Enzyme and Microbial Technology 108 (2018) 42–52

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

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

Fatin Myra Abd Manan, Nursyafreena Attan, Zainoha Zakaria, Aemi S. Abdul Keyon, Roswanira Abdul Wahab*

Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 Johor Bahru, Malaysia

ARTICLE INFO

Keywords:
Eugenol
Eugenyl benzoate
Rhizomucor miehei lipase
Esterification
Rsm
Kinetic of inhibition

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

* Corresponding author.
E-mail address: roswanira@kimia.fs.utm.my (R.A. Wahab).

http://dx.doi.org/10.1016/j.enzmictec.2017.09.004
Received 23 April 2017; Received in revised form 5 September 2017; Accepted 11 September 2017
Available online 12 September 2017
0141-0229/

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).

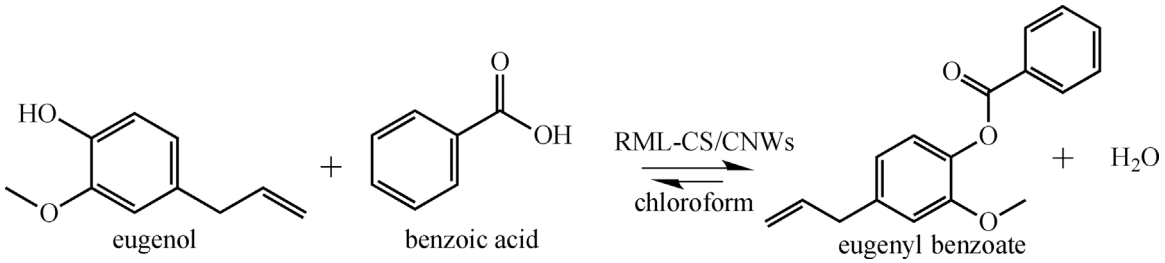
In this study, lipase activity was determined from the esterification reaction of eugenol and benzoic acid [25].A stock solution consisting of eugenol (0.33 M) and benzoic acid (0.16 M) in 85.95 mL chloroform was prepared for the analyses. The experiment was set up in a 15 mL screw-capped bottle containing 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



Scheme 1. Esterification reaction of eugenol and benzoic acid catalyzed by RML-CS/CNWs to produce eugenyl benzoate.

bottle that consisted of appropriate amounts of eugenol, benzoic acid and chloroform (solvent). The RML-CS/CNWs beads were added into the mixture and stirred magnetically in a horizontal paraffin oil bath at 250 rpm. Molecular sieves (20 mg/mL of substrates) were added into the reaction system as the dehydrating agent at the start of the third hour of the reaction. An aliquot of the mixture is withdrawn periodically and titrated with NaOH (0.02 M) using phenolphthalein as the indicator and each sampling was carried out in triplicate. The produced eugenyl benzoate was expressed in terms of percent conversion (%) i.e. percent of benzoic acid converted with respect to the total acid in the reaction mixture (Equation (2)).

$$\text{Conversion}(\%)=\frac{V_0\#\text{XPS\#ndash; }V_t}{V_0}\times 100 \quad (2)$$

where: V_0 = the volume of NaOH at initial time (t = 0) and V_t = the volume of NaOH at particular interval (t = t_1, t_2, t_3,…). The enzymatic synthesis to produce eugenyl benzoate is illustrated in Scheme 1.

2.7. Experimental design for optimizing the enzymatic synthesis of eugenyl benzoate using RSM

A 4-factor-3-level Box-Behnken experimental design with a total of 29 reactions was selected to assess the response pattern and to determine the optimum combination of factors. The interactions between these variables were assessed for, A (time), B (reaction temperature), C (substrate molar ratio (eugenol: benzoic acid)) and D (enzyme loading). Design Expert 7.1.6 software (Stat-Ease, Statistical Made Easy, Minneapolis, USA) was used for designing and analyzing the experimental data and the response of the experimental design was the percent conversion of eugenyl benzoate. Table 1 illustrates the independent variables and their levels, as well as the actual and coded values for the process optimization. The experimental runs were carried out in triplicate and the experiments were randomized to avoid bias. The general model equation used to predict the optimum values and elucidate the interaction between the variables is as follows (Equation (3)):

$$\text{Conversion}(\%)=\beta_0+\sum_{i=1}^{k}\beta_i x_i+\sum_{i=1}^{k}\beta_{ii}x_i^2+\sum_{i=j}^{k}\sum_{j=i+1}^{k}\beta_{ij}x_{ij} \quad (3)$$

where β_0 is a constant coefficient, *k* is the number of studied factors and optimized in the experiment, x_i and $x_{ij}$are the independent variables (factors), β_i, β_{ii} and β_{ij} are the interaction coefficients of linear, quadratic and second-order terms, respectively.

