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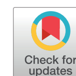
Production of benzyl cinnamate by a low-cost immobilized lipase and evaluation of its antioxidant activity and toxicity



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ABSTRACT

In this work was optimized the production of benzyl cinnamate by enzymatic catalysis using the immobilized lipase NS88011 and to evaluate its biological properties. The optimized condition for this system was 1:3 (acid:alcohol) molar ratio, 59 °C, biocatalyst concentration 4.4 $mg.mL^{-1}$ for 32 h, with a yield of 97.6 %. The enzyme stability study showed that the enzyme remains active and yields above 60 % until the 13th cycle (416 h), presenting a promising half-life. In the determination of the antioxidant activity of the ester, an inhibitory concentration necessary to inhibit 50 % of the free radical 2,2-diphenyl-1-picryl-hydrazyl DPPH (IC_{50}) of 149.8 $mg.mL^{-1}$ was observed. For acute toxicity against bioindicator *Artemia salina*, lethal doses (LD_{50}) of 0.07 and 436.7 $\mu g.mL^{-1}$ were obtained for the ester and cinnamic acid, showing that benzyl cinnamate had higher toxicity, indicating potential cytotoxic activity against human tumors.
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1. Introduction

The group of compounds dominating the world market for additives is aromas, flavorings, flavor enhancers, acidulants, and emulsifiers. The flavor industry stands out for accounting about 55 % of the billion dollars of the global chemical market, moving 3.85 billion dollars in 2015 and forecasting a compound annual growth rate (Generally Recognized as Safe - GRAS) of 6.2 % between 2016 and 2024 [1]. The segment of flavors, fragrances, and aromas is composed of products with high value compound when compared to other segments, also adding value to the raw material and being attractive for investments. This sector not only represents a multibillion-dollar market, but also a source of scientific development and constant innovation. In this sense, companies are seeking to produce new flavors and aromas, using more efficient, and low-cost processes [2].

Biocatalysis applied in the production of flavorings is a promising alternative, linked to green chemistry, which has been elected as one of the main areas of emerging research for sustainable manufacturing [3]. From the possible processes catalyzed by enzymes in the organic médium, the synthesis of esters is presented as a very promising aspect, as attested by the processes already industrially implemented. Among the esters of industrial interest are derivatives of cinnamic acid. Some cinnamic acid esters, besides being aromatic, have biological properties, besides being aromatic, have biological properties. In a study conducted by Zanetti et al. [4], geranyl cinnamate ester showed strong antimicrobial properties, being considered microbiologically active in relation to the two microorganisms tested: *Staphylococcus aureus* and *Escherichia coli*. Studies carried out by Dubey et al. [5] showed that ethyl cinnamate presents in *Ocimum gratissimum* essential oil (ethyl cinnamate chemotype) is active against the microorganisms *Scopulariopsis brevicaulis*, *Cryptococcus neoformans* and *Malassezia pachydermatis* and is suitable for dermatology and cosmetic use.

Another ester of industrial interest is benzyl cinnamate, which can be extracted from natural sources (propolis, *Bermuda buttercup* leaves and branches, *Myroxylon pereirae* resin) [6–8] or produced by esterification reactions [9,10]. It is known for having the ability to protect against UV radiation, been widely used in the formulations of sunscreens, fine fragrances, shampoos, soaps, cleaning products, and detergents, in addition to presenting antimicrobial activities [9]. The worldwide use of benzyl cinnamate is in the range of 10–100 metric tons per year [11]. However,

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there is a lack of reports in the literature about the studies of its toxicological properties. The results of synthesis by enzymatic catalysis were already described, indicating the need for high concentrations of enzyme, reducing the viability of the industrial production process. In this context, the present study aims to optimize the production of benzyl cinnamate by enzymatic catalysis in an organic solvent system using a low-cost commercial lipase NS 88011 and evaluate its toxicity and antioxidant activity.

2. Material and methods

2.1. Substrates and solvents

The benzilic alcohol and cinnamic acid (Sigma-Aldrich®) were used as substrates for esterification reaction and n-heptane (Vetec®) as solvent. The commercial lipase used in this work was *Candida antarctica* (NS 88011) immobilized on a hydrophobic polymeric resin (Novozymes®).

