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Enzymatic esterification of eugenol and benzoic acid by a novel chitosan-chitin nanowhiskers supported *Rhizomucor miehei* lipase: Process optimization and kinetic assessments

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ABSTRACT

A biotechnological route *via* enzymatic esterification was proposed as an alternative way to synthesize the problematic anti-oxidant eugenyl benzoate. The new method overcomes the well-known drawbacks of the chemical route in favor of a more sustainable reaction process. The present work reports a Box-Behnken design (BBD) optimization process to synthesize eugenyl benzoate by esterification of eugenol and benzoic acid catalyzed by the chitosan-chitin nanowhiskers supported *Rhizomucor miehei* lipase (RML-CS/CNWs). Effects of four reaction parameters: reaction time, temperature, substrate molar ratio of eugenol: benzoic acid and enzyme loading were assessed. Under optimum conditions, a maximum conversion yield as high as 66% at 50 °C in 5 h using 3 mg/mL of RML-CS/CNWs, and a substrate molar ratio (eugenol: benzoic acid) of 3:1. Kinetic assessments revealed the RML-CS/CNWs catalyzed the reaction *via* a ping-pong bi–bi mechanism with eugenol inhibition, characterized by a V_{max} of 3.83 mM min^{-1}. The Michaelis–Menten constants for benzoic acid ($K_{m,A}$) and eugenol ($K_{m,B}$) were 34.04 and 138.28 mM, respectively. The inhibition constant for eugenol ($K_{i,B}$) was 438.6 mM while the turnover number (k_{cat}) for the RML-CS/CNWs-catalyzed esterification reaction was 40.39 min^{-1}. RML-CS/CNWs were reusable up to 8 esterification cycles and showed higher thermal stability than free RML.

1. Introduction

Eugenol esters such as eugenyl benzoate, eugenyl palmitate and eugenyl myristate are prized for their sweet aroma as well as identified as potential future drugs against certain diseases [1] and a potent inhibitor of inflammation [2]. These esters of eugenol are preferred over the pure eugenol due to their lower cytotoxic and higher antioxidant activity [2]. Nonetheless, the current chemical route to synthesize eugenol esters is rather problematic. This is attributable to the high process temperature and utilization of homogeneous acid catalysts that causes the gradual corrosion of the reactor along with the large discharge of unwanted dissolved solids [3–5]. The use of such acid catalysts also increases the likelihood of product impurities, unsatisfactory product yield as well as the possible adverse impact on the environment [5]. In view of such predicament, the development of greener methods *via* the biotechnological route to produce eugenol esters proves relevant. For this study, the process protocol for a lipase-catalyzed synthesis to obtain satisfactory yields of a eugenol ester i.e. eugenyl benzoate was evaluated and statistically optimized.

The use of lipases (triacylglycerol lipases E.C. 3.1.1.3) as biocatalysts are particularly advantageous as they are versatile due to their high operating efficiency under mild reaction conditions while providing an energy-saving route [6]. Lipase from *Rhizomucor miehei* (RML) was the biocatalyst of choice for this study principally due to its proven specificity of reaction, high activity and suitability for catalyzing ester syntheses [7–9]. A matter of fact, there is still much room for improvement when concerning the process of enzyme-assisted synthesis of eugenyl benzoate. A previous study reported the enzymatic synthesis of eugenyl benzoate using immobilized lipase from *Staphylococcus aureus* [2], however, the source of the lipase was often associated with pathogenicity of microbial origin [10,11] and may incur issues with the safety of use. Hence, the study believes the use of the commercial RML as the biocatalyst for the esterification reaction to produce eugenyl benzoate would be more acceptable.

In this study, the easily deactivated free forms of RML [12,13] were covalently immobilized onto a novel support fabricated from chitosan (CS) and chitin nanowhiskers (CNWs) to afford the RML-CS/CNWs as the biocatalyst. The strategy was adopted in our study to insolubilize

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the RML so as to permit easy biocatalysts recovery and reuse for better productivity [14], aside from improving the operational stability as well as activity [15,16] of the lipase. In this study, the organic polymers of CS/CNWs were chosen as the matrix for RML immobilization due to their excellent biocompatibility, biodegradability, renewability, non-toxicity as well as high mechanical strength [17]. Moreover, earlier works utilizing similarly immobilized lipases to synthesize various commercially important esters have reported favorable improvements in their enzyme performance as well as product yields [18,19].

The process parameters for the RML-CS/CNWs-catalyzed esterification to synthesize eugenyl benzoate were statistically optimized using the method of response surface methodology (RSM). The software can predict the best reaction conditions that would maximize the yield of the ester *via* a statistically optimized model without requiring arduous and time-consuming experiments [20]. RSM merges the experimental designs with interpolation by first or second-order polynomial equations in a sequential testing procedure [21] that allows good estimation of the optimized parameters in the RML-CS/CNWs-catalyzed esterification process. Herein, the present study aimed to model the RML-CS/CNWs-catalyzed esterification of eugenol and benzoic acid using a three-level-four-factor Box-Behnken design (BBD). Relevant factors that would maximize the yield of eugenyl benzoate were assessed for incubation time, temperature, molar ratio of acid to alcohol and enzyme loading. The study also evaluated the reaction kinetics of the esterification reaction for a clearer insight into the mechanism of the reaction.

