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Eco-friendly methodology for efficient synthesis and scale-up of 2-ethylhexyl-*p*-methoxycinnamate using *Rhizopus oryzae* lipase and its biological evaluation

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Abstract Lipase-mediated synthesis of phenolic acid esters is a green and economical alternative to current chemical methods. Octyl methoxycinnamate, an important UVB-absorbing compound, was synthesized by the esterification of *p*-methoxycinnamic acid with 2-ethyl hexanol using *Rhizopus oryzae* lipase. A molar ratio of 1:2 of *p*-methoxycinnamic acid and 2-ethyl hexanol was found to give an optimum yield using cyclo-octane (50 ml) as reaction solvent, at a temperature of 45 °C, and 750 U of lipase, resulting in a yield of 91.3 % in 96 h. This reaction was successfully scaled up to 400-ml reaction size where 88.6 %bioconversion was achieved. The synthesized compound was found to have superior antioxidant activity as compared to ascorbic acid. The synthesized compound also exhibited good antimicrobial activity against *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella typhi*, *Staphylococcus aures*, *Candida albicans* (yeast), *Aspergillus niger*, *Alternaria solani*, and *Fussarium oxysporum* by well diffusion method in terms of zone of inhibitions (in mm).

Keywords *p*-Methoxy cinnamic acid · 2-Ethyl hexanol · Esterification · 2-Ethylhexyl-*p*-methoxycinnamate · Antioxidant activity · Antimicrobial activity

Introduction

Lipases are a group of hydrolases that are known to catalyze a plethora of reactions such as hydrolysis, esterification, and transesterification, which form the basis of many industrial processes [6]. These enzymes can act under mild conditions, are highly stable in organic solvents, show broad substrate specificity, and high regio/stereo-selectivity [13].

Esterification of cinnamic acid is a very important reaction catalyzed by lipase, which has potential for industrial application in the synthesis of flavor and fragrance compounds widely used in pharmaceutical, food, beverage, and cosmetic industries. Presently, these esters are synthesized through chemical route under extreme conditions of temperature and pressure, which have the hazards of operational insecurity in addition to environmental concerns [2]. Classical esterification is carried out in the presence of strong acidic catalysts such as concentrated sulphuric acid or PTSA (*p*-toluenesulfonic acid) using large amounts of reagents in benzene or toluene as the solvent [8, 14, 27]. Increasing interest in clean, efficient and economical technologies for sustainable chemistry has resulted in a search for enzymatic methods for the synthesis of various esters from aromatic acids. Such esters include widely used bioactive ingredients of cosmetic products such as 3-methylbutyl-4-methoxycinnamate, 2-ethylhexyl-4-methoxycinnamate, 2-ethylhexyl-4-(dimethylamino) benzoate, 2-ethylhexylsalicylate, 4-isopropylbenzylsalicylate, propyl-4-hydroxybenzoate, and butyl-4-hydroxybenzoate [28]. Direct esterification of phenolic acid (including cinnamic acid derivatives) with aliphatic alcohol using *Candida antarctica* and *Rhizomucor miehei* lipases in anhydrous organic solvents or solvent-free systems have been reported [10, 28].

Octyl methoxycinnamate, using as UV-B absorbing sunscreen agent extensively used in many new cosmetic sunscreen formulations, has been reported to be produced using complex synthetic procedures outlined in certain papers and patents [3, 24]. However, these methodologies

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suffer from disadvantages such as long reaction times, hard work-up, and the use of non eco-friendly chemicals such as acrylic solvents.

Realizing the industrial significance, esterification using *Rhizopus oryzae* lipase has been evaluated for synthesis of 2-ethylhexyl-*p*-methoxycinnamate, and the reaction conditions have been optimized. Here we propose a simple methodology for the synthesis of 2-ethylhexyl-*p*-methoxycinnamate as an improvement and simplification over classical methods, in the context of green chemistry.