2.2. Determination of esterification activity

The esterification activity (EA) of the NS 88011 lipase was quantified through the synthesis reaction of oleic acid and ethanol (mole ratio of 1:3 by volume) [12]. The reaction was started by the addition of 0.1 g of the enzyme to the reaction medium, in glass flasks with lids, kept in a shaker at 160 rpm for 40 min. Aliquots of 0.5 mL were extracted from the reaction medium in triplicate. 15 mL of an acetone-ethanol (1:1) (v/v) solution was added to each solution to stop the reaction. The amount of oleic acid consumed was determined by titration with 0.05 M NaOH until the medium reached pH 11. The blank assays of the samples contained 0.5 mL of the reaction medium and 15 mL of the acetone-ethanol solution. A unit of enzyme activity was defined as the amount of enzyme that consumes 1 mol of fatty acid in one minute, calculated by Eq. (1):

$$AE \ \left(U.g^{-1}\right) = \frac{(V_b - V_a) \times M \times 1000 \times V_f}{t \times m \times V_c} \quad (1)$$

Where:

AE: esterification activity (U/g), V_b: volume of the NaOH spent on titration of the blank sample (mL); V_a: volume of NaOH spent on titration of the sample withdrawn after 40 min (mL); *M*: Molarity of the NaOH solution (mol/L); V_f: total volume of reaction (mL); *t*: time (minutes); *m*: mass of the enzyme (g); and *Vc*: volume of the reaction aliquot taken for titration (mL).

2.3. Optimization of benzyl cinnamate production

The synthesis of benzyl cinnamate was performed from the preparation of a reaction mixture containing cinnamic acid and benzyl alcohol in temperature and enzyme concentration conditions pre-established by the Central Composite Rotational Design 2^2 (CCRD) with triplicate at the central point. Through preliminary tests, the molar ratio was fixed at 1:3 (100 μmol acid and 300 μmol alcohol) and the reaction time at 48 h. Statistica® 5.0 software (Statsoft Inc., USA) was used for statistical analysis and obtaining a coded empirical mathematical model for the production of benzyl cinnamate as a function of enzyme concentration and temperature, with a 95 % confidence level.

2.4. Determination of reaction conversion

Quantitative analyses of benzyl cinnamate produced were conducted in a gas chromatograph (Shimadzu GC-2010) equipped with a data processor using an Inowax capillary column of fused silica (30 m length × 250 mm i.d. × 0.25 mm thickness), flame ionization detector, with the following temperature program: 40–200 °C (20 °C/ min), 200 °C (25 min) with an injector and the detector temperature of 220 °C, injection in the split mode, and ratio of the split of 1:50. H_2 (40 mL.min^{-1}) was used as a carrier gas and a volume of 0.4 mL of sample diluted in n-hexane (1:10) was injected. The quantification of benzyl cinnamate was calculated based on the reduction of the area of limiting reagent based on reaction stoichiometry (Fig. 1) [13].

2.5. Determination of thermal deactivation constant (k_d) and half-life ($t_{1/2}$)

The determination of thermal deactivation constant (k_d) of lipase NS 88011 was calculated at the temperature of the optimal condition (59 °C) according to Arrhenius kinetic model (Eq. (2)), considering that enzyme follows first-order kinetics.

$$A = A_0 e^{(-k_d.t)} \quad (2)$$

Where:

A = final activity; A_0 = initial activity; t = time (hours); k_d = deactivation constant.

The half-life of the enzyme, which corresponds to the period necessary for the residual enzyme activity to decrease to 50 % of its initial value, was calculated by Eq. (3).

$$t_{1/2} = -\frac{\ln 0.5}{k_d} \quad (3)$$

Where:

$t_{1/2}$ = half-life; k_d = deactivation constant.

2.6. Operational stability of the enzyme

The cycles of enzyme reuse were determined from the esterification reaction carried out in the maximization condition obtained after performing the experimental design. After each reaction the enzyme was separated from the reaction medium, washed with 10 mL of n-hexane and left in a desiccator for 24 h at room temperature, and then used again in a new batch. The operational stability of the enzyme was evaluated in terms of production of benzyl cinnamate after each cycle of reuse.

