Continuous production of eugenol esters using enzymatic packed bed micro-reactors and evaluation of the products as antifungal agents

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Abstract

The enzymatic synthesis of biological active compounds is receiving increasing interest by academic and industrial researchers. Herein, we report on the continuous-flow enzymatic production of eugenol esters with packed bed micro-reactors prepared with the Novozym 435 and Lipozyme RM IM lipases. After optimization of the independent process variables, namely temperature (68 °C), flow rate (8 µL min^{-1}) and eugenol to isobutyric anhydride molar ratio (1:5) by means of experimental design methodologies, the unprecedented continuous-flow production of eugenyl isobutyrate has been achieved with steady-state conversions of 77 and 70%, using packed-bed microreactors prepared with Novozym 435 and Lipozyme RM IM, respectively. With acetic anhydride as the acylating agent, the same reactors, under the same conditions, afforded eugenyl acetate with steady-state conversions of 82% (Novozym 435) and 90% (Lipozyme RM IM). The two ester products have been tested against phytopathogenic and dermatophytic fungi, showing interesting activities against this last category of pathogens. The reported results demonstrated the suitability of the enzymatic packed-bed microreactors as the sustainable and scalable technology for the solvent-free continuous-flow production of biologically active esters of eugenol.

Keywords: Continuous esterification; Lipases; Eugenyl acetate; Eugenyl isobutyrate; Antifungal activity

1. INTRODUCTION

Eugenol is one of the key phenylpropenes, a class of natural compounds widely diffused in essential oils. Indeed, eugenol is a major aromatic constituent (up to approximately 80% by weight) of the essential oil of clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry - Myrtaceae) and is commercially available in large quantities.[1] Eugenol is commonly used as a fragrance and flavoring agent in a variety of cosmetics, pharmaceuticals and food products. Furthermore, it has shown antimicrobial, antifungal, antioxidant, anti-inflammatory, antispasmodic, antidepressant, antigenotoxic, and anticarcinogenic properties.[1] However, the efficiency of this compound in therapeutic treatments is limited by its poor water solubility.[2] Nevertheless, in virtue of its easy access and polyfunctional structure, it is considered as a useful starting material to produce valuable derivatives through chemical synthesis[1] and biotransformation.[3,4] Biotechnological approaches based on the use of hydrolytic enzymes as biocatalysts have been proved efficient for the preparation of many attractive esters,[5] but, despite this, the applications of hydrolases at industrial scale remain limited. In general, the developed systems suffer from high operational costs that make difficult their scaling-up. To make these processes economically profitable, not only the yield and the selectivity of the reactions need to be as higher as possible, but also the stability and reusability of the biocatalyst must be maximized. In this view, great attention has been given to the use of hydrolytic enzymes in non-conventional media and in solvent-free reactions while the employment of immobilized hydrolases under continuous-flow mode is still under-exploited.[6]

Among the hydrolases, lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), are the most commonly used enzymes to produce esters by either direct esterification or

transesterification reactions. Their use in industry[7,8] is increasing also because the ester produced through a lipase-mediated processes may be considered as *natural* if substrates are of natural origin.[9]

The eugenol esters have been mainly produced through traditional esterification reactions with acyl chlorides as acylating agents[1,10] although several enzymatic syntheses have been proposed as alternative processes. The lipase catalyzed eugenol esterification reactions are usually carried out either in solvent organic media or under solvent-less conditions.[11-13] The (2-methoxy-4-prop-2-enylphenyl) acetate [eugenyl acetate] showed increased stability with respect to the eugenol still preserving its potential as antifungal,[14] antimicrobial,[11,15] as well as antileishmanial[16] therapeutic agent. In addition, it has been proposed also as eco-friendly larvicidal compound against larvae of *Aedes aegypti* with lower toxicity and increased activity with respect to eugenol.[13,17] Finally, like eugenol, eugenyl acetate has been authorized for use in foods by the Joint Expert Committee on Food Additives (JECFA)[18] and the European Food Safety Authority (EFSA)[19] and it is currently listed in the European Union database of flavoring substances. Eugenyl acetate can be obtained naturally in the essential clove oil *Syzygium aromaticum*.[20] This essential oil can be obtained from flowers and leaves by steam distillation,[21] solvent extraction or extraction with supercritical CO_2,[22] however, generally, the concentration of eugenyl acetate is less than 10% of the essential oil.[20]

On the contrary, at the best of our knowledge, no data on the synthesis and biological activities of (2-methoxy-4-prop-2-enylphenyl) 2-methylpropanoate [eugenyl isobutyrate] has been ever reported until today. This eugenol ester has been detected in considerable concentrations in the root oil of *Bidens alba*[23] and in trace amount in the essential oil of aerial parts of *Scandix pectin-veneris*.[24]

In the present article, we describe the solvent-free enzymatic synthesis of eugenyl acetate and eugenyl isobutyrate under continuous-flow mode by means of packed bed micro-reactors. Two commercially available immobilized lipases, namely Novozym 435 and Lipozyme RM IM, have been evaluated for this purpose while the reaction parameters temperature, flow rate and substrates molar ratio have been optimized following an experimental design methodology. The novel product eugenyl isobutyrate was purified by column chromatography and characterized through ^{1}H- and ^{13}C-NMR analysis. Finally, the activity against various filamentous fungi of both the pure acetic and isobutyric esters of eugenol has also been evaluated.

