from 1.87 % to 8.88 %, using a hydrodistillation process to obtain essential oil of clove. Low yields are associated with factors such collection period of the materials, storage conditions and climate, interfering in the oil constitution.

The quality of an essential oil depends on several parameters such as solubility in different organic solvents, density, refraction index, among others. Table 2 presents the physical-chemical parameters obtained to the essential oil of clove, according USP 41 methodology [25]. Through verification of the parameters, it is possible to conclude that the results are satisfactory since all values of the analysis were within the specification limits provided in the regulatory standard.

**Table 2** Physical-chemical parameters obtained for the essential oil of clove

| Parameters                                | Result Obtained | Specification$^1$ |
|-------------------------------------------|-----------------|------------------|
| Density Relative (g·mL$^{-1}$)            | 1.041           | 1.038 – 1.060    |
| Refraction Index (20°C)                   | 1.531           | 1.527 – 1.535    |
| Color                                     | Light yellow    | -                |
| Appearance                                | Clear liquid    | -                |
| Odor                                      | Characteristic  | -                |
| Moisture (wt%)                            | 0.15            | -                |
| Solubility in Ethanol 70 vol%             | 1:2             | 1:2              |
Table 3 presents the chemical composition of the clove oil, considering the major components that compose the samples. The mass composition relative to the eugenol, the major component of clove oil, is close to that obtained in similar researches, ranging from 83.6 % to 88.4 % [8].

Table 3 Chemical composition of the clove oil used in the tests

| Parameters          | Mass Composition (wt%) |
|---------------------|-------------------------|
| Eugenol             | 86.87                   |
| β-Caryophyllene     | 11.70                   |
| Humulene            | 0.93                    |
| Eugenyl Acetate     | 0.11                    |

Eugenyl Acetate Synthesis

Selection of the Lipase

Preliminary tests were carried out in order to define which lipase, among the three available enzymes (lyophilized enzyme produced *Penicillium sumatrense* microorganism, Lipozyme® TL 100L and Lipozyme® CALB L), would present the best response in terms of raw material conversion. At 50 °C, eugenol to acetic anhydride molar ratio of 1:3, enzyme load of 5.5 wt.%, the lipase Lipozyme® TL 100L presented the higher conversion: 85.40 ± 3.2 % after 2 hours of reaction. In these same reaction conditions, the homemade lyophilized enzyme and Lipozyme® CALB L presented conversion of 73.4 ± 4.2 % and 58.3 ± 6.0 %, respectively. Based on these results, lipase Lipozyme® TL 100L was chosen as catalyst to be utilized in the optimization of the reaction process via statistical design. It is important to note the interesting conversion achieved by the lyophilized enzyme produced in laboratory, superior in comparison to the commercial lipase CALB L. This fact demonstrates that the homemade biocatalyst has potential to be applied in the process.

Influence of the Reaction Parameters in the Eugenyl Acetate Synthesis.

In the evaluation of the EA production using Lipozyme® TL 100L as reaction biocatalyst, a full 2³ factorial design with three replications of the central point was employed, investigating the effects of the eugenol to acetic anhydride molar ratio, temperature and enzyme load in the process performance. Table 1 presents the matrix of the statistical design with coded and real values of the independent variables and the experimental reaction conversions to EA. The highest conversion into EA occurred at 55 °C, eugenol to acetic anhydride molar ratio of 1:1 and 10 wt% of Lipozyme® TL 100L (assay 7), achieving 91.80 % of
conversion. The lowest conversion obtained occurred at 45 °C, eugenol to acetic anhydride molar ratio of 1:1 and 5 wt% of Lipozyme® TL 100L (38.12 % of conversion, assay 1), indicating that a catalyst load superior than 5 wt% is indicated to the reaction.

Results presented in Table 1 were statistically analyzed via Pareto Diagram, according shown in Figure 1. Significant variables presented p < 0.05. Coefficients with a positive sign indicate a synergic effect whereas negative coefficients indicate an antagonistic effect in the ester formation. The Pareto Diagram of standardized effects confirm that the molar ratio between eugenol and acetic anhydride had no significant effect in the EA synthesis, while reaction temperature and lipase load had a significant positive effect (p < 0.05) on ester production. However, it is possible observe that the interaction between the variables “eugenol to acetic anhydride molar ratio” and “reaction temperature” had a negative effect on ester production. Similar tendency was also observed by Silva et al. [4] that, however, used immobilized lipase to produce EA from clove oil.