2.7. Isolation of the ester

Benzyl cinnamate was isolated by column chromatography (CC) packed with silica gel 60 (70–230 mesh). As a mobile phase, a

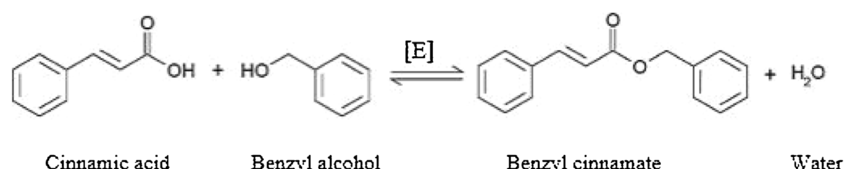

Fig. 1. Esterification reaction for the production of benzyl cinnamate.

mixture of solvents formed by hexane and ethyl acetate was used in different ratios (9:1; 8:2; 7:3) according to the methodology used by Jakovetić et al. [14].

2.8. Determination of antioxidant activity

The antioxidant activity of benzyl cinnamate and its precursors was determined by the spectrophotometric method, which is based on measuring the extinction of absorption of the radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH) at 515 nm following the methodology described by Vanin et al. [15].

The determination of antioxidant activity was performed in triplicate by a spectrophotometric method. The technique consisted of incubation for 30 min in 500 μL of an ethanol solution of DPPH 0.1 mmol/L with 500 μL of solutions containing increasing concentrations of benzyl cinnamate (2.5, 5, 7.5, 10, 25, 50, 75, 100 and 250 mg/mL) in ethanol. We proceeded similarly to the preparation of the solution called “control” but substituting 500 μL of sample to 500-μL ethanol solvent. To a solution called blanc, ethanol was used and the benzyl cinnamate in the same concentration range studied. The percent uptake of the DPPH radical was calculated in terms of the percentage of antioxidant activity (AA%) by Eq. (4):

$$AA\% = 100 - \{[(Abs._{sample} - Abs._{blanc}) \times 100] \div Abs._{control}\} \tag{4}$$

The determination was made in a UV–vis spectrophotometer (515 nm) (Agilent Technologies brand, model 8453E). After evaluating the optimal concentration range, we calculated the concentration of the benzyl cinnamate needed to capture 50 % of the free radical DPPH (IC_{50}) by linear regression.

2.9. Determination of toxicity against A. salina

The toxicity test was carried out in triplicate runs, using the methodology described by Meyer et al. [16] and adapted by Cansian et al. [17]. Solutions containing different concentrations of cinnamic acid or benzyl cinnamate (0.5–100 $\mu g.mL^{-1}$) were prepared in saline water containing 0.1 % dimethylsulfoxide (DMSO), 10 mL of solutions were transferred to a petri dish and 50 *A. salina* nauplii were added. After 24 h, live and dead nauplii were counted. From the mortality data, the value of LD_{50} (lethal dose of the sample for 50 % of the population) was obtained from the linear regression. Tests only with saline water solution and saline water containing 0.1 % DMSO were also performed to confirm that the mortality of nauplii occurred due to the toxicity of the compound and not due to lack of food.

3. Results and discussion

3.1. Optimization of benzyl cinnamate production

From preliminary tests and literature data [9], the molar ratio of reagents was fixed at 1:3 (acid to alcohol) and reaction time at 48 h. Table 1 shows the CCRD 2^2 matrix with the actual and coded values and the responses in terms of conversion into benzyl cinnamate, using 10 mL of solvent (n-heptane). From the results presented in Table 1 it is possible to observe that the highest yield was obtained in assay 6 (97.4 %) for the enzyme concentration of 4.4 $mg.mL^{-1}$ and temperature of 60 °C. Increased yield with increased temperature can be attributed to esterification being an endothermic reaction, however, very high temperatures can lead to thermal inactivation of the enzyme, since lipases are vulnerable to heat. The increase in ester production with increased enzyme concentration is related to the increase in active sites available for the formation of the acyl-enzyme complex.

The statistical data allowed obtaining a coded empirical mathematical model for the production of benzyl cinnamate as a function of enzyme concentration and temperature (Eq. (5)).

$$C(\%) = 75,5 + (30.55 \times E) - \left(10.37 \times E^2\right) - (6.72 \times T) - \left(20.6 \times T^2\right) \tag{5}$$

Where:

C = conversion; E = enzyme concentration; T = temperature.

