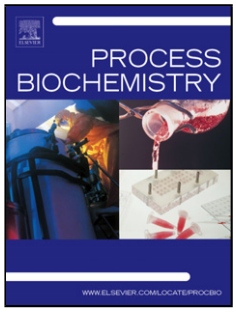

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Synthesis of eugenyl acetate by immobilized lipase in a packed bed reactor and evaluation of its larvicidal activity

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Abstract

Eugenol esters, including eugenyl acetate, has been intensively investigated because of its beneficial antioxidant, antimicrobial, antitumor and potential larvicidal properties. Recent studies verified that small amounts of eugenol esters are effective against the development of larvae of *Aedes egytpti*. Packed Bed Reactors (PBR) have been employed for the synthesis of a variety of esters, as it is essential to assess some process parameters such as molar ratio of substrates, operating temperature and reaction residence time. Based on these aspects, the objective of this study was to evaluate the effect of molar ratio of eugenol and acetic anhydride, reaction temperature and substrates flow rate on the synthesis of eugenyl acetate esters in a packed bed reactor, using Lipozyme TL IM lipase as catalyst, and to determine the larvicidal activity of the obtained ester against larvae of *Aedes aegypti*. The optimal condition was obtained with flow rate of 0.1 $mL.min^{-1}$, 55 °C and 1:5.82 (eugenol:acetic anhydride) molar ratio, affording a conversion value of about 93%. Further, the potential toxicity of *Aedes aegypti* larvae increased under the effect of eugenyl acetate, presenting a LC_{50} of 0.102 $mg.mL^{-1}$, which demonstrates its usability as a natural compound that can be employed in commercial larvicidal formulations.

Keywords: eugenol, eugenyl acetate, lipase, packed bed reactor.

Highlights

- ✓ Eugenol esters are effective against the development of larvae of *Aedes egytpti;*
- ✓ Conversion of eugenyl acetate of 80% was obtained in PBR reactor;
- ✓ Eugenyl acetate showed moderate larvicidal activity (LC_{50} = 0.102 $mg.mL^{-1}$);
- ✓ Potential of eugenyl acetate ester as larvicidal agent.

1. Introduction

According to the Brazilian Ministry of Health only the first half of 2016 were recorded more than 1,500,535 cases of dengue, 271.824 cases of Chikungunya fever, 215.319 cases of fever by *Zika* virus and 10,867 cases of microcephaly in newborns, which has worried the authorities sanitary. The *Aedes aegypti* mosquito is the main transmitter of these diseases in tropical countries of South America [1, 2]. The main mosquito control method is done using insecticides and larvicides, which are sprayed in favorable locations to larval reservoirs. The main synthetic larvicides marketed are composed of substances such as pyrethroids, organophosphates, carbamates and organochlorines [3]. However, prolonged use of synthetic larvicides causes an increase of the young mosquito resistance, besides impacts to the environmental [4].

Eugenyl acetate (4-allyl-2-methoxifenol acetate) is a derivative of eugenol, which can be obtained naturally in the essential clove oil *Syzygium aromaticum* [5]. This essential oil can be obtained by flowers and leaves by steam distillation [6], solvent extraction or extraction with CO_2 supercritical [7], however, generally, the concentration of eugenyl acetate is less than 10% of the essential oil. This is a pale yellow liquid compound, [8] considered safe to be used in food products by Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) and European Food Safety Authority (EFSA) [9,10]. This phenylpropanoid has been studied for presenting potential bioactive properties such as

antimicrobial [11, 12], medical [13,14] and larvicidal [8]. Moreover, recent studies verified that small amounts of eugenyl acetate are effective against the development of *Aedes aegypti* mosquito larvae [15, 8].

Enzymatic synthesis processes of esters become an alternative to replace chemical synthesis processes because they occur in milder conditions of temperature, pH and pressure producing less harmful effluents to the environment. In addition, it fits perfectly into the concept of green chemistry, for being an environmentally friendly process type with the use of fewer reagents in absence of solvents [16-20]. The obtained eugenyl acetate ester can occur through acetylation reaction catalyzed by immobilized lipases in solvent free systems in batch reactors [11, 12]. However, this procedure has some disadvantages such as the necessity for further separation steps of the catalyst from the reaction medium [19]. Thus, the Packed Bed Reactor (PBR) appear as a more advantageous alternative esters production via enzymatic catalysis compared to the batch mode. This is because PBR possess ease of operation in continuous mode, enzymes stability guarantees, possibility of catalyst recycling, contrariwise, the absence of further complex separation and purification steps of the product in the final process, due to the remaining immobilized enzyme in the reactor bed [19,20]. The absence of separation steps of the immobilized enzyme at the end of the process, due to its remaining in the reactor bed, decreases the costs with purification steps of the final product. It turns interesting the use of this reactor type in an economic point of view for industrial application [21, 22].