The statistical analysis of the results presented on Table 1 allowed the obtaining of a model for EA conversion as a function of eugenol to acetic anhydride molar ratio, temperature and lipase load. The model was statistically validated (p < 0.05) by analysis of variance (ANOVA), according presented on Table 4, where a coefficient of determination (R²) of 99.15, proving that the model obtained, with a level of reliability of 95 %, is statistically valid.

**Table 4** ANOVA for validation of the model that describes the production of eugenyl acetate using the lipase Lipozyme TL 100L
| Source of Variation                                      | Sum of Squares | Degrees of Freedom | Mean Square | F<sub>Calculated</sub> | p-value |
|---------------------------------------------------------|----------------|--------------------|-------------|------------------------|---------|
| (1) Eugenol to ac. anhydride molar ratio (mol·mol<sup>-1</sup>) | 14.55          | 1                  | 14.55       | 5.25                   | 0.1491  |
| (2) Temperature (°C)                                    | 1,516.10       | 1                  | 1,516.10    | 5466                   | 0.0018  |
| (3) Lipase load (wt%)                                   | 814.67         | 1                  | 814.67      | 293.75                 | 0.0034  |
| 1 by 2                                                  | 247.87         | 1                  | 247.87      | 89.37                  | 0.0110  |
| 1 by 3                                                  | 53.41          | 1                  | 53.41       | 19.26                  | 0.0482  |
| 2 by 3                                                  | 50.96          | 1                  | 50.96       | 18.37                  | 0.0503  |
| Lack of Fit                                             | 17.61          | 2                  | 8.80        | 3.17                   | 0.2395  |
| Pure Error                                              | 5.55           | 2                  | 2.77        |                        |         |
| **Total**                                               | **2720.68**    | **10**             |             |                        |         |

a Significant at “Prob > F” less than 0.05;  
b Insignificant at “Prob > F” more than 0.05.

Results presented in the Table 1 and 4 as well as the Pareto diagram (Figure 1) shows an interactive effect between the considered variables. Thus, an independent analysis of the parameters would not be appropriate. Therefore, contour surfaces between the parameters is necessary to find the region with the best response and thus determine the optimum conditions of the system, where the EA conversion is maximum. These results are presented in the Figure 2. The Figure 2a shows the conversion in EA as function of reaction temperature and lipase load, Figure 2b presents the conversion in esters as a function of eugenol to acetic anhydride molar ratio and temperature and Figure 2c simulates the ester conversion ranging with the eugenol to acetic anhydride molar ratio and lipase load. From these results, it is possible to observe that the EA conversion is benefited with the simultaneous increase of the enzyme load and reaction temperature, especially with temperatures higher than 52 °C. It was also possible to observe that at temperatures below 48 °C, even an increase in the eugenol to acetic anhydride molar ratio was not enough for satisfactory EA conversions to be achieved, requiring higher temperatures to be used in the system. Similar situation was observed when the EA conversions were analyzed ranging the eugenol to acetic anhydride molar ratio and lipase load. With lipase loads inferior than 7 wt%, even using a high eugenol to acetic anhydride molar ratio, it was not possible to obtain satisfactory conversions, corroborating the hypothesis presented previously in the Pareto Diagram (Figure 1) that this variable is
not significant for the EA conversion. Such finding allowed us to deduce that a minimum eugenol to acetic anhydride molar ratio of 1:1 is the ideal to be used in the process. On the other hand, as expected, an increase in the lipase load applied to the process returned satisfactory EA conversion.

Therefore, based on the observed results, the reaction conditions that yielded the highest EA conversion were: 55 °C, eugenol to acetic anhydride molar ratio of 1:1 and 10 wt% of lipase load.

**Time Course of the Eugenyl Acetate Synthesis**

With the optimization of the reaction parameters considered in the EA production, the behavior of reaction temperature, Lipozyme® TL 100L load and eugenol to acetic anhydride molar ratio were evaluated in a time course to the acetylation process. Samples at 20, 40, 60, 120, 180, 240, 300 and 360 min were collected using the optimized reaction conditions previously obtained (55 °C, eugenol to acetic anhydride molar ratio of 1:1 and 10 wt% of lipase). The results obtained are presented on Figure 3.