2. EXPERIMENTAL

2.1. Chemicals and consumables

Commercial eugenol (Aldrich, 99% purity; Milan, Italy), isobutyric anhydride (Aldrich, 97% purity; Milan, Italy) and acetic anhydride (Riedel-de Haën, 99% purity; Milan, Italy) were used as substrates for the esterification reactions. Analytical standard of eugenyl acetate is commercially available from Fluka (Sigma-Aldrich; Milan, Italy) and HPLC grade methanol was from Sigma-Aldrich (Milan, Italy). The commercial lipases Lipozyme RM IM, produced by *Rhizomucor miehei*, immobilized in phenolic resin (Declared activity - 275 IUN/g) and Novozym 435, produced by *Candida antarctica*, immobilized in acrylic resin (Declared activity - 8000 PLU/g) were kindly supplied by Novozymes S.A (Madrid, Spain).

2.2. Eugenyl ester production under continuous-flow mode

2.2.1. Micro-reactors preparation

Two 5 cm long stainless-steel column micro-reactors with an internal diameter of 0.5 cm were packed by gravity with the immobilized enzymes. To fill the reactors 585 mg of Lipozyme RM IM or 572 mg of Novozym 435 were employed. The void volumes of the packed reactors (ε) were calculated according to Shang et al.[25] and were 0.225 mL and 0.304 mL for Lipozyme RM IM and Novozym 435, respectively.

2.2.2. Experimental apparatus for the continuous-flow synthesis of the eugenol esters

The experimental setup employed to carry out the esterification reactions under continuous-flow mode, was the same previously described by Lerin et al.[4] and is described in Figure 1. It consists of an HPLC pump (Agilent 1100 series, Agilent Technologies; Waldbronn, Germany) to feed the packed bed microreactor with adjustable ratios of the substrate. A thermostatic bath (Grant – TC 120; Cambridge, England) was used to control the temperature (±0.1 °C) of the micro-reactor. At defined times, a remote-controlled switching valve allowed to redirect 1 µL of the micro-reactor effluent to the HPLC which was equipped with a C18 column (C18-RP Symmetry from Waters (Guyancourt, France), 15×0.21 cm ID, particle size: 3.5 µm) and a diode array detector (DAD, Agilent 1100 series; Waldbronn, Germany). A binary mobile phase composed of methanol:water (70:30, v/v) with a flow rate of 0.1 mL min^{-1} and a temperature column of 30 °C were the conditions adopted for the chromatographic analysis. Products and substrates were monitored at 302 nm (detector was calibrated to avoid saturation).

2.2.3. Effect of the flow rate on eugenyl isobutyrate synthesis

To evaluate the effect of the flow rate on eugenol esterification, different flow rates (20, 10, 8 and 6 µL min^{-1}) were applied with both the enzymatic micro-reactors (Lipozyme RM IM or Novozym 435) by keeping constant the eugenol to isobutyric anhydride molar ratio (1:5) and temperature (50 °C). The conversion was considered in the steady state condition for each flow rate. Residence times were calculated according to Dalla Rosa et al.[26] At flow rates of 6, 8, 10 and 20 µL min^{-1}, residence times for Novozym 435 reactor were 99, 74, 59 and 29 min, respectively whereas, the same flow rates, with the Lipozyme RM IM reactor, gave residence times of 73, 55, 44 and 22 min, respectively.

2.2.4. Optimization of eugenyl isobutyrate synthesis

The effect of the process variables eugenol to isobutyric anhydride molar ratio (from 1:0.77 to 1:9.23) and temperature (from 46.9 to 68.1 °C) were evaluated through a 2^2 Central Composite Rotational Design (CCRD), with triplicate of the central point,[27,28] using the reactor packed with Novozym 435 and a flow rate of 8 µL min^{-1}. The time-course of the reaction was followed for 6.5 hours by measuring the conversion every 30 min in all runs. The conversion used for the statistical analysis was the mean conversion between the last 5 points (4.5, 5.0, 5.5, 6.0 and 6.5 h) of each run. Statistical analyses were performed using Statistica® 12 (Statsoft Inc., Tulsa, OK, USA) and level of significance of 95% ($p<0.05$).

2.2.5. Reactor operational stability during the synthesis of eugenyl isobutyrate

The operational stability of the enzymatic reactors (Novozym 435 and Lipozyme RM IM) was evaluated under the optimized CCDR conditions determined for the

Novozym 435 one (Table 1, run 8 - molar ratio of 1:5, temperature of 68 °C and flow rate of 8 µL min^{-1}) for 26 hours reaction time.

2.2.6. Reactors operational stability during the synthesis of eugenyl acetate

As above, to determine the operational stability of the Novozym 435 and the Lipozyme RM IM reactors during the synthesis of eugenyl acetate, 26 hours experiments were performed under the above optimized conditions (1:5 of substrate molar ratio, 68 °C, 8 µL min^{-1} flow rate).