In this context, the proposal for the use of environmentally friendly larvicides substances is an alternative to minimize the effects caused by conventional larvicides. Thus, the objective of this study is to optimize the synthesis process of eugenyl acetate ester in packed bed reactor

(PBR) with Lipozyme TL IM enzyme as a catalyst and evaluate the larvicidal activity of the ester against to the *Aedes aegypti* mosquito larvae.

2. Materials and methods

2.1. Materials

The clove essential oil (*Syzygium aromaticum* (L.) Merr. & L.M.Perry - *Myrtaceae*, (Ferquima Indústria e Comércio de Óleos Essenciais– Brazil), obtained by steam distillation of clove leaves). According to the company that supplies the essential oil, the main compounds present in essential oil are eugenol (83.4 %), eugenyl acetate (0.04%), β-cariophyllene (11.4 %), α-humulene (3.1%). The clove essential oil and acetic anhydride (Vetec, 98% purity) were used as substrates for the acetylation reactions. Commercial lipase Lipozyme TL IM (produced via *Thermomyces lanuginosus* by submerged fermentation and immobilized on silica gel) was kindly donated by Novozymes (Brazil, Araucaria- PR). The standard sample of eugenyl acetate was obtained from Sigma-Aldrich (Fluka, 99% purity).

2.2. Eugenyl acetate synthesis in fixed packed bed reactor (PBR)

Eugenyl acetate was obtained by the acetylation of acetic anhydride with eugenol (clove essential oil) in solvent-free system. The enzymatic synthesis of eugenyl acetate was conducted in a fixed packed bed reactor (PBR) with a nominal capacity of 10 mL and 55 mm in length and with a 15 mm diameter. The system was composed of 9 g of immobilized enzyme column packed added to the reactor, and then the reactor column was subjected to temperature stabilization with the aid of the thermostatic bath circulation (MICROQUIMICA, Santa Catarina-Brazil). Thus, the feed solution containing the substrate and the acyl donor grouping for the acetylation reaction was pumped with the aid of a peristaltic pump (Watson-

Marlow, São Paulo-Brazil) with upward flow in the power flow established for each assay. Fig. 1 shows scheme of the experimental set up employed under present investigation.

2.3. Effect of flow rate in the enzymatic acetylation reaction of eugenol $_{in}$ PBR

Feed rates of the study tests for the conversion of eugenyl acetate ester in packed bed reactor with Lipozyme TL IM were made varying the flows in 0.1, 0.8 and 1.35 mL.min^{-1}, and 1:3 eugenol:acetic anhydride molar ratio, and temperature (55 °C). The void ratio was calculated via the following equation [23]. All assays were performed in triplicate.

$$\varepsilon = \frac{V_{void}}{V} \qquad \textbf{(Eq. 1)}$$

Where: V_{void} is the void volume of packed-bed reactor (mL) and V is the bed volume of the whole reactor (mL). The void volume was measured by injecting distilled water to the packed-bed reactor. The residence time was calculated according to the following equation [24]:

$$\tau = \frac{\varepsilon \times V_t}{q} \qquad \textbf{(Eq. 2)}$$

Where: ε denotes the void fraction, V_t is the total bed volume (mL) and q is the substrates (eugenol + acetic anhydride) flow rate (mL.min^{-1}). The pump was manually calibrated in accordance with the conditions established for each assay. The residence time is the time required for the solution go through the entire reactor bed, and was determined by the ratio between the flow rate and the volume of the reactor [22]. Thus due to various feed flow rates each assay had a defined residence time.

2.4. Optimization of eugenol acetylation reaction in fixed packed bed reactor with Lipozyme TL IM

From the results obtained for the tests to assess the proper feed flow rate for the ester conversion, the effects of temperature and molar ratio were evaluated using a Central Composite Rotatable Experimental Design (CCRD) 2^2, whose coded levels and actual values of the variables are shown in Table 1. The ANOVA statistical analysis was performed with the help of STATISTICA 13 program. The calculation of the relative error deviation of experimental and predicted conversion values of CCRD is demonstrated in Eq. (3).

$$\% \text{ Relative error deviation} = \frac{\%\text{Exp. Conversion} - \%\text{ Pred.Conversion}}{\%\text{ Exp.Conversion}} \times 100 . \quad \textbf{(Eq. 3)}$$

2.5. Ester analysis

2.5.1. urier Transform Infrared Spectroscopy (FTIR) analysis

Samples of product (eugenyl acetate) was subjected to FTIR analysis and the spectra were obtained using a Care 600 Series FTIR Spectrophotometer (Agilent Technologies, USA). The amount of 10 wt % of the sample was mixed and ground with 100 wt % of potassium bromide and then compressed into a pellet under a pressure of 80 kN, for about a minute, using Graseby Specac Model: 15.011. For each sample, the mean of 20 scans in the range 400-4000 cm^{-1} and the resolution of 4 cm^{-1} was done. The measurements of the samples were normalized by the air background.