2. Materials and methods

2.1. Materials

Rhizomucor miehei lipase (RML) (≥20,000 U/g) was purchased from Sigma–Aldrich (St. Louis, USA) and used as received. For the esterification reaction, substrates, eugenol (Merck, Germany) and benzoic acid (QRec, New Zealand) were of > 99% purity. Chitosan (CS) in the form of ground flakes, chitin from shrimp shell, *N*-hydroxysuccinimide (NHS), 2-(*N*-morpholino)ethanesulfonic acid (MES) salt and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC) were purchased from Sigma-Aldrich (St. Louis, USA). Other chemicals such as chloroform, sodium hydroxide (NaOH) pellet, glacial acetic acid, and phenolphthalein used were analytical grade chemicals purchased from QRec (New Zealand).

2.2. Preparation of chitin nanowhiskers by acid hydrolysis

The CNWs was prepared by dissolving chitin (30 g) in 900 mL H_2SO_4 solution (3 M) and incubated at 95 °C for 12 h under vigorous stirring. The suspension was diluted with copious amounts of deionized water and subsequently centrifuged (15 min, 8 000 rpm). The washing process was repeated several times until a neutral pH (pH = 7) of the resultant CNWs final suspension was achieved. The CNWs were lyophilized and stored in a refrigerator [22].

2.3. Development of reinforced Chitosan/Chitin nanowhiskers beads (CS/CNWs)

Powdered CS (1 g) was added into a 50 mL acetic acid solution (2.0% v/v) and stirred for 1 h at room temperature. The CNWs (30%, w/w of CS) was gradually added into the CS solution until a homogenous CS/CNWs suspension was obtained. Chemical crosslinking was achieved by incorporating the tannic acid (30 mg of tannic acid per 1 g of CS) into the suspension and was further stirred till homogeneity. The CS/CNWs suspension was dropped into a NaOH (1 M) solution using a dropper under continuous stirring at 150 rpm. The initial CS/CNWs beads formed in the mixture were stirred for an additional 2 h before washing with distilled water. The beads were repeatedly wash until a neutrality (pH = 7) was attained and, subsequently air-dried at room temperature overnight.

2.4. Covalent immobilization of Rhizomucor miehei lipase on CS/CNWs beads

Crosslinking of the free RML using EDAC as the cross linker was carried out according to the method described by Raghavendra et al. [23] with several modifications. The CS/CNWs beads (1 g) were suspended in 20 mL of 50 mM MES buffer (pH 6.1) and stirred at 150 rpm for 10 mins before filtering through a plastic sieve to remove the buffer. The beads were transferred into a solution of EDAC (7.0 mL of 40 mg/mL) and stirred for a further 1 h. A 7.0 mL solution of NHS (48 mg/mL) was added under fast stirring and the mixture was stirred at room temperature for another 1 h. The solution was decanted and the CS/CNWs beads were washed with 50 mM MES buffer (pH 6.1) to remove the excess EDAC, NHS and urea by-product. The activated CS/CNWs beads were transferred into the immobilization solution containing 50 mM MES buffer (pH 6.1), 20% (v/v) cyclohexane and free RML (10 mg/mL). The CS/CNWs beads were stirred in the mixture overnight at room temperature to obtain the RML-CS/CNWs. The RML-CS/CNWs beads were washed with copious amount of 50 mM MES buffer (pH 6.1) to remove any unbound lipase and finally washed with cyclohexane. The RML-CS/CNWs beads were air-dried and stored at 4 °C until further use.

2.5. Determination of protein content, immobilized protein and lipase activity of free RML and RML-CS/CNWs

The concentration of protein in the enzyme solution, before and after the immobilization, was quantified by the Bradford method using bovine serum albumin (BSA) as the protein standard and Bradford reagent protein dye [24]. Different concentrations of BSA were prepared using a stock solution (1 mg/mL) and the absorbance was measured at 595 nm by a spectrophotometer (HITACHI U-3210), using preparations without BSA as blank. All determinations were performed in triplicates. Determination of immobilized protein (IP) was calculated by determining the difference between the initial protein concentration and final protein concentration present in the solution for every gram of support used in immobilization ($mg{\cdot}g^{-1}$ of support).

In this study, lipase activity was determined from the esterification reaction of eugenol and benzoic acid [25].A stock solution consisting of eugenol (0.33 M) and benzoic acid (0.16 M) in 85.95 mL chloroform was prepared for the analyses. The experiment was set up in a 15 mL screw-capped bottle containing 3 mL of stock solution with the appropriate quantity of RML. The reaction mixture was incubated in an oil bath (50 °C) for 2 h, followed by the addition of 1 mL of methanol as the quenching agent and then directly titrated. The mixture was titrated with NaOH (0.03 M), using phenolphthalein as the indicator. The blank containing 3 mL of the stock solution devoid of the enzyme was titrated to determine the total acid content of the reaction mixture. Calculation of lipase activity was made using Equation (1). One international unit of activity was defined as 1 μmol of benzoic acid consumed in the esterification per min (1 IU) under assay conditions.

$$\text{Activityfor RML-CS/CNWs}=\frac{(V_{NaOH}-V_0)\times N_{NaOH}\times 1000}{\text{massof biocatalyst}\times t} \quad (1)$$

Where: V_{NaOH} = volume of NaOH needed to titrate the samples,
V_0 = volume of NaOH needed to titrate the blank,
N_{NaOH} = normality of NaOH used,
t = time of the esterification reaction (120 mins)

2.6. Esterification of eugenol and benzoic acid catalyzed by the RML-CS/CNWs

The standard reaction was carried out in a 15 mL screw-capped