2.3. Quantification of eugenyl esters by HPLC

For the quantification of the esters, the calibration curves were built using standard solutions of eugenol (from 0 to 25 mM), eugenyl isobutyrate and eugenyl acetate (from 0 to 33 mM) in methanol:water (70:30, v/v). The validation of the method was performed by ^{1}H-NMR analysis according to Lerin et al.[4]

2.4. Purification and spectroscopic characterization of the eugenyl isobutyrate

A 1.0 mL sample of reactor effluent was diluted with a 5% (w/v) solution of $NaHCO_3$ (10 mL; Sigma-Aldrich; Milan, Italy) and the resulting mixture was extracted twice with ethyl acetate (2 x 5 mL; Sigma-Aldrich; Milan, Italy). The combined organic extracts were evaporated under reduced pressure and the residue was loaded on a chromatographic column (20 x 3 cm) packed with silica gel (60 Å, 70-230 mesh, 63-200 µm particle size; Aldrich; Milan, Italy) and conditioned with a cyclohexane/ethyl acetate (Sigma-Aldrich; Milan, Italy) 15:1 mixture. Elution with the same mixture afforded pure eugenyl isobutyrate which showed the following ^{1}H- and ^{13}C-NMR spectra (acquired with

a 300 MHz Varian Gemini spectrometer; California, United States): ^{1}H, $CDCl_3$, δ (ppm): 6.95 (d, 2H, *J* = 8.0 Hz, Ar), 6.80-6.74 (m, 3H, Ar), 6.0-5.90 (m, 1H, C<u>H</u>=CH_2), 5.12-5.05 (m, 2H, CH=C<u>H</u>$_2$), 3.80 (s, 3H, OCH_3), 3.38 (d, *J* = 6.7 Hz, 2H, CH_2), 2.82 (ddd, 1H, *J* = 7.0 Hz, C<u>H</u>$(CH_3)_2$), 1.31 (d, 6H, *J* = 7.0Hz, 2 CH_3); ^{13}C, $CDCl_3$, δ (ppm): 175.4, 150.9, 138.7, 138.2, 137.1, 122.4, 120.6, 116.0, 112.7, 55.8, 40.1, 34.0, 19.1 (2 C).

2.5. Evaluation of antifungal activity

Tests to assess the susceptibility of various filamentous fungi to eugenol, eugenyl acetate and eugenyl isobutyrate were performed.[29] The phytopathogenic fungi used in this study were *Alternaria* spp., *Botrytis cinerea* strain ATCC 48339, *Fusarium oxysporum* strain ATCC 12581 and *Penicillium crustosum*, purchased from SIAPA-ISAGRO, Milano, Italy. The dermatophytes used were *Microsporum gypseum* Guiart & Grigoraki strain 3999 from Institute of Hygiene and Epidemiology-Mycology (IHME), Brussels, Belgium and *Trichophyton mentagrophytes* R. Blanch strain 160.66 from Centraal Bureau voor Schimmelcultures (CBS), Baarn, The Netherlands. The cultures were maintained in the laboratory as agar slants on a suitable culture medium, that is, potato dextrose agar (PDA; Difco; Milan, Italy), for the phytopathogenic fungi, and Sabouraud dextrose agar (SDA; Difco; Milan, Italy), for the dermatophytes.

To evaluate biological activity, cultures of each fungus were obtained by transplanting mycelium discs, 8 mm in diameter, from a single culture in stationary phase to Petri dishes containing the suitable agar medium (PDA or SDA) supplemented with the compound to be tested. Each compound was dissolved into dimethyl sulfoxide (DMSO; Sigma-Aldrich; Milan, Italy), and a proper volume of the solution was aseptically added to the medium at 45 °C in order to obtain a final concentration of 0.062,

0.125, 0.25, 0.50, 0.75 or 1 mg mL^{-1}. The final concentration of DMSO in the medium was adjusted to 0.1% (v/v). Controls were set up with equivalent quantities of DMSO (0.1% v/v). The cultures were incubated at 24 ± 1 °C and the growth rate was determined by measuring the colony diameter on the sixth day after the transfer of the fungus onto plates containing the substance to be tested. At this time the percentage growth inhibition in comparison with the control was evaluated for each fungus. Three replicates were used for each concentration. The percentage of growth inhibition was expressed as the mean of values obtained in three independent experiments. For comparison, the same concentrations of the commercial fungicide tricyclazole (Beam, Dow AgroSciences; Bologna, Italy) were tested. Three replicates were used for each concentration.

The Minimum Inhibitory Concentrations (MICs) were defined as the lowest concentration of compound that completely inhibited (MIC_{100}) or to reduce 50% (MIC_{50}) the visible fungal growth at the end of a 6 days incubation. The Minimum Fungicidal Concentrations (MFCs) were determined by subculturing of mycelium discs from each plate without visible growth onto medium suitable for each organism. The plates were incubated at 24 °C for 5 days. The MFCs were the lowest concentrations that did not permit growth on the plates.

Susceptibility was expressed as Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC).

3. RESULTS AND DISCUSSION

3.1. Enzymatic synthesis of eugenyl isobutyrate under continuous-flow mode

Based on the current literature[7,8,12] the commercial immobilized Novozym 435 and Lipozyme RM IM lipases were identified as potential catalysts for the esterification of eugenol. Thus, the unprecedented enzymatic production of eugenyl isobutyrate under continuous-flow mode was investigated using the same experimental apparatus described in our previous article on the chemocatalyzed synthesis of eugenyl acetate.[4]

3.1.1. Flow rate effect

One of the main process parameters to be optimized in continuous-flow syntheses is the feed flow. As the feed flow decreases, the residence time (RT) increases and consequently higher conversions are achieved, on the other hand, the increase in feed flow has a negative effect on the conversion. Therefore, we investigated the effect of flow rates ranging from 6 to 20 μL min^{-1} on the eugenyl isobutyrate production, using an eugenol to isobutyric anhydride molar ratio of 1:5 and a temperature of 50 °C. The eugenyl isobutyrate conversion at steady-state as a function of flow rate is shown in Figure 2. It can be observed that Novozym 435 reactor afforded slightly best results compared to the Lipozyme RM IM one in all the flow conditions explored. The results suggested using the Novozym 435 reactor and a flow rate of 8 μL min^{-1} as promising starting point for optimization studies since these conditions afforded a satisfactory conversion (65%) in a reasonable RT (74 min). In fact, with a 25% increase in retention time (flow rate of 6 μL min^{-1}) a small improvement of the conversion (3%) occurred. On the other hand, higher flow-rates determined too high loss of conversion with both the commercial lipases (about 35 and 31% conversion at 20 μL min^{-1} with Novozym 435 and Lipozyme RM IM, respectively).