Materials and methods

Microbial culture condition and materials

Rhizopus oryzae, obtained from our laboratory culture collection, was maintained on potato dextrose agar (PDA). *p*-Methoxycinnamic acid, 2-ethyl hexanol and dialysis tubing (10 kDa) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Production conditions

Production of lipase was carried out in 2-l Erlenmeyer flasks containing 400 ml of optimized production medium (% w/v: soyabean oil (emulsified with 0.2 % gum acacia), 1.5; cane molasses, 1.0; maize starch, 1.0; soyabean meal, 2.0; $CaCl_2{\cdot}2H_2O$, 0.10; KH_2PO_4, 0.50, pH 9.0) inoculated with 8×10^7 spores and incubated at 30 °C, 200 rpm for 96 h. The culture broth was subjected to filtration and centrifugation to remove the biomass, followed by fourfold concentration by ultrafiltration using 10-kDa cellulose acetate membrane (Millipore, Bedford, MA, USA). This partially purified and concentrated lipase was precipitated using the protocol of Shu et al. [17] and lyophilized. This preparation had a lipase activity of 3.6 U/mg and was used for the present study.

Determination of lipase activity

The lipase activity of both the immobilized and the free lipase was measured by the procedure described by Winkler and Stuckmann [22]. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 μmol *p*-nitrophenol per minute under assay conditions.

Esterification of *p*-methoxycinnamic acid

The esterification of *p*-methoxycinnamic acid with 2-ethyl hexanol (used as acyl acceptor) using *R. oryzae* lipase was carried out in 250-ml round-bottom flasks containing 50 ml of reaction mixture. Experimentally, *p*-methoxycinnamic acid (5 g) was mixed with 2-ethyl hexanol (10 g) and 50.0 ml of *n*-hexane in 250-ml screw-capped vials in individual sets. Lyophilized lipase (500 IU) was added to the reaction mixture and incubated at 45 °C, 200 rpm for 96 h. The reaction mixture without enzyme was set as the control. Samples (10 μl) were withdrawn after 96 h for analysis. The products formed were quantitatively analyzed by HPLC method.

Analysis of esters (reaction products) by HPLC

Samples were filtered to remove the enzyme and analyzed using a Waters HPLC system (San Ramon, CA, USA) consisting of a pump and system controller (Model 600), with a manual injection valve with a 20-μl sample loop, a degasser system, a quartet pump, and a multiple wavelength UV-detector. A C-18 nucleosil column was used at room temperature with a solvent system of 0.5 % methanol in chloroform and detection at 290 nm.

Purification of the reaction product

After completion of the reaction, lipase was filtered off using Whatman No. 1 filter paper. The reaction filtrate was concentrated in a rotary vacuum evaporator (Buchi R 215, Switzerland), and the target product was purified by column chromatography using chloroform: methanol (96:2, v/v) as the mobile phase. A 400 × 20-mm glass tube packed with 25 g of silica gel (100–200 mesh) was used as the chromatography column. About 30 mg of the crude product was dissolved in the solvent (2 ml), and a small amount (0.3 ml) of this solution was applied to the chromatography column. The column was eluted at a flow rate of 1–2 ml/min. The eluates were collected and analyzed by HPLC.

Nuclear magnetic resonance (NMR) analysis

Purified reaction product was subjected to ^{1}H NMR analysis. The spectra were recorded with a Bruker Avance II 400-MHz spectrometer at 294.3 K, and the data processing was performed with Top Spin 1.3 software.

Evaluation of antioxidant activity

The 2-ethylhexyl-*p*-methoxycinnamate obtained after enzymatic reaction were subjected to DPPH assay using the method of Turkmen et al. [20] with slight modification. The appropriate dilution of test samples was prepared with 70 % ethanol. A test sample of 50 μl and 1.95 ml of 100 mM DPPH radical in ethanol were mixed by vortexing and the reaction mixture was incubated at 25 °C in the dark for 30 min. The process of decolorization was recorded at 520 nm using a Shimadzu UV-2450 UV–VIS

spectrophotometer (Kyoto, Japan). Ascorbic acid was used as the positive control and antioxidant standard.

Yen and Duh [26] determined the antioxidant activity that was expressed as percentage inhibition of the DPPH radical using the following equation:

$$AA\% = 100 - \left\{ \left[\left(Abs_{sample} - Abs_{blank} \right) \times 100 \right] / Abs_{control} \right\}$$

Antimicrobial activity

The antimicrobial activity of synthesized compound was evaluated using agar well diffusion method [7] with minor modification. Diluted inoculums of 0.1 ml (10^5 CFU/ml) of the *E. coli*, *Klebsiella pneumonia*, *Salmonella typhi* (Gram-negative bacteria) *Staphylococcus aureus* strain (Gram-positive bacteria) were swabbed on nutrient agar plates. They were also evaluated for their in vitro antifungal potential against *Aspergillus niger*, *Alternaria solani*, *Fussarium oxysporum*, and *Candida albicans* as examples of yeast strains. The growth inhibition zone present after incubation of plates aerobically in an upright position at 37 ± 2 °C for 24–48 h determines the antimicrobial effect of the sample.