The terms in the regression equations were assessed using analysis of variance (ANOVA) for statistical significance of the response and, only variables with p value ≤ 0.05 are deemed significant and fitted. The fitness of the model was evaluated using several numeral methods while the major and interactive effects between the dependent and independent variables in the model was expressed by the value of coefficient of determination, R^2. Other main indicators to signify the significance and adequacy of the model generated in this study cover the model lack of fit, F-value and probability value (*Prob* > P), as well as Adjusted coefficient of determination (*Adj,* R^2) and Adequate precision (*Adeq prec.*). Surface and contour plots were employed to illustrate the effect of two interactive variables on the response i.e. conversion (%) of eugenyl benzoate based on the model equation.

Table 1
The actual and coded independent variables for the Box-Behnken design for the RML-CS/CNWs-catalyzed synthesis of eugenyl benzoate.

Variable	Units	Symbol	Coded levels		
			−1	0	+1
Time	h	A	3	5	7
Temperature	°C	B	40	50	60
Molar ratio (eugenol: benzoic acid)		C	2:1	3:1	4:1
Enzyme loading	mg/mL	D	1.5	3	4.5

2.8. Determination of kinetic parameters for the enzymatic synthesis of eugenyl benzoate

The kinetic parameters for the RML-CS/CNWs-catalyzed synthesis of eugenyl benzoate was assess by first carrying out initial rate kinetics [26]. Initial rates of reaction of esterification were determined by varying the concentrations of benzoic acid (100, 125, 150, 175, 200 and 225 mM) and eugenol (100, 150, 200, 250, 300 and 350 mM) substrates. Esterification reactions were performed using the optimized reaction conditions established in the RSM study and the kinetic data were calculated using the following Equation (4).

$$v=\frac{v_m[A][B]}{K_{m,B}[A]+K_{m,A}[B]\left(1+\frac{[B]}{K_{i,B}}\right)+[A][B]} \quad (4)$$

where *v* is the reaction rate; v_m is the maximum reaction rate; $K_{m,A}$ and $K_{m,B}$ are the Michaelis–Menten's constants for substrates, benzoic acid and eugenol, respectively; $K_{i,B}$ is the inhibition constant for eugenol; and [A] and [B] are the concentrations of benzoic acid and eugenol, respectively.

2.9. Reusability and thermal stability

The assessments were carried using the optimized esterification condition determined by RSM. After completing each esterification cycle, the RML-CS/CNWs biocatalyst was rinsed with cyclohexane three times and left to dry in a desiccator overnight. The biocatalysts were then re-suspended in a fresh aliquot of substrates and assayed. For calculating lipase activity in the subsequent reactions, the activity in the first reaction was taken as 100%. Relative activity was defined as the ratio of lipase activity to its initial activity. For the thermal stability assessment, thermal stability of free RML as that of RML-CS/CNWs (3 mg/mL) were compared by pre-incubating each biocatalysts in chloroform immersed in a water bath at temperatures 30, 40, 50, 60 and 70 °C. Each biocatalysts was transferred into a flask containing the substrates, eugenol: benzoic acid (3:1) and stirred at 250 rpm. The relative activities were estimated by comparing the activities of both the free RML and RML-CS/CNWs.

Table 2
Experimental conditions of the various runs of the Box-Behnken design in coded and actual terms for the obtained actual and predicted responses.

Variable						
Run	Time (h)	Temperature (°C)	Substrate molar ratio	Enzyme loading (mg/mL)	Actual conversion (%)	Predicted conversion (%)
1	5(0)	50(0)	3(0)	3(0)	66.0	65.08
2	5(0)	60(+1)	4(+1)	3(0)	48.2	47.80
3	5(0)	40(−1)	3(0)	1.5(−1)	40.8	40.61
4	5(0)	50(0)	3(0)	3(0)	65.6	65.08
5	7(+1)	40(−1)	3(0)	3(0)	52.5	52.58
6	7(+1)	50(0)	3(0)	4.5(+1)	53.0	53.35
7	3(−1)	50(0)	3(0)	4.5(+1)	60.6	59.85
8	3(−1)	50(0)	4(+1)	3(0)	52.9	53.28
9	5(0)	40(−1)	4(+1)	3(0)	48.3	48.00
10	7(+1)	50(0)	3(0)	1.5(−1)	42.8	43.34
11	5(0)	60(+1)	3(0)	1.5(−1)	40.3	40.51
12	5(0)	50(0)	3(0)	3(0)	64.8	65.08
13	3(−1)	60(+1)	3(0)	3(0)	53.2	53.28
14	5(0)	50(0)	3(0)	3(0)	64.0	65.08
15	5(0)	60(+1)	3(0)	4.5(+1)	55.8	56.03
16	5(0)	60(+1)	2(−1)	3(0)	49.6	49.69
17	5(0)	40(−1)	3(0)	4.5(+1)	56.5	56.33
18	3(−1)	40(−1)	3(0)	3(0)	49.5	49.88
19	3(−1)	50(0)	2(−1)	3(0)	45.7	46.16
20	5(0)	50(0)	2(−1)	4.5(+1)	55.1	54.98
21	5(0)	50(0)	4(+1)	1.5(−1)	37.2	37.48
22	5(0)	40(−1)	2(−1)	3(0)	49.7	49.89
23	5(0)	50(0)	2(−1)	1.5(−1)	40.2	39.92
24	7(+1)	50(0)	4(+1)	3(0)	43.8	43.38
25	7(+1)	50(0)	2(−1)	3(0)	54.6	54.26
26	7(+1)	60(+1)	3(0)	3(0)	49.0	48.78
27	5(0)	50(0)	3(0)	3(0)	65.0	65.08
28	3(−1)	50(0)	3(0)	1.5(−1)	39.2	38.64
29	5(0)	50(0)	4(+1)	4.5(+1)	53.2	53.65