3.1.2. Optimization of the eugenyl isobutyrate synthesis

After determination of the best flow rate, a 2^2 CCRD to evaluate the effect of temperature and substrate molar ratio on the eugenyl isobutyrate production was carried out (Table 1) by keeping the flow rate of 8 µL min^{-1} and using the Novozym 435 reactor. Table 1 presents the matrix of the experimental design with the real and coded values and the responses in terms of eugenyl isobutyrate conversion on the steady-state conditions (after 3.5 h). From this table, one can verify that higher conversions were obtained in runs 4 (72%) and 8 (77%), which were performed with higher temperatures (65 and 68 °C) and substrate molar ratio of 1:5 and 1:8, respectively. The good reproducibility of the results can be seen in the experiments corresponding to the central point (runs 9, 10 and 11). The high selectivity of this continuous-flow enzymatic synthesis was demonstrated by the absence of by-products in all runs.

The results were subjected to statistical analysis, which resulted in an empirical mathematical model (Eq. 1) expressing the conversion (%) of the eugenyl isobutyrate as a function of temperature (°C) and substrate molar ratio. Model validation was done through analysis of variance (ANOVA). The good correlation coefficient obtained (0.96) and the value of F (calculated F (23.8) greater than tabulated $F_{0.95;5;5}$ (5.05)) show that the coded model is predictive ($p < 0.05$) proving that it was capable of well representing the experimental data of eugenyl isobutyrate conversion in the range of factors investigated, as illustrated by the predicted conversion (column 5 of Table 1) and the standard deviation (RED) (column 6 of Table 1), and making it suited for the construction of response surface presented in Figure 3.

$$C = 57 + 9.25 * RM - 6.15 * RM^2 + 13.94 * T + 0.64 * T^2 + 0.25 * RM * T \quad (1)$$

Where: *C* represents the conversion of eugenol to eugenyl isobutyrate (%), *T* is the temperature (°C) and *MR* the substrate molar ratio.

Observing the model, it can be concluded that, in the studied ranges of temperature and substrate molar ratio, the process is optimized. Furthermore, it can be noticed that the variables and their interaction have a positive significant effect ($p < 0.05$) on the conversion of eugenyl isobutyrate. The increase in temperature and an excess of isobutyric anhydride seem to promote a good reaction system. Experiments performed at temperature above 70 °C showed instead a sharp drop in the conversion (data not shown), probably due to the loss of enzyme activity. Thus, the optimized conditions to produce eugenyl isobutyrate indicated by this study are those of the run 8 of Table 1, namely flow rate of 8 µL min^{-1}, eugenol to isobutyric anhydride molar ratio of 1:5 and temperature of 68 °C.

3.1.3. Operational stability of Novozym 435 and Lipozyme RM IM reactors during eugenyl isobutyrate continuous-flow synthesis

The stability of the enzymatic reactors was studied, as this is an important consideration for the industrial scale-up of the process. The optimized conditions determined for the continuous-flow production of the isobutyric ester (molar ratio of 1:5, temperature 68 °C, feed flow rate 8 µL min^{-1}) were employed to assess the stability of both, the Novozym 435 and the Lipozyme RM IM reactors. As shown in Figure 4, the maximum conversion of eugenol to its isobutyric ester with the Novozym 435 reactor reached a value of 77% after 4 h. This conversion was kept constant for about 4 h (4 to 8 h), then it decreased to a conversion of 46% up to 24h (31% conversion drop), remaining stable for the next 3h. A similar trend was shown by the process conducted with the

Lipozyme RM IM reactor. In this case, the higher conversion (70%) was achieved after 4 h and maintained for about additionally 4 h (4 to 8 h). Following, the conversion lowered reaching 56% after 24 h (14% conversion drop), remaining constant for the following 3 hours.

This decrease in conversion to ester may be related to instability, inhibition (by product or substrate) of the enzyme or the extraction of water essential for lipase. According to Lerin et al.,[30] the enzymatic activity and conversion into product are directly dependent on the substrates and solvents used in the reaction. Substrates and by-products change the pH of the reaction and, as it is known, that enzymes are pH-dependent biocatalysts working at an optimum pH range. With the pH out of this optimum the protein can lose its tertiary structure and, consequently, could lose its biocatalytic activity. Enzyme activity depends on the acidity of the microaqueous layer around the enzyme, which can be modified if any substrate or product is solved in it. Novozym 435 when exposed to different pHs showed a loss in its enzymatic activity. The further away from the optimal pH, the greater the loss of enzymatic activity.[31] In this study, isobutyric anhydride was used as the acylating agent, releasing isobutyric acid at the end of the reaction, resulting in acidification of the reaction medium and, therefore, may lead to loss of the tertiary structure of the enzyme.