2.5.2. Eugenyl acetate quantification

The reaction conversion to eugenyl acetate esters was determined by the method described by [11] with modifications. Samples were analyzed by gas chromatography (Shimadzu GC-2010, São Paulo-Brazil) equipped with a data processor, using a capillary column of fused

silica ZB-WAX (30 m×250 μm×0.25 μm) and flame ionization detector (FID), with programmed temperatures of 40 °C (8 min), 40-150 °C (10 °C.min^{-1}), 150-220 °C (10 °C.min^{-1}), and 220 °C (5 min), injector temperature of 250 °C, detector at 250 °C, injection in split mode, ratio of split 1:100, N_2 (56 kPa and 2 mL.min^{-1}) as carrier gas, injected in volume 1 μL, and sample diluted in dichloromethane (2:10). Reaction conversion was calculated measuring the reduced area of the limiting reagent based on the reaction stoichiometry.

2.5.3. Gas Chromatography and Mass Spectrometry GC-MS analysis

The determination of the chemical composition of the clove oil and the eugenyl acetate sample obtained through the acetylation reaction catalyzed by the enzyme Lipozyme TL IM was performed according to the modified methodology of Guan et al. [7]. 1 μL of samples solution (1% v/v in chloroform) was analyzed by a gas chromatograph equipped with a mass spectrophotometer (GC/MS, model 7890 A, mass detector 5975C, Agilent Technologies, USA), attached to a HP-5MS column (30 m x 0.25 mm internal diameter x 0.25 μm film thickness, Agilent Technologies, USA). The carrier gas was helium with flow rate of 1 mL.min^{-1}, split ratio of 1:100, injector temperature of 240 °C and detector temperature of 250 °C, while column temperature was linearly programmed from 70 to 180°C at a rate of 3 °C.min^{-1}. The main components of essential oil and eugenyl acetate were identified by comparing their mass spectra and retention times with NIST 11 mass spectral library available on equipment. This analytical procedure was performed at Laboratório Central de Análises – UFSC.

2.6. Larvicidal activity

2.6.1. Rearing of *Aedes aegypti*

Aedes aegypti eggs were collected as methodology described by Santos et al. [25] in the city of Aracaju, Sergipe state, Brazil, and laboratory reared at the Federal University of Sergipe insectary at 26 ± 2 °C and 60 ± 10% relative humidity under a 12:12 h light:dark cycle. They are known to be resistant to temephos. Eggs of Rockefeller were kindly donated by the Laboratório de Fisiologia e Controle de Artrópodes Vetores (Laficave, Fiocruz, Rio de Janeiro, Brazil). Adults were provided with a 10% sucrose solution ad libitum. Assay eggs were obtained attached to paper strips. All bioassays were conducted in a walk-in environmental chamber with these environmental conditions.

2.6.2. Larvicidal assay

Assays were carried out following the methodology described by Thangam and Kathiresan [26] with some modifications. Third-instar larvae were used in the experiment. The concentration ranges were determined by a previous concentration response curve with 20 larvae. It was added 20 mg of the extract containing 98% eugenyl acetate, 1.2% β-caryophyllene and 0.8% α-humulene to a 1 mL Eppendorf vial and dispersed in Tween-80 (0.1 mL). DMSO (0.3 mL) was added, and the mixture was stirred until a clear stock solution was obtained. Natural mineral water (0.6 mL) was added to make a standard solution (20 $mg.mL^{-1}$).

The stock solution was used to make 20 mL water solutions with five concentrations ranging from 0.0005 to 2 $mg.mL^{-1}$. Twenty larvae were collected with a Pasteur pipette, placed on a 25 mL graduated cylinder. The volume was completed to 20 mL with natural mineral water and transferred to disposable cups containing variable volumes of the stock

solution. A mortality count was conducted 24 h after treatment. Controls were prepared with Tween-80 (0.1 mL), DMSO (0.3 mL) and water (19.6 mL). Three replicates were used for each concentration and the control.