Significant parameters were considered for Analysis of Variance (ANOVA), the correlation coefficient R (0.99) and calculated F (25.4 times higher than the tabulated F) statistically validated the empirical model for the production of benzyl cinnamate in the studied conditions ($p < 0.05$), allowing the construction of the response surface and the contour plot, which are found in Fig. 2(a and b).

The relative error deviation between the experimental values and those predicted by the model were calculated ($RED = \left[\frac{Yexp - Ymodel}{Yexp}\right] x100$). For the better condition to benzyl cinnamate production (run 6, Table 1), relative error deviation between values predicted by the model and those obtained experimentally was only 0.5 %.

3.2. Evaluation of the reaction time of benzyl cinnamate production

Having as one of the objectives the reduction of the reaction time, a complementary kinetic study was performed in the optimal condition obtained through the empirical model. As can be

Table 1
Central Composite Rotational Design 2^2 (CCRD) matrix with real and coded values and response in terms of benzyl cinnamate conversion.

Run	Enzyme concentration ($mg.\,mL^{-1}$)	Temperature (°C)	Experimental conversion (%)	Predicted conversion (%)	RED* (%)
1	−1 (0.94)	−1(53)	19.8	20.7	4.5
2	1 (3.77)	−1(53)	87.8	81.8	6.9
3	−1 (0.94)	1(67)	6.5	7.26	11.0
4	1 (3.78)	1(67)	62.8	68.4	0.8
5	−1.41 (0.36)	0(60)	12.7	11.8	7.5
6	1.41 (4.36)	0(60)	97.4	98.0	0.5
7	0 (2.36)	−1.41 (50.13)	40.1	44.0	9.8
8	0 (2.36)	1.41 (69.87)	29.2	25.1	14.1
9	0 (2.36)	0 (60)	75.5	75.5	0.0
10	0 (2.36)	0 (60)	71.5	75.5	5.7
11	0 (2.36)	0 (60)	79.5	75.5	5.1

*- relative error deviation.