Scale-up of esterification of *p*-methoxycinnamic acid

Process optimization was initially carried out in 250-ml screw-capped flasks containing 100 mM of *p*-methoxycinnamic acid in 50-ml reaction volume. Further experiments were planned wherein the synthesis procedure was scaled up to 100, 200, and 400-ml reaction mixture size. A control experiment in 50-ml reaction size was also carried out. The amount of the substrate (*p*-methoxycinnamic acid) acyl acceptor (2-ethyl hexanol) and lipase concentration was used in the ratio as optimal for 50-ml reaction size. The reaction was performed under the optimized conditions obtained so far.

Results and discussion

Optimization of reaction parameters for synthesis of 2-ethylhexyl-*p*-methoxycinnamate

The effect of solvents

To study the effect of solvent for maximum reaction, flasks containing 5 g of *p*-methoxycinnamic acid, 10 g 2-ethyl hexanol and 500 IU of the enzyme in 50 ml of different organic solvents (*n*-hexane, tetrahydrofuran (THF), toluene and cyclo-octane) were placed in an incubator shaker at 45 °C for 96 h. The synthesis of 2-ethylhexyl-*p*-methoxycinnamate was found to depend significantly on the organic solvent used for the reaction (Fig. 1). A maximum

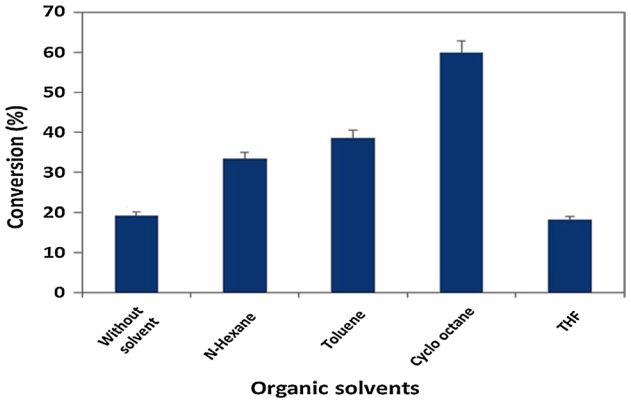


Fig. 1 The effect of solvent on the synthesis of 2-ethylhexyl-*p*-methoxycinnamate

bioconversion of 60.0 % was obtained in the presence of cyclo-octane, as compared to 56 % obtained by Guyot et al. [5]. However, minimum conversion of 30.1 % was observed with *n*-hexane (control). The presence of substrate–solvent interactions determines the availability of substrate to an enzyme, and it varies with the organic solvent used [26]. The choice of solvent therefore affects the synthesis of the product, in this case 2-ethylhexyl-*p*-methoxycinnamate. On the basis of this result, cyclo-octane was used as reaction medium throughout this study. Inactivation of enzyme molecules by polar solvents has been cited as the reason for low enzymatic activities and bioconversion yields obtained in polar media [21]. The use of non-polar solvents in this study circumvents this problem. The esterification of *p*-methoxycinnamic acid with 2-ethyl hexanol is in the same range as that obtained by Stamatis et al. [19] using *n*-octanol. Sharma and Kanwar [16] reported the synthesis of methyl cinnamate in the presence of DMSO solvent using immobilized lipase from *B. licheniformis* MTCC-10498. However, recovery of product from DMSO is difficult due to its high boiling point, thus complicating the work-up. The use of cyclo-octane leads to a considerably simpler workup. Yang et al. [25] reported the esterification of phenolic acids in the presence of ionic liquid-assisted solubilization using *Candida antarctica* lipase B, but the process suffered from scalability problems due to the expensive solvent used. In comparison, cyclo-octane is more cost-effective and amenable to scale-up.