Probit analysis was conducted on mortality data collected after 24 h exposure to different concentrations of testing solutions to establish the lethal concentration for 50 % mortality (LC_{50}) and 95% confidence interval values for the eugenyl acetate. In all cases where deaths had occurred in the control experiment, the data were corrected using Abbott's Eq. (4). In order to verify whether the variation in potency following a modification in structure was statistically significant, the results were further analyzed using ANOVA analysis of variance, followed by Tukey's test. A significance level of 5% was set for the analysis [27].

$$\%\text{Deaths} = 1 - \frac{test}{control} \times 100 \qquad \textbf{(Eq. 4)}$$

Where: *% Deaths* is mortality rate experiment, *Test* is number of deaths in the test and *Control* is number of deaths in control.

3. Results and discussion

3.1. Effect of flow rate in the enzymatic acetylation of eugenol in PBR

The residence time is the one of the most important parameter to obtain high conversion values of product of interest in packed bed reactors [21, 22]. Thus, for the synthesis of eugenyl acetate through acetylation reaction of eugenol by employing Lipozyme TL IM

enzyme as catalyst in a continuous mode fixed packed bed reactor, a preliminary study was conducted of the feed flow rate. Assays were performed with flow rates of 0.1 $mL.min^{-1}$, 0.8 $mL.min^{-1}$ and 1.35 $mL.min^{-1}$ with retention time of 55, 7 and 4 min, respectively, with temperature of 55 ºC and 1:3 eugenol:acetic anhydride molar ratio. Conversions ranged from 83.5 % to 62.4 %, the highest conversion obtained was with the value of 0.1 $mL.min^{-1}$ feed flow rate as demonstrated in Fig. 2.

In reactors, operating in continuous production system may experience different behaviors depending on the flow solution within the bed. In this study, the flow rate values greater than 1 $ml.min^{-1}$ can cause the reduction of ester conversion due to increased shear stress of the fluid, which can change the structure of the lipase. Behavior confirmed in this work, a drop of approximately 20% conversion in the test with the flow of 1.35 $mL.min^{-1}$ was observed. With respect to smaller flow values imply longer residence times, which increases the conversion of the ester due to increased contact time between the substrate and the enzyme, what increases mass transfer [28]. The similar behavior was verified by Dahlan, Kamaruddin and Najafpour [28] and Dalla Rosa et al. [20] in the synthesis of esters by esterification reaction catalyzed by immobilized lipase in packed bed reactor, where the increasing residence time was also increased the conversion of esters.

There are few studies of essential oils esters production with Lipozyme TL IM enzyme in fixed packed bed reactors. Nevertheless, Yang et al. [29] achieved an interesterification of 56.18% in the medium chain triglycerides synthesis from soybean oil using Lipozyme RM IM and Lipozyme TL IM as catalysts, and Sutili et al. [30] in the acetylation reaction of ketal of the fructose and palmitic acid achieved 91.1% using Lipozyme TL IM as catalyst with few feed flow values in the fixed packed bed reactor.

Knowing the mechanisms of the reaction under study, we know that autocatalysis can occur, so we performed tests in the conditions studied without the biocatalyst and observed that the autoconversion was not greater than 5% in all tests (results not shown). Thus, a 5% conversion value was discounted for all other conversion calculations.

3.2. Optimization of eugenol acetylation reaction in fixed packed bed reactor

The optimization of the production of eugenyl acetate in fixed bed packed reactor (PBR) was made using the value of feed flow rate of 0.1 $mL.min^{-1}$. Thus, a CCDR 2^2 was done too, added with 3 center points to evaluate the effects of the molar ratio of clove oil (eugenol) to acetic anhydride and the temperature for the synthesis of the ester in a PBR. Table 1 shows the matrix and the results of the experiment in real and predicted terms of eugenyl acetate conversion.

The maximum eugenyl acetate conversion achieved were in assay 6 with an average conversion values of 93% within the first 3 h of reaction with 0.1 $mL.min^{-1}$ flow rate, 1:5.82 eugenol: acetic anhydride molar ratio and 55 °C. Run 3 (1:1 eugenol: acetic anhydride molar ratio) presented 29.37% and the run 5 (1:0.18 eugenol: acetic anhydride molar ratio) presented 10.22% of eugenyl acetate conversion, the smallest values of ester conversion. All reactions showed stability from 2 h of reaction.

In general, when the reaction mixture contains low substrate concentrations, there is a decrease in donor agent disposal of acyl grouping for the reaction which implies low yield even at elevated temperatures [31, 32], situation confirmed in this study, by run 5.