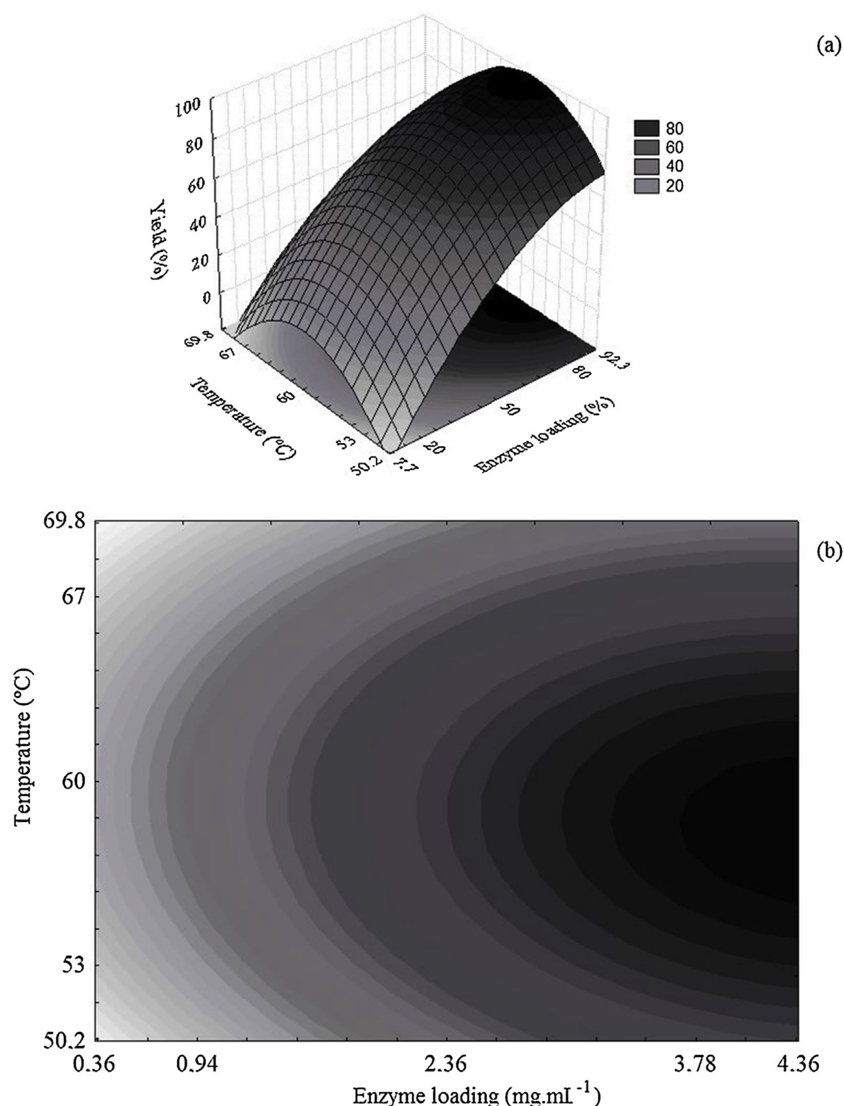

Fig. 2. Response surface (a) and contour plot (b) for the production of benzyl cinnamate as a function of enzyme concentration and temperature.

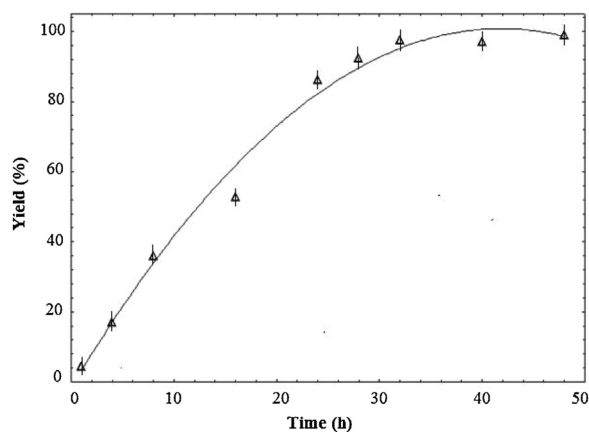

Fig. 3. Kinetics of benzyl cinnamate production using 1:3 M ratio (acid:alcohol), temperature 59 °C, enzyme concentration 4.4 mg.mL^{-1}.

observed (Fig. 3) yields above 90 % were obtained after 28 h of reaction, reaching a maximum of 97 % after 32 h. Therefore, the condition to maximum production of benzyl cinnamate was defined as molar ratio 1:3 (acid to alcohol), temperature 59 °C, 4.4 mg.mL^{-1} enzyme (NS 88011), solvent n-heptane, at 32 h.

There are some reports in the literature about the production of benzyl cinnamate by enzymatic esterification with yields above 90 %. Zhang et al. [18] used the response surface methodology to optimize the production of benzyl cinnamate the optimal condition was: 31 mg.mL^{-1} of commercial lipase Lipozyme TL IM, molar ratio 1:2.6 (acid:alcohol), the temperature of 40 °C, reaction time 27 h in isooctane media. Wang et al. [9], used 30 mg. mL^{-1} of Lipozyme TL IM lipase, solvent isooctane, and the same molar ratio and temperature reported by Zhang et al. [18] during 24 h of reaction. Sun and Tian [19] were able to reduce the amount of Novozym 40086 lipase to 20 mg.mL^{-1} and reaction time to 11.3 h. In our study, it was possible to reduce the amount of biocatalyst 5 times when compared with Sun and Tian [19]. This is an important factor to be considered, since enzymes have a high commercial value, and their reduction can considerably decrease the cost of the process.

3.3. Thermal deactivation constant (k_d) and half-life time ($t_{1/2}$)

The use of enzymes in industrial processes requires an understanding of the operational stability of the enzyme, so

thermal deactivation constant and half-life are basic parameters to be known before designing a process. To demonstrate these parameters the enzymatic activity of lipase was measured at time 0 and after 32 h of reaction at 59 °C (maximized condition). The results are presented in Table 2.

The high enzymes half-life suggests that it can be used for about 505.9 h maintaining 50 % of its initial enzymatic activity, demonstrating that lipase NS 88011 has conditions to be industrially employed in the production of benzyl cinnamate due to its high and efficiency and considerable half-life. We did not find in the literature data on the thermal deactivation constant and half-life time for the production of benzyl cinnamate using other free or immobilized lipases.