From the results obtained, it should be note the interesting enzymatic activity of the lipase Lipozyme® TL 100L after 1 hour of process, reaching more than 60% of feedstock conversion. As the process advance, the increments in conversion decreased according the reaction equilibrium is achieved. With the use of the optimized reaction conditions, it was possible to achieve more than 90% conversion in 5 h of process. Data about the application of lipases in liquid formulation for EA synthesis from clove oil are rare. Few researches about this topic are published, where those that involve enzymatic process, use immobilized lipases as reaction catalyst. Silva et al. [4] investigated the ability of the commercial immobilized lipase Lipozyme® TL IM to catalyze the acetylation of essential oil of clove with acetic anhydride in a solvent-free system. At 70 °C, eugenol to acetic anhydride molar ratio of 1:5 and 5 wt% of lipase, a conversion of 92.86% was obtained by these authors after 3 h of reaction. In another similar research, Chiaradia et al. [27] reported data of an eugenyl acetate production by esterification of eugenol and acetic anhydride in a solvent-free system using the immobilized lipase Novozym® 435 as catalyst: at 50 °C, eugenol to acetic anhydride of 1:3 and 5.5 wt% of enzyme, a conversion of 99% was achieved after 6 h of reaction.

**Characterization of the Eugenyl Acetate**

The chemical structure of the purified EA obtained at optimized reaction conditions was confirmed by the proton nuclear magnetic resonance (1H-NMR) and infrared spectroscopy (FT-IR) analysis.

The results of the FT-IR analysis and the information related to the identified attributions and functional groups are present in Figure 4. The evaluated spectrum shows bands at 2,939 and 2,841 cm⁻¹ that are attributed, respectively, to the asymmetric and symmetric axial deformation of the CH₂ group, while the
existing one at 3,005 cm\(^{-1}\) is attributed to the =C-H stretch of the aromatic ring structure. In the spectrum it is possible to observe the characteristic presence of the carbonyl band of the ester bound with the aromatic ring at 1,761 cm\(^{-1}\), suggesting that the molecule is, in fact, EA due to the addition of the acyl group in the eugenol molecule. In addition, the spectra present two axial stretch bands of the ester at 1,184 cm\(^{-1}\) (high intensity) and 1,267 cm\(^{-1}\) (medium intensity). The bond stretches of the C-O between 1,214 and 1,033 cm\(^{-1}\) are assigned to the methoxy group. In 1,419 and 1,368 cm\(^{-1}\) there was a folding of CH\(_2\) and CH\(_3\) [28]. According Engel et al. [28], the stretch of the double aliphatic/aromatic carbon bond is around 1,680 – 1,600 and 1,600 to 1,475 cm\(^{-1}\), respectively. In Figure 4, such stretches occurred in 1,604 and 1,507 cm\(^{-1}\), originating from C = C (aliphatic) and C = C (aromatic). The aromatic compound of the EA molecule presented characteristic bands around 906, 822 and 747 cm\(^{-1}\), which are attributed to off-plane folding, where these occur at 900 – 690 cm\(^{-1}\) and are used to define aromatic ring replacement pattern [28].

Table 5 presents results of \(^1\)H and \(^{13}\)C NMR analysis, demonstrating the molecular structure of EA ester, obtained through acetylation reaction. The ester was submitted to detailed analysis of \(^1\)H NMR spectra for 1 and 2 D. Through the NMR spectrum of \(^1\)H (Supplementary Information 1), DEPT 135 (Supplementary Information 2) and Table 5, was observed a dubbing at \(\delta\)H 7.00 ppm \((\text{H of position 6 carbon, d, } J = 8.0 \text{ Hz})\), a voice over at \(\delta\)H 6.96 ppm \((\text{H of position 3 carbon, d, } J = 1.8 \text{ Hz})\) and a double dublet at 6.78 \((\text{H of position 5 carbon, dd, } J = 8.0 \text{ and } 1.8 \text{ Hz})\), indicating a tri-substituted aromatic ring. The position of the substituters in the aromatic ring was performed based on the values of chemical displacements, multiplicity and long-distance correlations. The atoms of the functional group that are closer to the oxygen atom have less shielding (higher value of ppm of chemical displacement), than the atoms closest to a carbon atom (lower value of ppm chemical displacement) [28]. Thus, of the 12 carbon signals expected for EA that are observed in the DEPT 135 spectrum, the carbon signal of position 12 is highlighted, according to Table 5, which corresponds to the carbonyl group of the ester with the highest displacement of 169.0 ppm. Additionally, a dublet was observed at \(\delta\)H 3.38 \((\text{H of position 7 carbon, d, } J = 6.8 \text{ Hz})\) and two multiplets in \(\delta\)H 6.00 \((\text{H of carbon of position 8, m})\) and \(\delta\)H 5.12 \((\text{H carbon of position 9, m})\) combined with the correlations of the HMBC (Supplementary Information 3). It is possible to assign this behavior to the isoprenic unit (alilic group) as one of the substituents linked to the aromatic ring. The presence of two singlets in \(\delta\)H 3.76 \((\text{H of carbon from position 10, s})\) and 2.25 \((\text{H of carbon of position 11, s})\) are attributed respectively to hydrogens of the methoxy group and hydrogens of the acetoxi group, associated with data from DEPT 135. It is allowed to characterize the molecular structure of the EA obtained in the acetylation of eugenol with acetic anhydride using the enzyme Lipozyme\(^\text{®}\) TL 100L as catalyst. Santos et al. [29] analyzed the molecule of EA through NMR, verifying the peaks of the additional acetyl group (C-CH\(_3\)) with lower chemical displacement value, and -COCH\(_3\) with higher ppm value were observed in the spectra.