The increment of the reaction temperature from 50 to 60 °C did not possess effect on the synthesis of eugenyl acetate ester causing the fall of the ester conversion percentage, even in assays with higher substrate availability. This reduction may have been caused due to a

denaturation of the enzyme [32]. On the other hand, Lipozyme TL IM enzyme is stable in a temperature range of 50-70 °C, but used in fixed packed bed reactors the enzyme is more stable at moderate temperatures, between 50-60 °C [29, 30], confirming that higher conversion values are obtained at temperatures around 55 °C. Damnjanović et al. [33] also observed this behavior in the geranyl butyrate synthesis using nonspecific lipase immobilized AY (Type VII, L 1754) and temperature 35 °C presented the best thermostability performance, obtaining 78.9% of ester conversion in 10 h of reaction. Dahlan, Kamaruddin; Najafpour [28] observed a similar behavior by studying the effect of temperature in the biosynthesis of citronellyl butyrate using immobilized lipase from *Candida rugosa* in packed bed reactor, they observed that temperatures above 50 °C in this system there was a drop in ester conversion. In that way, it appears possible to obtain high ester conversion values using milder temperatures with longer reaction, conditions that favor the application of fixed packed bed reactors compared to batch processes.

Successively, data obtained from the central composite rotatable experimental design for conversion as a function of temperature and eugenol to acetic anhydride molar ratio were analyzed by analysis of variance (ANOVA), and was used to assess the goodness of fit of the coded model (Table 2). Based on F-test, the conclusion can be drawn that the model is predictive. In fact, the F calculated value ($F_{calc.}$) obtained was 5 times greater than the F tabulated value ($F_{tabl.}$) with a regression coefficient satisfactory enough (0.96). This study led to the Equation 5. Therefore, the coded model expressed by Equation (5) has been used to generate the contour plot of Fig. 3 and to predict the effects of experimental conditions on eugenyl acetate production (column 5 of Table 1). Results of experimental design and data of Table 1 suggest that it is convenient to use an eugenol to acetic anhydride molar ratio of 1:5.82 and temperature of 55 °C to achieve high conversions (93.1%) of eugenyl acetate.

$$\%AE = 73.71 + 25.94 \times MR - 12.91 \times MR^2 \quad \textbf{(Eq. 5)}$$

The contour plot analysis proved to be a useful tool for obtaining esters in a fixed packed bed reactor. By evaluating the flow and subsequent experimental design with variables such as molar ratio (eugenol: acetic anhydride) and reaction temperature, it was possible to obtain the optimum condition for the synthesis of eugenyl acetate ester in a fixed packed bed reactor with Lipozyme TL IM [30, 34, 35].

3.3. Operational stability of Lipozyme TL IM in the production eugenyl acetate in Packed-Bed Reactor (PBR)

The operational stability of the immobilized enzyme in a packed bed reactor is an important parameter to be evaluated with a view to industrial production because the ester directly influences the cost [21]. Thus, the stability enzymatic (Lipozyme TL IM) in the reaction of acetylation of eugenol was carried out using the optimal conversion conditions, with flow rate of 0.1 $ml.min^{-1}$, temperature of 55 °C and molar ratio of 1:5.82 (eugenol:acetic anhydride) for 28 hours of reaction. As can be seen in Fig. 4, the conversion of eugenyl acetate decreased considerably after 9 hours, reaching less than 30% after 24 hours of reaction. This loss in the conversion of esters is related by some authors to the inhibition of enzymatic activity by the excess of acetic anhydride [36] or by the deformation of the enzyme support caused by the shear forces inside the reactor [22]. Other authors state that the ease of bed obstruction, the appearance of flow path preferences and inefficiencies in heat and mass transfer can also influence the loss of ester conversion [28].

3.4. Eugenyl acetate identification

3.4.1. Fourier Transform Infrared Spectroscopy (FTIR) analysis

The wavelength values obtained in the analyzes of eugenyl acetate they are represented in Fig. 5, where is observed the presence of the characteristic carbonyl band of the ester linked to the aromatic ring at 1766 cm-1, confirming that only the acyl group was added to the eugenol molecule [37]. The spectra obtained in this study were similar to those found by Affonso et al. [38], in the quantification and characterization of the main components of ethanol extract of *Syzygium aromaticum* clove oil.

3.5. Larvicidal assay

The extract containing 98% eugenyl acetate, 1.2 % β-caryophyllene and 0.8 % α-humulene was tested for its larvicidal activity. Both terpenes cited have larvicidal potential, but some studies reported that β-caryophyllene (1.202 $mg.mL^{-1}$) and α-humulene (0.018 $mg.mL^{-1}$) have an LC_{50} value greater than eugenyl acetate (0.107 $mg.mL^{-1}$) [39,40,15].

In this study, it was obtained a LC_{50} of 0.102 $mg.mL^{-1}$ with a 95% confidence interval (0.095 to 0.110 $mg.mL^{-1}$), that way the amount of eugenyl acetate easily removed 50 % of the *Aedes aegypti* mosquito larvae. Pandey et al. [8] and Barbosa et al. [15] obtained LC_{50} of 0.050 $mg.mL^{-1}$ and 0.113 $mg.mL^{-1}$, respectively in assays of eugenyl acetate toxicity front larvae of the same species of mosquito.