3.4. Operational stability of lipase NS 88011

The residual activity of the immobilized enzyme is one of the most important factors for industrial applications that provide important information for evaluating the process economics. To confirm the capacity enzyme reuse and the half-life obtained, operational stability evaluation of enzyme was performed in several operational cycles in the optimized condition: 1:3 M ratio (acid to alcohol), the temperature of 59 °C, enzyme concentration 4.4 $mg.mL^{-1}$, solvent n-heptane, reaction time 32 h. The results obtained are shown in Fig. 4.

It was possible to observe that conversions above 80 % were obtained until the 7th operational cycle and above 60 % until the 13th cycle, which corresponds to 416 reaction hours confirming the good operational stability of NS 8801 lipase.

Despite being a *C. antarctica* fraction B immobilized, the same fraction commercially known as Novozym 435 lipase, NS 88011 lipase is immobilized on a low-cost hydrophobic support [20], a very relevant factor given the importance of using a raw material with lower cost so that the process can be used in industrial scale to obtain the interest product.

3.5. Identification of the ester

The reaction mixture chromatographic profile before (a) and after the esterification reaction (b) and the benzyl cinnamate standard (c) are shown in Fig. 5(a, b and c), respectively, where it is possible to observe the almost complete reduction of the cinnamic acid peak (retention time: 17.6 min.) and the appearance of a new peak (retention time: 27.5 min.) that matches the retention time of the peak presented by the benzyl cinnamate standard (Fig. 5(c)), confirming that the desired product was obtained. Benzyl cinnamate was isolated by Column Chromatography (CC) and analyzed by Gas Chromatography (GC). For the antioxidant activity and toxicity assays, the purified fraction was used.

3.6. Antioxidant activity

Evaluating the antioxidant capacity of ester precursors (cinnamic acid and benzyl alcohol) it was found for both that the highest concentration tested was not efficient to capture 50 % of free radicals (200 $mg.mL^{-1}$ for cinnamic acid and 200 $\mu L.mL^{-1}$ for benzyl alcohol), so these data were not presented. The antioxidant

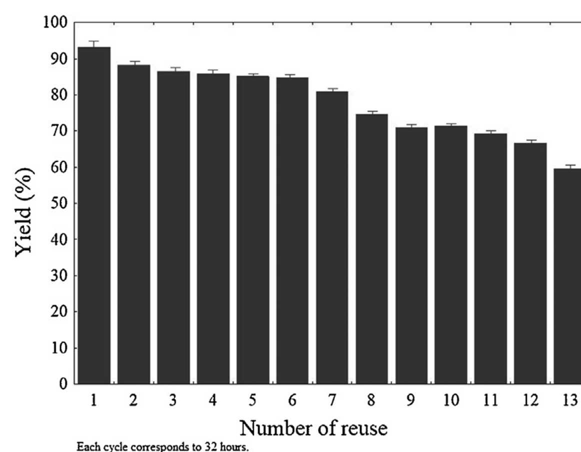

Fig. 4. Operational stability of the enzyme NS 88011 in the production of benzyl cinnamate.

capacity of benzyl cinnamate ester increased linearly with increasing ester concentration, and the highest antioxidant capacity was 79.6 % at 250 $mg.mL^{-1}$. The correlation between antioxidant activity and benzyl cinnamate concentration ($y = 0.3048x + 4.3501$; $R^2 = 0.9938$) provided an IC_{50} of 149.77 $mg.mL^{-1}$. However, this concentration is high compared to the excellent antioxidants such as ascorbic acid and BHT (IC_{50} = 0.002 $mg.mL^{-1}$ and IC_{50} = 0.005 $mg.mL^{-1}$, respectively) [21] indicating low benzyl cinnamate antioxidant activity.

3.7. Toxicity against A. salina

The *in vitro* toxicity test against *A. salina* is fast and low-cost bioassay and has been one of the most widely used tools for preliminary toxicity assessment and can be used for screening new drugs. The results obtained in the toxicity test for cinnamic acid and benzyl cinnamate are presented in Table 3. It is possible to verify that mortality increases with the increase in the concentration of the compound in both cases, reaching 100 % of dead nauplii with a concentration 3500 $\mu g.mL^{-1}$ for cinnamic acid and 96.8 % in the concentration of 0.7 $\mu g.mL^{-1}$ for benzyl cinnamate. The correlation between mortality and logarithm of concentration cinnamic acid ($y = 18{,}624\ln(x) - 63.343$; $R^2 = 0.95$) and benzyl cinnamate ($y = 17{,}3181\ln(x) + 97.01$; $R^2 = 0.96$), provided values of LD_{50} = 439.6 $\mu g.mL^{-1}$ and LD_{50} = 0.07 $\mu g.mL^{-1}$, respectively, demonstrating increased toxicity in 6280 times after esterification.