Another factor that may be contributing to the loss of enzyme activity is the fact that isobutyric anhydride and isobutyric acid have a *log P* of 1.75 and 0.94, respectively, indicating that these substances are hydrophilic and may cause denaturation of the enzyme. In fact, according to the *log P* hydrophobicity sensor, defined as the logarithm of the partition coefficient in a standard octanol-water two-phase system, enzymatic activities are low in relatively hydrophilic solvents having log P < 2, are quite variable

for solvents having *log P* between 2 and 4 (eugenol - *log P* 2.61 and eugenyl isobutyrate log - *P 3.42*), and are high in hydrophobic solvents for which *log P* > 4. The reason is that solvents having *log P* < 2 strongly distort the essential water-biocatalyst interactions, thereby inactivating or denaturing the biocatalyst.[32] Thus, the acylating agent and the by-product (isobutyric acid) of the reaction may have a deleterious effect on the enzymatic activity, leading to decreased conversions.

3.2. Operational stability of Novozym 435 and Lipozyme RM IM reactors during eugenyl acetate continuous-flow synthesis

Once demonstrated that the unprecedented synthesis of eugenyl isobutyrate can be efficiently achieved under continuous-flow mode with the aid of Novozym 435 or Lipozyme RM IM packed bed micro-reactors, we verified the efficiency of the same experimental setup in the eugenyl acetate synthesis, as well. We were encouraged in doing this by previous works[11-13,15] that described the enzymatic synthesis of eugenyl acetate under batch and continuous-flow mode in solvent-free systems with experimental conditions (substrate molar ratio and temperature) very similar to those determined in this work. Thus, we conducted the tests to assess the operational stability of both the Novozym 435 and Lipozyme RM IM reactors using eugenol to acetic anhydride molar ratio of 1:5, feed flow rate 8 µL min^{-1} and temperature of 68 °C. Figure 5 described the results of these experiments. The Novozym 435 and Lipozyme RM IM reactors gave maximal conversions of 90 and 82% after 4 and 3.5 h, respectively. Regarding the operational stability, Lipozyme RM IM showed to be more stable during the 26 hours of study, presenting about 6% loss in the conversion with respect to the higher value achieved. The

Novozym 435 instead, showed a loss of about 16% in the conversion, during the same period.

Most of the experimental works on esterification synthesis have shown that acetates have no kind of inhibition on lipases no matter the type of lipase.[33] However, the loss or decrease of the enzymatic activity may occur due to several factors, as explained in the previous item. The use of an acyl donor (acetic anhydride) and the byproduct (acetic acid) of the reaction strongly hydrophilic, with *log P* of -0.27 and -0.17, respectively, can withdraw the essential water to the biocatalyst leading to the denaturation of the enzyme.[32] For Romero et al.,[31] a negative impact of high acetic anhydride concentrations on Novozym 435 lipase activity may lead to a loss of 40% of maximum activity when using pure acetic anhydride and suggests that this is a competitive inhibition. The acidity of the medium is also an extremely important factor for enzymatic activity. Romero et al.[31] observed a strong inhibition of Novozym 435 lipase when exposed to very low concentrations of acetic acid. In addition, according to Gomes et al.,[34] the increase in temperature leads to an increase in the speed of the reaction per enzyme unit. However, the use of the enzyme at an elevated temperature for a prolonged time may result in enzyme deactivation.

However, the use of continuous flow production reduces the deleterious effects on enzymes due to the low accumulation of substrate and reaction products in the reactor, as can be observed in Figure 5, maintaining high enzymatic activity and high conversions for longer periods. Another important factor for the maintenance of enzymatic activity and high conversion is the type of support in which the enzyme is immobilized and its interaction with the reaction substrates and products.

3.3. Antifungal analysis

Eugenol and its derivatives are largely used in perfumes and mouthwashes and as dental analgesics and have been well studied for their antimicrobial properties in food industry.[35,36] The activity of eugenol as control agent for post-harvest fruit pathogens has been demonstrated by Amiri et al.,[35] while Pinto et al.[37] reported the activity of eugenol against dermatophytic fungi, including strains with decreased susceptibility to fluconazole. Like for other phenolic compounds, the antifungal activity of eugenol was considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport and coagulation of cell contents.[38] Nevertheless, the low stability to the light and high temperatures partially limits the therapeutic use of eugenol. For this reason, the esterification of the phenolic hydroxyl group has been suggested to create more stable bioactive derivatives.[3] Under this perspective, the enzymatic approach herein described can be considered of great interest. Thus, we decided to evaluate the antimicrobial activity of the two ester derivatives against four phytopathogenic and two dermatophytic fungi, comparing the results with those obtained with eugenol. Starting from the assumption that natural products having antimicrobial activity with minimum inhibitory concentrations (MICs) $\leq$ 1.00 mg mL^{-1} are generally considered noteworthy,[39] both the eugenol esters object of this work did not appear as good candidates for the control of infections by the phytopathogenic fungi *Alternaria* sp., *B. cinerea*, *F. oxysporum* and *P. crustosum* since any inhibition of these microorganism's growth was observed at esters concentration $\geq$ 1 mg mL^{-1}. On the contrary, eugenyl acetate and eugenyl isobutyrate provided interesting results in the control of the dermatophytes *M. gypseum* and *T. mentagrophytes* (Table 2). In fact, even though with lower activity with respect to the eugenol, they can be considered potential

therapeutic agents since they showed MIC_{50} significantly lower than the limit of 1 mg mL^{-1}.

4. CONCLUSIONS

The present study highlights how the synergic application of innovative synthetic methodologies like biocatalysis, flow-chemistry and experimental design can contribute to the development of sustainable processes to produce bioactive molecules. The continuous-flow synthesis of eugenyl isobutyrate has been herein addressed with good yields using packed bed micro-reactors containing the Novozym 435 or Lipozyme RM IM lipases. The same experimental set-up has been successfully used to produce eugenyl acetate as well. The eugenyl isobutyrate has been characterized (^{1}H- and ^{13}C-NMR) for the first time and both the isobutyryl and the acetyl esters of eugenol have been evaluated for their antifungal activity giving encouraging results in the control of the dermatophytes *M. gypseum* and *T. mentagrophytes*.