Terpenes and phenypropanoids derivated of other essential oils had been studied due to their larvicidal potential effect. The larvicidal activity of different essential oils was tested [41], for example in *Syzigium aromaticum* (L.) Merr. & Perry, *Lippia sidoides* Cham, and *Hyptis martiusii* Benth against *Aedes aegypti* larvae and got a LC_{50} of 0.0214, 0.0195 and 0.0185 $mg.mL^{-1}$, respectively. Govindarajan et al. [42] evaluated the essential oil toxicity of *Plectranthus barbatus* and its main constituents, against larvae of the *Anopheles subpictus* malaria vector, *Aedes albopictus* and *Culex tritaeniorhynchus* dengue vector principal cause

of Japanese encephalitis. Lethal concentration values were obtained (LC_{50}) 0.084, 0.0944 and 0.0872 mg.mL^{-1} and 90% (LC_{90}) values of 0.165, 0.170 and 0.179 mg.mL^{-1}. However, it is suggested that the larvicidal effect conferred by the essential oils is not only given to one major component but to a synergistic effect of many components that enhance the action against the larvae. Eugenyl acetate compound constitutes only 5% of essential clove oil, its hydrophobicity (verified in this study) favors the larvicidal activity, together with the presence of an aromatic ring and a side chain with a allylic double bond turning the compound more reactive, able to act on cell wall membranes of the larvae [8].

Although some LC_{50} values are lower than those obtained in that work are, it is noted that there is not a thorough study of obtaining these compounds or derivatives via enzymatic synthesis. Some studies use acid catalysis for the synthesis of compounds derived of essential oils, which is often detrimental for employing elevated temperatures and forming effluents.

4. Conclusions

Continuous synthesis in the packed bed reactor with lipase Lipozyme TL IM showed to be a promising alternative for obtaining eugenol esters. It was possible to obtain the optimum condition for the acetylation reaction of eugenol, reaching a value of 93.1% of eugenyl acetate conversion. The ester proved to be a potential larvicidal with LC_{50} of 0.102 mg.mL^{-1} against *Aedes aegypti* mosquito larvae. Confirming the potential of this ester as environmentally friendly larvicidal agent, by the production and application to be completely safe for the environment unlike conventional larvicidal.

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Figure Captions

Fig. 1. Experimental setup used to eugenyl acetate synthesis (1) substrate mixing chamber; (2) peristaltic pump; (3) Packed Bed Reactor (4) substrate inlet (5) product outlet (6) sampling; (7 e 8) cooling/heating water.

Fig. 2. Effect of flow rate (0.1, 0.8 and 1.35 $mL.min^{-1}$, with retention time of 55, 7 and 4 min, respectively) on eugenyl acetate production in PBR at fixed values of temperature (55 ºC) and eugenol:acetic anhydride molar ratio (1:3).

Fig. 3. Contour plot of eugenyl acetate conversion as a function of temperature and eugenol to acetic anhydride molar ratio.

Fig. 4. Evaluation of operational stability of Lipozyme TL IM in the reaction of acetylation of eugenol using the optimal conversion conditions, with flow rate of 0.1 $mL.min^{-1}$, temperature of 55 °C and molar ratio of 1:5.82 (eugenol:acetic anhydride) for 28 hours of reaction

Fig. 5. Infrared spectra for the sample of eugenyl acetate obtained by acetylation catalyzed by Lipozyme TL IM.

Table 1 - Matrix of the Central Composite Rotatable Experimental Design 2^2 (coded and real values) with responses in terms of eugenyl acetate conversion.

Run	MR	T (ºC)	% Experimental conversion	% Predicted conversion	% RED
1	-1 (1:1)	-1 (50)	37.6	34.8	7.3
2	1 (1:5)	-1 (50)	79.0	86.7	-9.7
3	-1 (1:1)	1 (60)	29.4	34.8	-18.7
4	1 (1:5)	1 (60)	77.9	86.7	-11.3
5	-1.41(1:0.18)	0 (55)	2.9	11.4	-12.2
6	1.41 (1:5.82)	0 (55)	93.1	84.6	9.1
7	0 (1:3)	0 (55)	84.6	73.7	12.8
8	0 (1:3)	-1.41(1:0.18)	65.3	73.7	-12.8
9	0 (1:3)	1.41 (1:5.82)	73.6	73.7	-0.10
10	0 (1:3)	0 (55)	73.2	73.7	-0.69
11	0 (1:3)	0 (55)	74.2	73.7	0.68