**Table 5** \(^1\)H and \(^{13}\)C NMR data (DMSO-d\(_6\), 600 and 150 MHz) of eugenyl acetate ester (\(\delta\) in ppm and \(J\) in Hz)
## Eugenyl Acetate

| Position (Carbon) | Hydrogen Displacement ($\delta$H) | Carbon Displacement ($\delta$C) |
|-------------------|----------------------------------|-------------------------------|
| 1                 | -                                | 138.1                         |
| 2                 | -                                | 151.2                         |
| 3                 | 6.96 (d$^1$; 1.8)                | 113.2                         |
| 4                 | -                                | 139.2                         |
| 5                 | 6.78 (dd$^2$; 1.8; 8.0)          | 120.7                         |
| 6                 | 7.00 (d$^1$; 8.0)                | 123.0                         |
| 7                 | 3.38 (d$^1$; 6.8)                | 39.8                          |
| 8                 | 6.00 (m$^3$)                     | 137.9                         |
| 9                 | 5.12 (m$^3$)                     | 116.3                         |
| 10                | 3.76 (s$^4$)                     | 20.7                          |
| 11                | 2.25 (s$^4$)                     | 56.0                          |
| 12                | -                                | 169.0                         |

$^1$ d: doublet; $^2$ dd: double doublet; $^3$ m: multiplet; $^4$ s: singlet.

## Conclusions

In this work, eugeny1 acetate synthesis from clove oil through liquid lipase-mediated acetylation was investigated. Among the enzymes tested, the lipase Lipozyme® TL 100 L presented the better performance in terms of feedstock conversion. Via statistical design, the best condition found for the EA production was 55 °C, eugenol to acetic anhydride molar ratio of 1:1, enzyme load of 10 wt% and time of 2 hours of reaction, where a conversion of 91.80 % was obtained. The variables that had a significant influence on the ester synthesis were temperature and catalyst load. Infrared spectroscopy and nuclear magnetic resonance confirmed that the compound formed is eugenyl acetate. Considering that data about the enzymatic synthesis of EA using lipases in liquid formulation are scarce, the results of this work serve as basis for future research about the topic. The satisfactory conversions reported support the fact that the soluble lipases-catalyzed synthesis of eugenyl acetate from clove oil is an interesting alternative to the conventional chemical route.

## Declarations
**Conflicts of Interest:** The authors have no conflicts of interest to declare that are relevant to the content of this article.

**Ethical Statement:** This article does not contain any studies with human participants or animals.

**Consent to Participate:** All authors agree mutually with the participation and publication of this work and declare that this is an original research.

**Data Availability Statement:** All data generated or analyzed during this study are included in this published article (and its supplementary information files).

**Authors’ Contributions:**

*Leandro Santolin:* Investigation; Writing (original draft preparation)

*Karina G. Fiametti:* Conceptualization; Investigation; Supervision

*Viviane da Silva Lobo:* Data Curation; Resources

*João H. C. Wancura:* Writing (review and editing), Validation

*J. Vladimir Oliveira:* Validation; Resources

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**Figures**
Figure 1

Pareto diagram presenting the effect of molar ratio, temperature and enzyme load on EA production
Figure 2
Response surface for EA conversion varying (a) temperature and lipase load (b) eugenol to acetic anhydride molar ratio and temperature and (c) eugenol to acetic anhydride molar ratio and lipase load
Figure 3

Time course of the eugenyl acetate synthesis. Assays performed in triplicate.
Figure 4

FT-IR spectrum for the eugenyl acetate sample

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