The effect of molar ratio of p-methoxycinnamic acid to 2-ethyl hexanol

One of the most important factors that affect the yield of esters is the molar ratio of substrate (*p*-methoxycinnamic acid) to acyl acceptor (2-ethyl hexanol). The effect of molar ratio of *p*-methoxycinnamic acid to 2-ethyl hexanol was

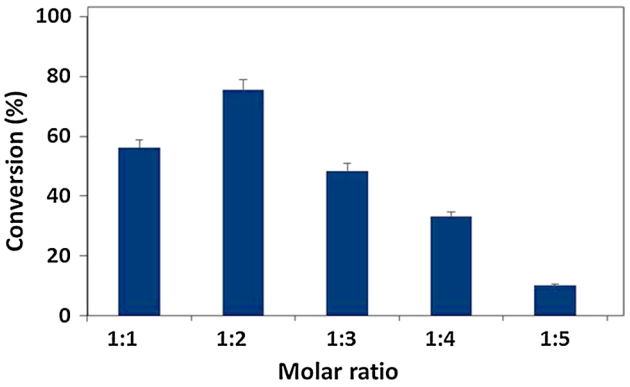


Fig. 2 Effect of different molar ratio (*p*-methoxycinnamic acid:2-ethyl hexanol) on the synthesis of 2-ethylhexyl-*p*-methoxycinnamate

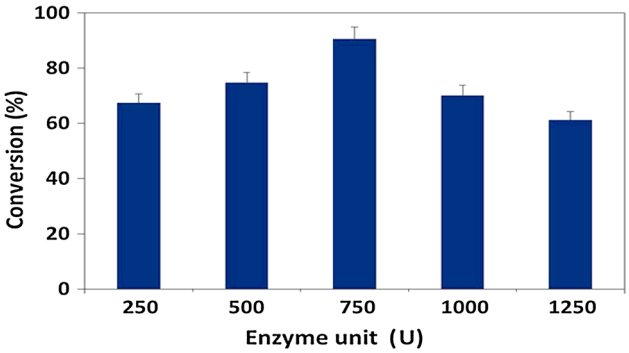


Fig. 3 Effect of different lipase concentration on the synthesis of 2-ethylhexyl-*p*-methoxycinnamate

studied in the range of 1:1–1:5. The reaction conditions were the same as for earlier experiments. In principle, an excess of acyl donor should not be required for an enzyme-catalyzed reaction, and an equimolar ratio of *p*-methoxycinnamic acid to 2-ethyl hexanol should be sufficient to give an optimum yield of 2-ethylhexyl-*p*-methoxycinnamate. However, decreasing the molar ratio through an incremental increase in the concentration of 2-ethyl hexanol resulted in an initial increase in the bioconversion yield (Fig. 2). A maximum bioconversion of 76.2 % was achieved using a molar ratio of 1:2 after 96 h of reaction. Lowering the molar ratio further led to a decrease in yield. The lowest bioconversion yield observed was 62.2 % a molar ratio of 1:5. This could be due to inactivation of lipase by higher concentrations of 2-ethyl hexanol, since alcohols such as methanol and ethanol are known to inactivate enzymes [18].

The effect of lipase concentration

The synthesis of 2-ethylhexyl-*p*-methoxycinnamate was performed by adding different concentrations of *R. oryzae* lipase (250–1,000 U) in 50 ml of reaction mixture containing 5 g *p*-methoxycinnamic acid and 2-ethylhexonol in *n*-hexane at 45 °C for 96 h under shaking conditions (200 rpm). Figure 3 depicts that the maximum conversion of 91.3 % was obtained in the presence of 750 U of lipase after 96 h. However, further increase in the amount of lipase (above 750 U) did not enhance the yield. This result is in agreement with those obtained in the study conducted by Pang et al. [11] and Yadav and Devendran [23]. This might have resulted because of the aggregation of enzyme at high concentration, which reduces the accessibility of enzyme particle to reactant, thereby decreasing the bioconversion rate. However, in contrast to these reports and the present study, Lee et al. [9] obtained 90.0 % conversion yield using much higher concentrations (60 mg) of Novozyme 435 for only 30 mg of the substrate in 10-ml reaction size.