3. Results and discussion

3.1. Determination of immobilized protein and lipase activity of RML-CS/CNWs and rationale of immobilization

For the enzyme-assisted esterification reaction, chloroform was used as the solvent as solubility of the starting material, benzoic acid in the reaction system has always been a problem. It has been shown that solid benzoic acid is only well solubilized in either chloroform or ethanol. In fact, molar solubility of benzoic acid decreases in the order of ethanol, chloroform, toluene, heptane, cyclohexane, and pentane [27]. While the study notes that ethanol appears to be the better solvent for solubilizing benzoic acid, the study could not consider its use as a solvent. Ethanol has an alcohol functionality that would compete for the same active sites on RML-CS/CNWs. Hence, its utilization can lead to formation of two types of esters as the end products, namely, eugenyl benzoate and ethyl benzoate. Such outcome must therefore, be avoided. Hence, chloroform was the best solvent for the esterification reaction.

Evaluation of immobilization parameters revealed that approximately 29% of the protein initially offered was covalently bound to the surface of CS/CNWs beads, yielding maximum protein loading and esterification activity of 8.12 $mg{\cdot}g^{-1}$and 537 ± 0.32 U/g of support, respectively. The study would like to note that auto-esterification was not observed in the blank assays. The catalytic activity of the prepared RML-CS/CNWs was substantially higher compared to the free RML (390 ± 0.58 U/g), indicating that the covalent immobilization of RML onto CS/CNWs support *via* EDAC as the zero-length crosslinker had enhanced the catalytic activity of the lipase. The findings, hence affirmed our hypothesis that the CS/CNWs supports was biocompatible for covalent attachment of RML and had improved activity of the lipase. Based on the initial activity of RML-CS/CNWs, it was evident that the immobilized lipase was capable of synthesizing higher yields of eugenyl benzoate over the free RML.

For this study, the relatively cheap tannic acid was used to crosslink the CS biopolymer to the CNWs nanofillers to reduce internal flexibility of the biopolymer, hence to yield a mechanically improved CS/CNWs support for covalent immobilization of RML. Tannic acid symmetrically deforms or protonates the amino groups in CS into $NH_3^+$and in due course, the dimeric tannic acid is hydrolyzed [17,28] hence forming a strong crosslinking network with CS via multiple interactions which include hydrogen bonds and electrostatic interactions. The well dispersed CNWs in the interior of the tannic acid-crosslinked CS increase the number of intermolecular hydrogen bonds between CNWs and CS. The technique appeared fruitful as the resultant CS/CNWs beads were considerably tougher than beads prepared from CS alone. Meanwhile, the study selected the zero-length crosslinker, EDAC [23] to covalently bind RML to the surface of CS/CNWs as the substance is not a part of the chemical scaffold that links RML to the support. The role of EDAC was to activate the surface functional groups on RML and CS/CNWs for binding *via* a Schiff base mechanism to afford the RML − CS/CNWs [29]. In this milieu, RML immobilized on the CS/CNWs supports were expectedly more rigid and better stabilized over its free forms. Correspondingly, cyclohexane was used during the immobilization step to activate the RML molecules. The hydrophobicity of the solvent can favorably induce the phenomenon of lid opening [30]. This increases possibility that a large population of the RML molecules were immobilized on the CS/CNWs supports in their active (open) forms.