Amarante et al. [22] considered a compound with low toxicity when it presents LD_{50} higher than 500 $\mu g.mL^{-1}$, moderate between 100 and 500 $\mu g.mL^{-1}$, and very toxic when lower than 100 $\mu g.mL^{-1}$. Many studies have shown that toxicity of a compound against *A. salina* is correlated with cytotoxic activity against human tumors; McLaughlin et al. [23] reported that this bioassay led to discovery a new class of active antitumor agents (*Annonaceous acetogenins*) and is also related to activity against *Ttypanosoma cruzi*, a protozoan that causes Chagas disease [24,25], with viricidal, antifungal and antimicrobial activity [26]. The oral toxicity of benzyl cinnamate in Osborne-Mendel rats were studied

Table 2
Thermal deactivation constant and half-life of NS 88011 lipase at a temperature of 59 °C and reaction time 32 h.

Enzyme activity ($U.g^{-1}$)	Thermal deactivation constant (k_d) (h^{-1})	Half-life ($t_{1/2}$) (h)
1679	$1.4{\cdot}10^{-3}$	505.9

The enzyme activity in time 0 was 1754 $U.g^{-1}$.

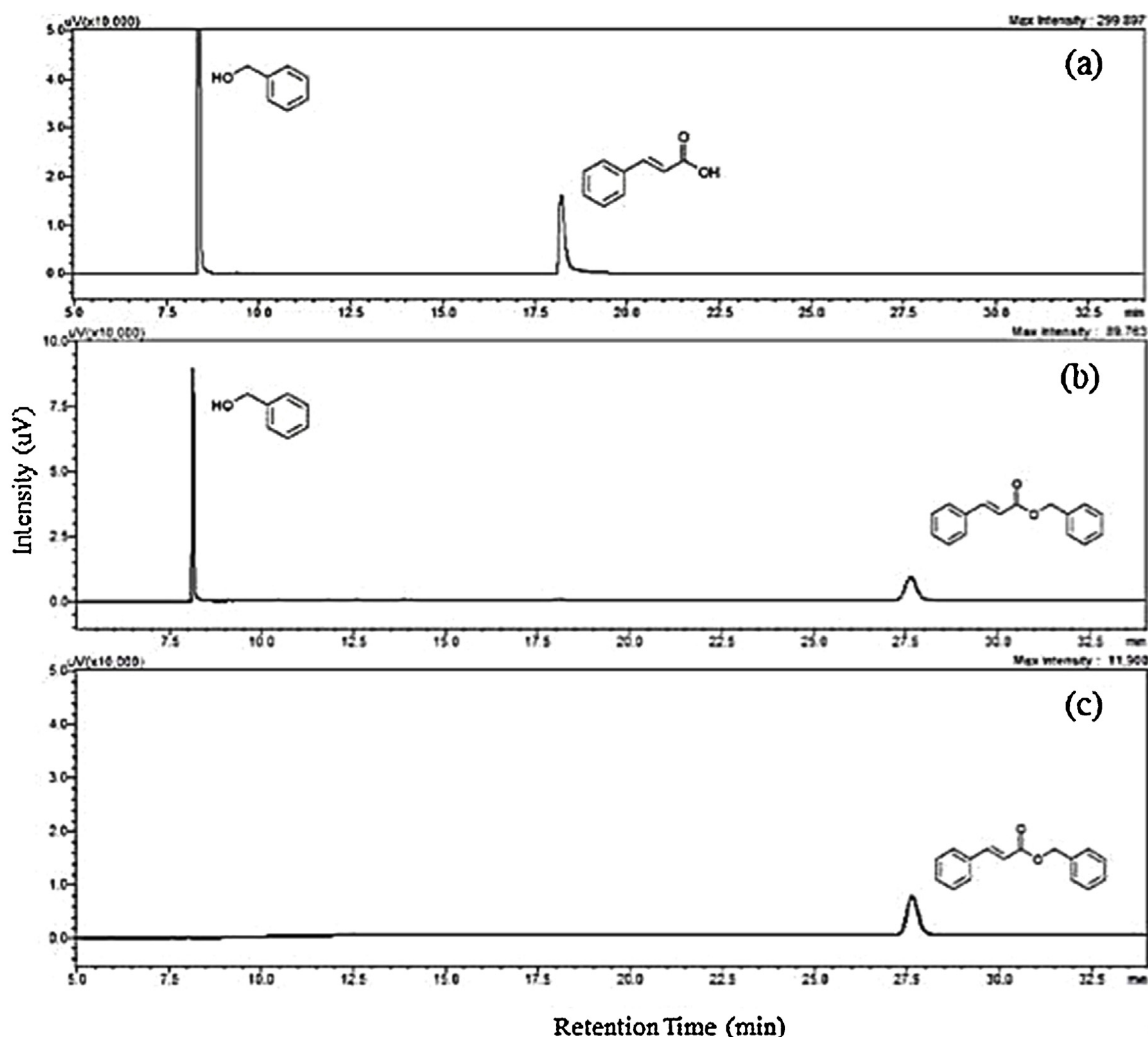

Fig. 5. Reaction mixture chromatograms before (a) and after (b) the esterification reaction and the benzyl cinnamate standard (c).

Table 3
Mean mortality of A. salina in different concentrations of cinnamic acid and benzyl cinnamate.

Concentration (μg. mL^{-1})	Mortality (%)	
	Cinnamic acid	Benzyl cinnamate
0.005	0	12.90 ± 1.13
0.01	0	19.62 ± 1.87
0.025	0	26.39 ± 0.98
0.05	0	40.32 ± 1.80
0.075	0	47.69 ± 1.78
0.1	0	58.59 ± 2.36
0.25	0	65.63 ± 1.91
0.5	0	92.15 ± 2.27
0.75	0	96.81 ± 1.43
30	8.53 ± 0.93	100 ± 0.00
75	16.14 ± 1.35	100
225	33.63 ± 1.83	100
300	38.20 ± 1.29	100
420	48.55 ± 2.48	100
480	52.48 ± 1.92	100
600	54.87 ± 1.76	100
1000	56.14 ± 2.32	100
2000	71.28 ± 1.84	100
2450	84.50 ± 1.33	100
2800	88.40 ± 1.57	100
3500	100 ± 0.00	100

and demonstrated that after 19 weeks of ingestion of concentrations from 0.1–10000 ppm, there was no death or adverse clinical signs, with no microscopic or macroscopic changes in tissues [27,28].