Product analysis

The position of acylation in enzymatically prepared ester was determined by NMR. ^{1}H spectra were recorded on a *Jeol* alpha-400 spectrometer at 400 and 100.6 MHz, respectively. DMSO-d*6* was used as a solvent. The structural analysis carried out using ^{1}H NMR and showed the following analytical data: ^{1}H NMR (400 MHz, DMSO d_6): δ7.6 (d, $J = 7.33$, 2H), 7.4 (s, $J = 8.7$, 1H), 6.89 (s, $J = 7.33$, 2H), 6.3 (d, 1H), 3.79 (d, 3H), 4.2 (m, 2H), δ 2.0 (s, 1H), δ 1.59 (d, 2H), δ 1.30 (d, 2H), δ 1.21 (t, 4H), 0.88 (m, 6H).

It is clear from the NMR data that there is multiplet corresponding to six protons at 0.88 belonging to two methoxy groups. A further nine protons at 2.0, 1.59, 1.30, and 1.21 ppm belonged to methyl ($–CH_2$) adjacent to methyl group ($–CH_3$), while a quartet at 4.2 ppm represents the two protons on the ethyl group, which is present adjacent to methyl group and bonded to the oxygen atom. Another major peak at 3.79 ppm corresponds to three protons due to the methoxy directly attached to the aromatic ring. The

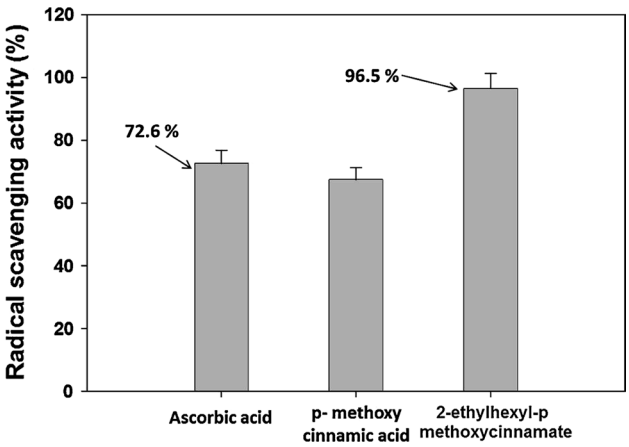
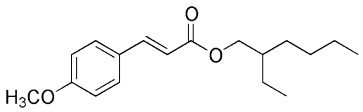


Fig. 4 Comparison of antioxidant activity between ascorbic acid and 2-ethylhexyl-*p*-methoxycinnamate sample by DPPH method

Table 1 Antimicrobial activity of 2-ethylhexyl-*p*-methoxycinnamate against pathogenic bacteria, yeast, and fungi

S. no.	Compounds	Inhibition zone (mm)							
		Bacteria					Fungi		
		B1	B2	B3	B4	B5	F1	F2	F3
1.	OMC 	10	12	8	10	8	15	20	16
2.	A (Ampicillin)	6	N	8	14	N	N	N	N
3.	T (Tetracycline)	2	N	9	8	N	N	N	N
4.	Co (Trimoxazole)	10	N	12	6	N	N	N	N
5.	S (Streptomycin)	14	8	6	8	N	N	N	N

B1, *E. coli*; **B2**, *K. pneumonia*; **B3**, *S. typhi*; **B4**, *Staphylococcus aureusl*; **B5**, *C. albicans* (Yeast); **F1**, *A. niger*; **F2**, *A. solani*; **F3**, *F. oxysporum*

doublet resonating at 6.3 ppm (1H, d) represents the proton attached to the C-2 position which proton attach to C-3 position further downfield at 7.6 ppm (2H, d). A doublet at 6.9 ppm (2H, d, $J6 = J8 = 8.4$ Hz) corresponds to the proton bonded to C-6 and C-8 while the doublet at 7.6 ppm (2H, d, $J = 7.33$, Hz) represents two protons attached to C-5 and C-9. A similar structural pattern by NMR was also reported by Pattanaargsan and Limphong [12].

Antiradical activity

It was observed that 2-ethylhexyl-*p*-methoxycinnamate has 96.5 % of free radical scavenging activity as compared with ascorbic acid, which showed 72.6 % (Fig. 4). The result showed that the product formed, 2-ethylhexyl-*p*-methoxycinnamate, can be used as an antioxidant compound. A similar study by Rice-Evans et al. [15] also reported that higher antioxidant activity was observed in the compounds whose structures had a large number of hydroxyl groups. A similar phenomenon was also reported by Faria et al. [4] wherein they studied the structure–activity relationships and observed that the *O*-dihydroxy groups in B ring and –OH groups are usually related to antioxidant properties of the flavonoids.