*MR= Molar Ratio eugenol: acetic anhydride.
*% RED= relative error deviation

Table 2- Results of regression analysis of Central Composite Rotatable Experimental Design 2^2.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	$F_{calculed}$
Regression	6522.42	5	1304.48	27.03
Residual	241.33	5	48.27	
Total	6763.75	10		

*Regression coefficient: R^2=0.96

*F 0.95; 5;5 =5.05, significant at the level *p*= 95%

Figure 1

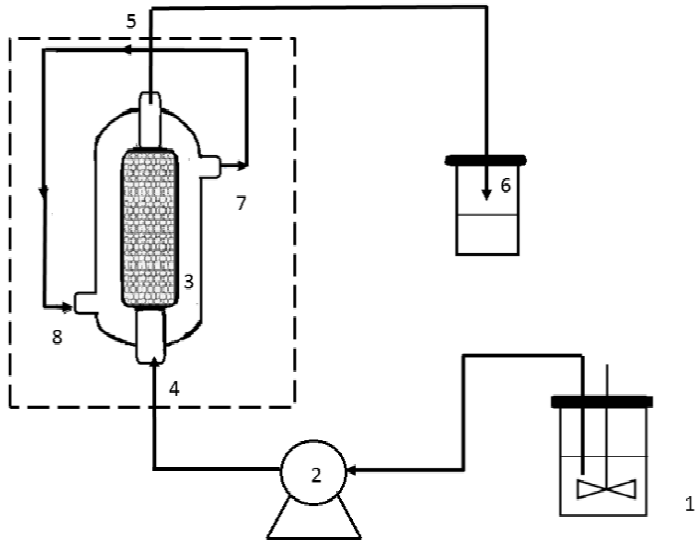