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CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

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Figure Captions

Figure 1 Schematic diagram of the experimental set up for the continuous-flow synthesis of eugenyl esters.

Figure 2 Effect of flow rate on eugenyl isobutyrate conversion. The reactions were performed at 50 °C using an eugenol to isobutyric anhydride molar ratio of 1:5.

Figure 3 Contour plot of eugenyl isobutyrate conversion as a function of temperature and substrate molar ratio. Experimental data and conditions shown in Table 1.

Figure 4 Evaluation of the stability of the enzymatic reactors during eugenyl isobutyrate synthesis. The reactions were performed at 68.07 °C using an eugenol to isobutyric anhydride molar ratio of 1:5 and a flow rate of 8µL min^{-1}. The standard deviation was ≤ 1.5% for all the assays.

Figure 5 Evaluation of the stability of the enzymatic reactors during eugenyl acetate synthesis. The reactions were performed at 68.07 °C using an eugenol to acetic anhydride molar ratio of 1:5 and flow rate of 8µL min^{-1}. The standard deviation was ≤ 1.5% for all the assays.

Table 1 The matrix of the 2^2 full CCRD experimental design (coded and real values) for optimization of eugenyl isobutyrate production.

Run	Molar ratio[a]	Temperature (°C)	Experimental Conversion [b] (%)	Predicted Conversion [c] (%)	RED[d] (%)
1	-1 (1:2)	-1 (50)	31	28.5	7.10
2	1 (1:8)	-1 (50)	42	46.5	-10.24
3	-1 (1:2)	1 (65)	60	55.9	6.37
4	1 (1:8)	1 (65)	72	74.9	-3.72
5	-1.41 (1:0.77)	0 (57.5)	27	31.7	-18.83
6	1.41 (1:9.23)	0 (57.5)	63	57.8	8.79
7	0 (1:5)	1.41 (46.9)	40	38.6	3.46
8	0 (1:5)	1.41 (68.07)	77	77.9	-1.20
9	0 (1:5)	0 (57.5)	57	57.0	0.01
10	0 (1:5)	0 (57.5)	57	57.0	0.01
11	0 (1:5)	0 (57.5)	57	57.0	0.01

[a] Eugenol to isobutyric anhydride. [b] Determined by HPLC analysis. [c] Calculated according to equation 1. [d] Relative Error Deviation - $RED = \left(\frac{Exp.\ Conv. - Predict.\ Conv.}{Exp.\ Conv.}\right) x100.$

Table 2 Susceptibility of some dermatophytic fungi to eugenol, eugenyl acetate and eugenyl isobutyrate.

Compound	Target fungal species					
	M. gypseum			*T. mentagrophytes*		
	MIC_{50}	MIC_{100}	MFC	MIC_{50}	MIC_{100}	MFC
Eugenol	0.087	0.125	0.500	0.093	0.250	0.250
Eugenyl acetate	0.136	0.250	1	0.112	0.250	0.500
Eugenyl isobutyrate	0.356	1	>1	0.538	1	>1

MIC: Minimum Inhibitory Concentration (mg mL^{-1}) were defined as the lowest concentration of compound that completely inhibited (MIC_{100}) or to reduce 50% (MIC_{50}); MFC: Minimum Fungicidal Concentration (mg mL^{-1}).

Figure 1

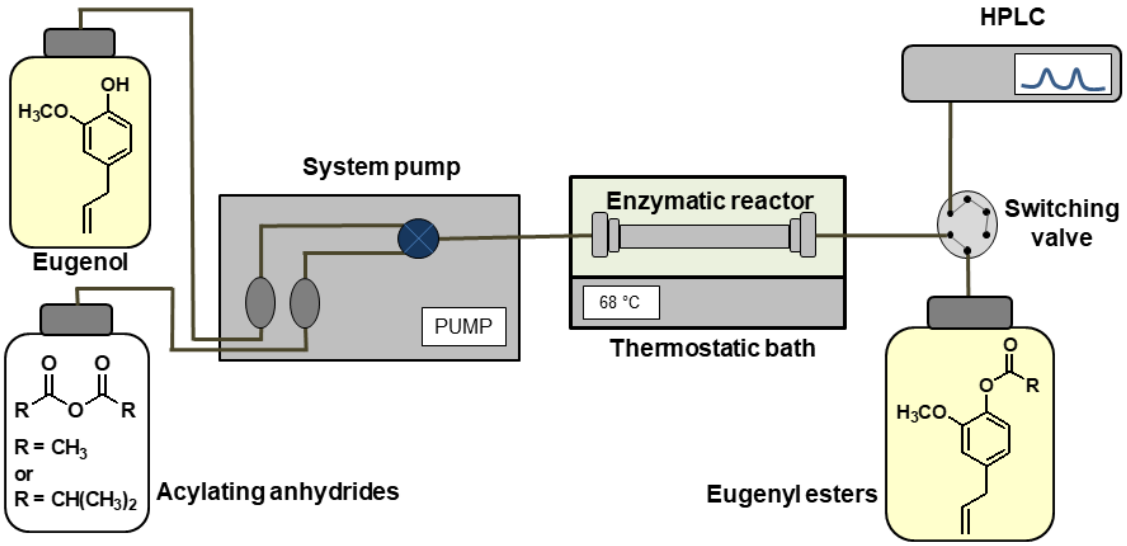

HPLC
OH
H3CO
Eugenol
System pump
PUMP
Enzymatic reactor
68 °C
Thermostatic bath
Switching valve
O
O
R
O
R
R = CH3
or
R = CH(CH3)2
Acylating anhydrides
Eugenyl esters