4. Conclusions

The response surface methodology proved to be effective in optimizing the production of benzyl cinnamate, making it possible to obtain an empirical mathematical model for the production of the ester in the function of enzyme concentration and temperature. From the mathematical model, it was possible to obtain the optimal temperature levels (59 °C) and enzyme concentration (4.4 mg.mL^{-1}) allowing a significant reduction in the use of the biocatalyst. Through the kinetic study of the reaction, it was also possible to reduce the reaction time from 48 to 32 h, reaching a conversion of 97.6 %. Lipase NS 88011 demonstrated efficacy in reaction catalyzing, showed excellent half-life, and provided good yields (above 60 %) after the thirteen reuse cycles. Thus, it was possible to evidence an alternative process with optimal responses that can be applied in the scheduling of enzymatic production of benzyl cinnamate. As for the antioxidant activity, there was an increase in the ability to capture free radicals after esterification, presenting IC_{50} of 149.77 mg.mL^{-1}. The toxicity of benzyl

cinnamate (LD_{50} = 0.07 μg.mL^{-1}) was 6280 times higher than that of cinnamic acid (LD_{50} = 439.6 μg.mL^{-1}) indicating it as a potential natural biological agent, justifying further studies in the search for new drugs and pest control agents.

CRediT authorship contribution statement

Suelen Paloma Piazza: Investigation. **Bruna Maria Puton:** Investigation. **Rogério Marcos Dallago:** Formal analysis. **Débora de Oliveira:** Writing - review & editing. **Rogério Luis Cansian:** Formal analysis, Writing - review & editing. **Marcelo Mignoni:** Formal analysis. **Natalia Paroul:** Supervision, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2021.e00586.

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