Figure 2

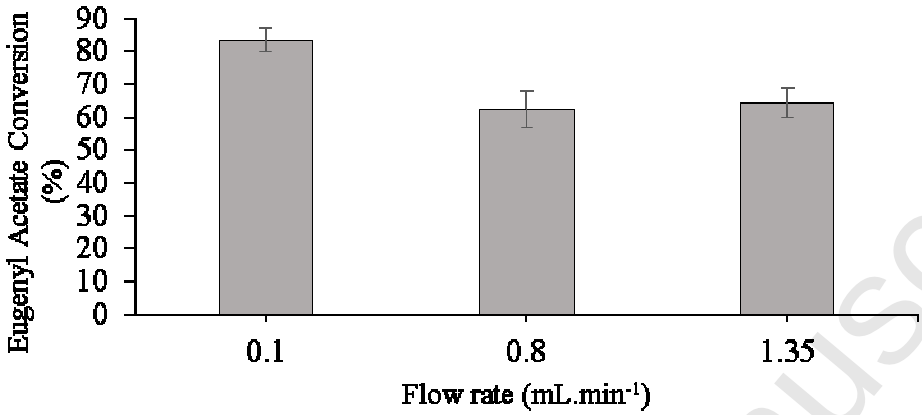


Figure 3

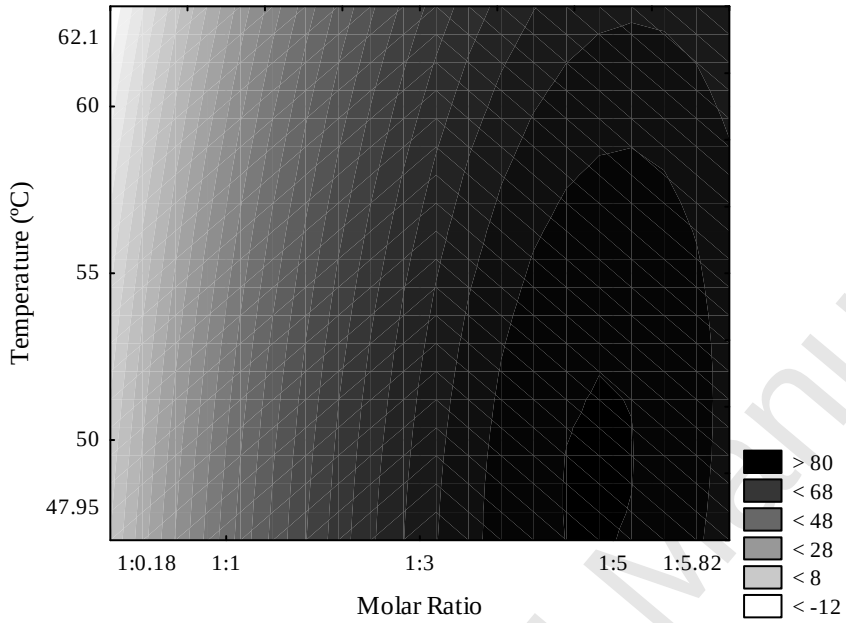


Figure 4

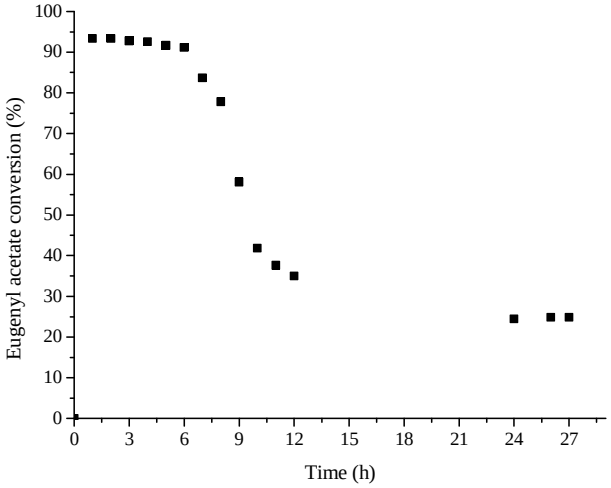
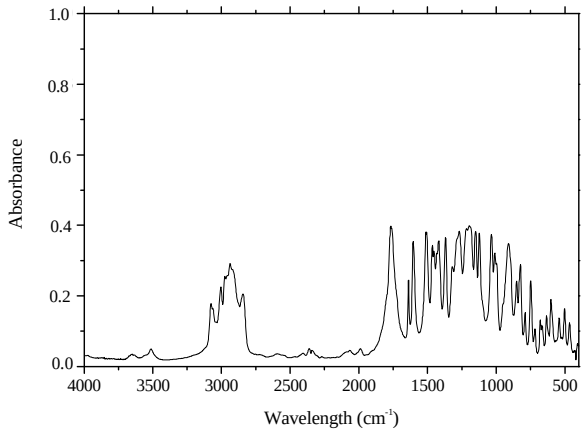
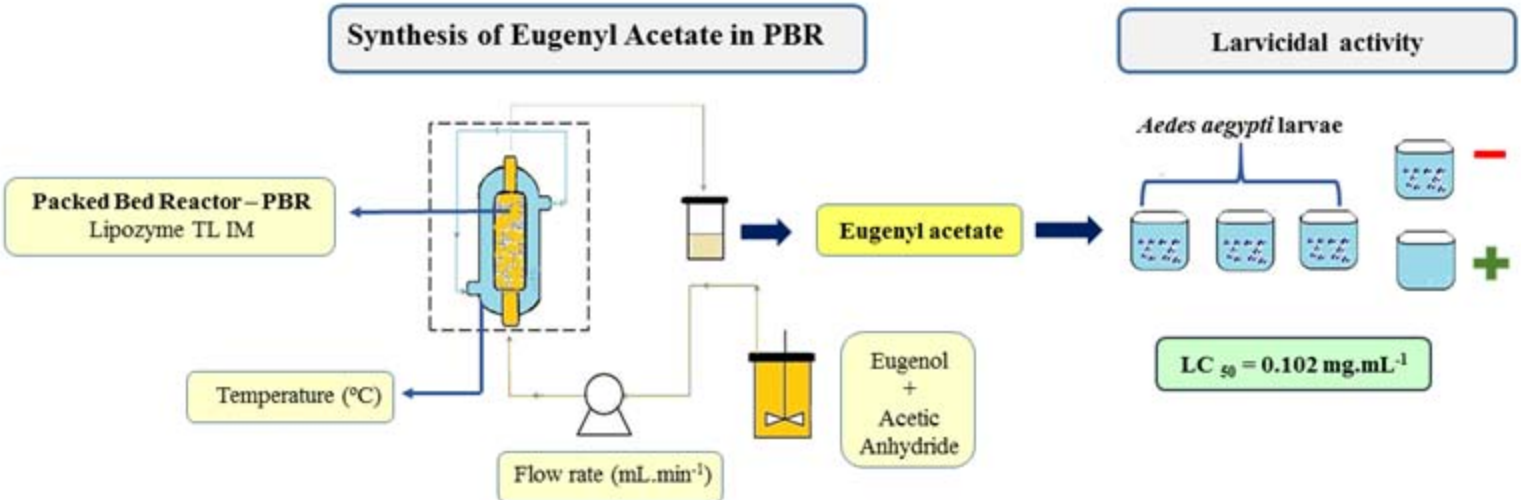


Figure 5




Synthesis of Eugenyl Acetate in PBR
Larvicidal activity
Packed Bed Reactor – PBR
Lipozyme TL IM
Temperature (°C)
Flow rate (mL.min-1)
Eugenol
+
Acetic
Anhydride
Eugenyl acetate
Aedes aegypti larvae
LC 50 = 0.102 mg.mL-1