Figure 2

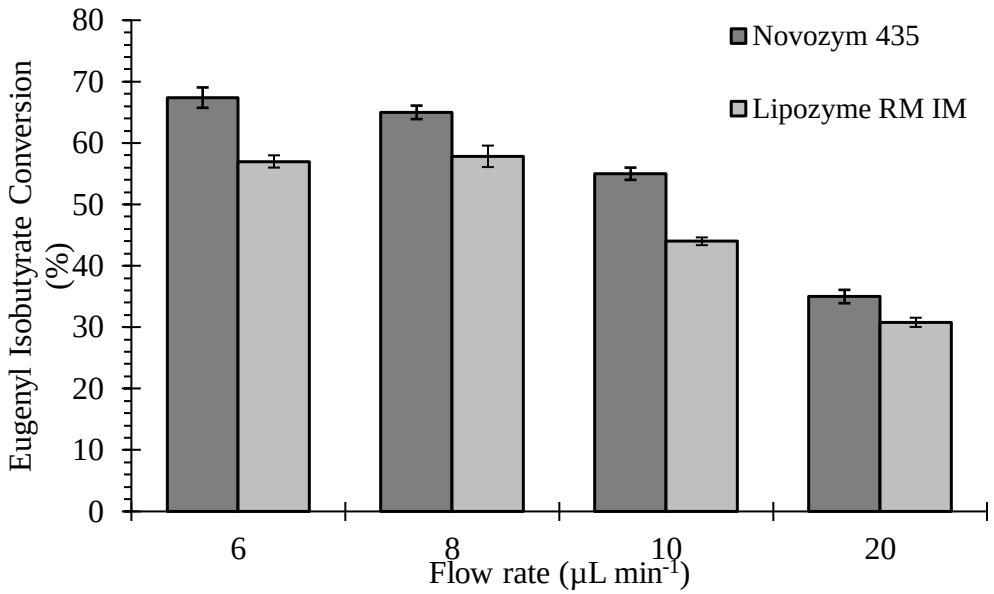

Eugenyl Isobutyrate Conversion (%)
80
70
60
50
40
30
20
10
0
6
8
10
20
Flow rate (µL min-1)
Novozym 435
Lipozyme RM IM

Figure 3

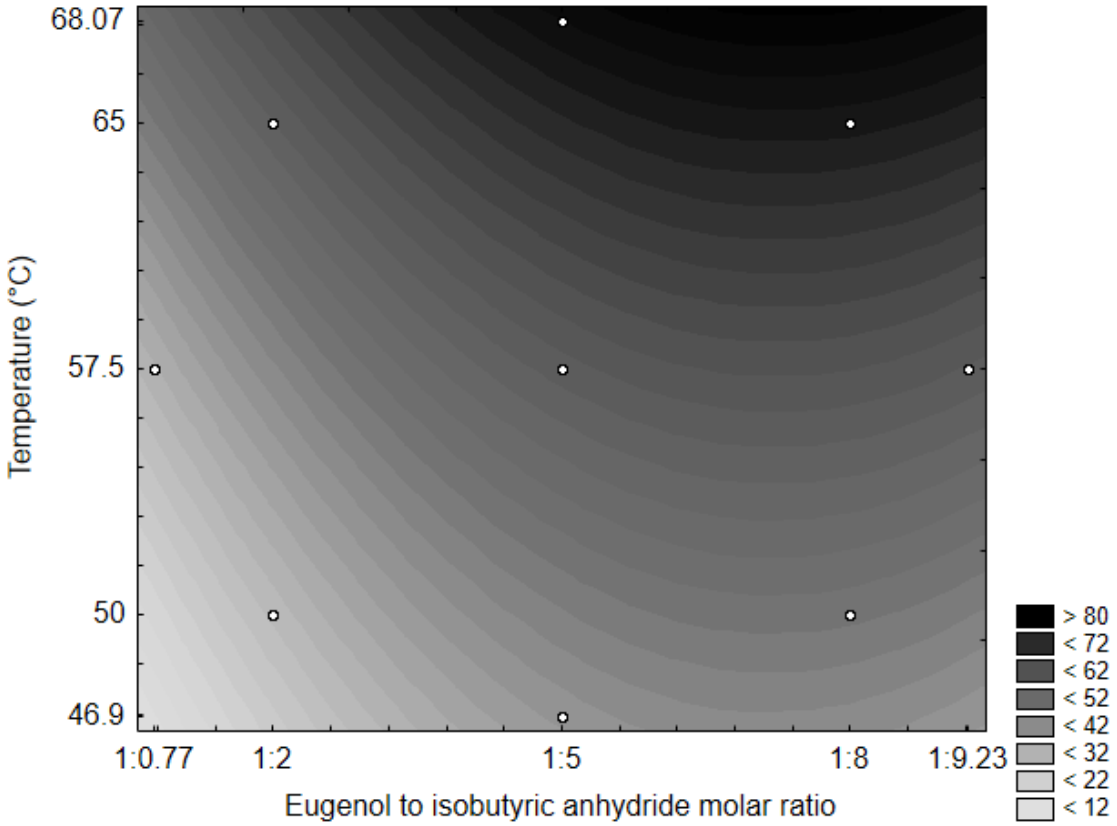

68.07
65
57.5
50
46.9
Temperature (°C)
1:0.77
1:2
1:5
1:8
1:9.23
Eugenol to isobutyric anhydride molar ratio
> 80
< 72
< 62
< 52
< 42
< 32
< 22
< 12