Antimicrobial evaluation

For antimicrobial evaluation, wells of 5-mm diameter were punched into the agar plates with the help of a sterilized cork borer (5 mm) and these were loaded with a 100 µl volume with a concentration of 1.0 mg/ml of each compound reconstituted in the dimethyl sulphoxide (DMSO). The plates were incubated aerobically in an upright position at 37 ± 2 °C for 24–48 h. Antimicrobial activity was evaluated by measuring the zone of inhibition (mm) against the pathogenic microorganism strains. The experiments were performed in triplicate. DMSO was used as a negative control.

The synthesized 2-ethylhexyl-*p*-methoxycinnamate was screened for their antibacterial activity against five bacterial strains, yeast, and fungi. The antimicrobial results are shown in Table 1. 2-ethylhexyl-*p*-methoxycinnamate was identified as a potent antifungal agent against fungal strains, yeast strain, and also inhibition showed against bacterial strains. These studies are interesting in the perspective of the antimicrobial drug from the tested sample.

Scale-up of the synthesis of 2-ethylhexyl-*p*-methoxycinnamate

The esterification reaction was initially carried out in 250-ml screw-capped flasks each containing 50 ml of the reaction mixture. It was thus felt necessary to scale-up the synthesis of 2-ethylhexyl-*p*-methoxycinnamate. It is clearly evident from Fig. 5 that 2-ethylhexyl-*p*-methoxycinnamate synthesis could be scaled up to various reaction sizes with almost the same conversion. In this investigation, 400 ml of solvent was used for 50.0 g of reaction substrate with maximum product yield (88.6 %). However, as compared to the present study, Chen et al. [1] used 1,000 ml of solvent for 25.0 g of reaction substrate. Moreover, in the

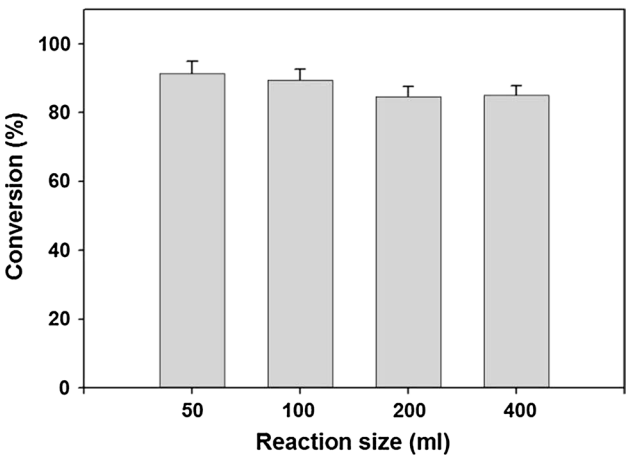


Fig. 5 Scale-up of the esterification reaction for the synthesis of 2-ethylhexyl-*p*-methoxycinnamate in different batch sizes

present investigation, the substrate showed high solubility in the solvent used, i.e., cyclo-octane, and there is also an easy product recovery from the reaction mixture. However, slight decrease in the 2-ethylhexyl-*p*-methoxycinnamate synthesis was noticed when the reaction was performed in the 400 ml of reaction size.

Conclusions

Synthesis of 2-ethylhexyl-*p*-methoxycinnamate from *p*-methoxycinnamic acid was successfully carried out using *R. oryzae* lipase, and optimal synthesis conditions were determined to be a reaction temperature of 45 °C, using cyclo-octane as solvent and 2-ethyl hexanol as an acyl donor and 750 U of lipase. A maximum bioconversion rate of 91.3 % was achieved in 50-ml reaction size. Further, when the reaction was scaled up using optimal reaction conditions, a product yield of 88.6 % was obtained in 400-ml reaction size containing 50 g of substrate. The process is simple and eco-friendly as there are no toxic waste products and the 2-ethylhexyl-*p*-methoxycinnamate can be used directly for various end uses. It can be further anticipated that these preliminary results could help in designing better molecules with enhanced antimicrobial activity.

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