Figure 4

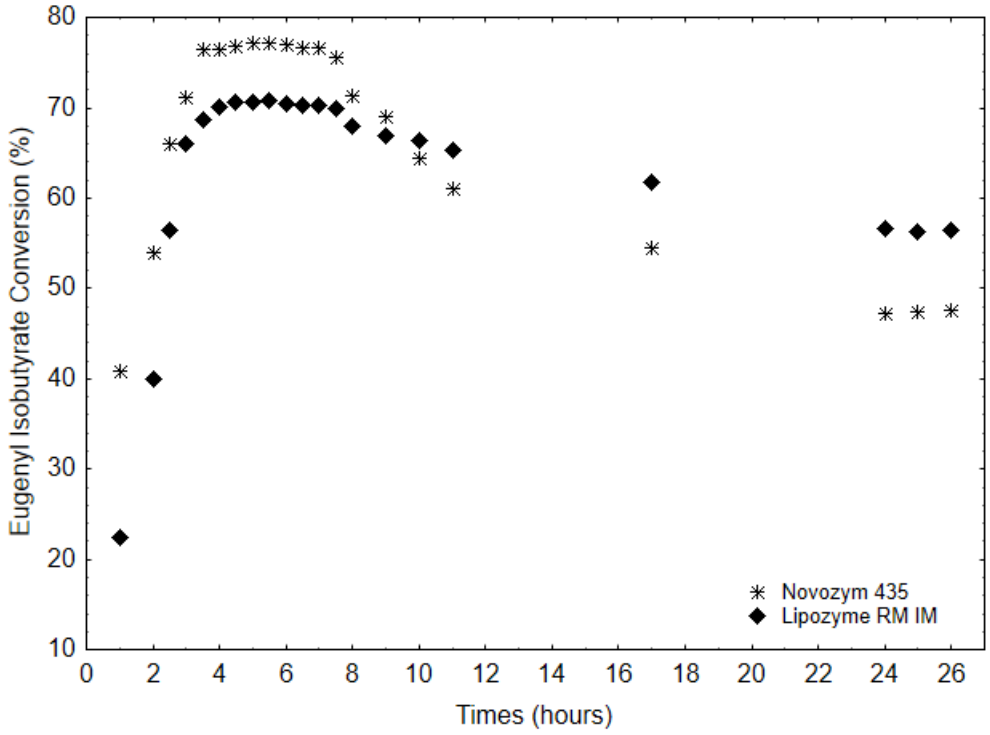
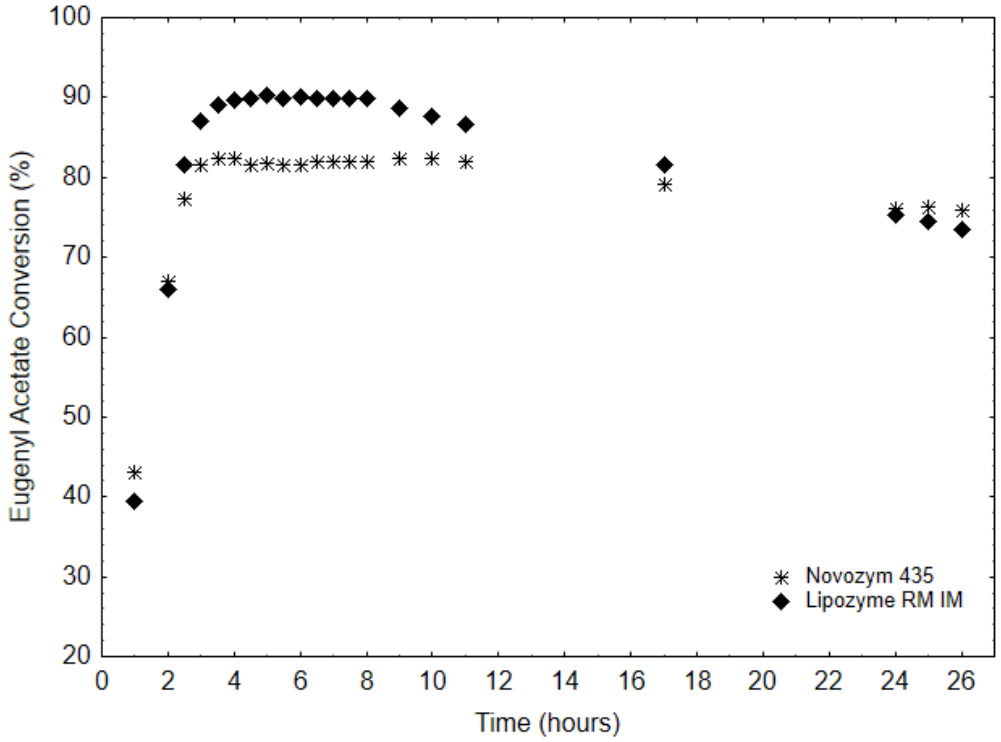

Eugenyl Isobutyrate Conversion (%)
Times (hours)
Novozym 435
Lipozyme RM IM

Figure 5


Eugenyl Acetate Conversion (%)
Time (hours)
Novozym 435
Lipozyme RM IM