3.2. Fitting of the response model

RSM is a powerful statistical tool for optimizing process parameters. The technique effectively minimizes the number of experiments while being capable of delivering statistically acceptable optimum conditions [31]. Our earlier preliminary screening experiments revealed four influential variables that significantly affected the activity of RML-CS/CNWs to produce high yields of eugenyl benzoate (data not shown). The variables included reaction time, temperature, substrate molar ratio of eugenol: benzoic acid and enzyme loading; in which their pre-

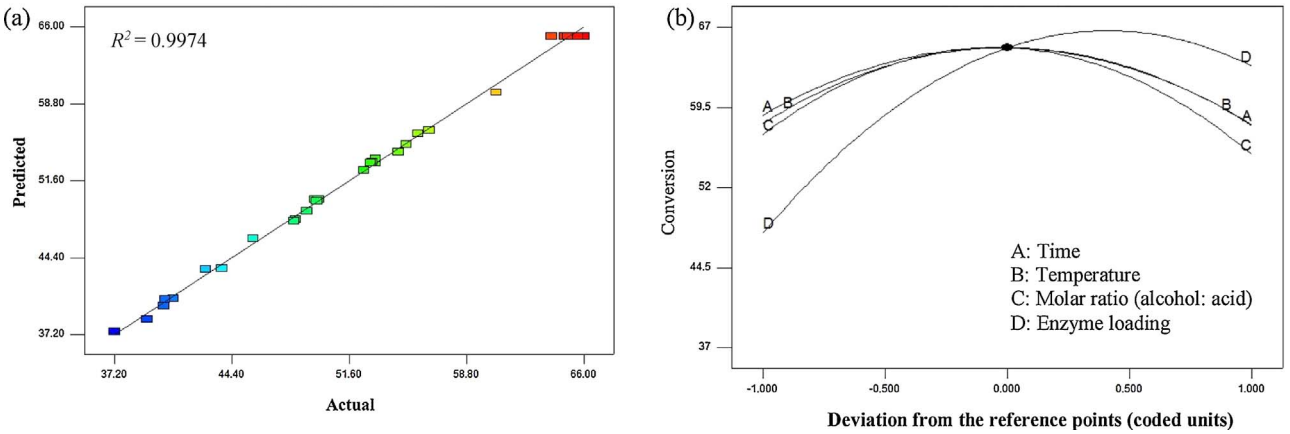


Fig. 1. a) Comparison between the predicted and actual values obtained for the BBD and the b) deviation from the reference point for the effects of time (A), temperature (B) and substrate molar ratio (C) and enzyme loading (D) to yield the highest percent conversion of eugenyl benzoate catalyzed by the RML-CS/CNWs.

determined logic limits in the screening experiments were consequently used for our BBD experimental design to obtain the best conditions for maximizing the percent conversion of eugenyl benzoate. Table 2 illustrates the rank of the independent variables, levels and experimental design in both coded and decoded terms along with the obtained actual and predicted values of the experiment.

The experimental data were regressed to fit the second-order polynomial equation to various models (linear, two factorial, quadratic and cubic) to assess the fitness of the BBD model. The study found the enzymatic synthesis of eugenyl benzoate catalyzed by the RML-CS/CNWs was well described by a quadratic polynomial model. The obtained experimental data closely agreed with the predicted values, reflected in the close scattering of experimental data near the trend line (Fig. 1a). The very high R^2 = 0.9974 implies an excellent correlation and a satisfactory model for predicting the best conditions [32] to maximize the yield of eugenyl benzoate. Over 99% of the variability in the response can be accounted by the model and a very high index of signal to noise ratio (*Adeq precision* = 62.15) further corroborates its reliability. The close agreement between the values of the *Adjusted* R^2 (0.9947) and R^2 further confirms the validity of the model.

The results for the ANOVA are presented in Table 3. It should be noted that a *P* value < 0.05 indicates a significant model term while *P* value < 0.0001 suggests the term is highly significant. P-value is an indicator for significance and interaction ability of each variable, in which variables showing lower P-values exhibit greater significance [33]. In contrast, a very large F-value signifies the magnitude of a given term to affect the outcome of the response, conversion (%) eugenyl benzoate. Hence, comparison between the F-values of the terms allows the study to rank the various interactions in order of their effect on the response. Here, the ANOVA for the regression model is highly significant due to a very small P-value (< 0.0001) and a corresponding large F-value (376.37) (Table 3). The F-value of the model (376.37) is substantially greater than the tabular $F_{0.05(14,14)} = 2.48$, implying that the degree of freedom relative to the residual is highly significant at the 95% confidence level. A high P value (0.8285) and small F value for the lack of fit (F-value = 0.5) in relevance to the tabulated $F_{0.05(10,4)}$ of 5.964 describes that the lack of fit is insignificant relative to the pure error. The above data conclusively show the constructed model is adequate to represent the experimental domain.

Table 3
ANOVA for the quadratic polynomial model of the BBD for the enzymatic synthesis of eugenyl benzoate.

	Source of Variation	Sum of Squares	Degree of freedom	Mean Square	F value	P-value
Conversion (%)	Model	2008.78	14	143.48	376.37	< 0.0001**
	Residual	5.34	14	0.38		
	Lack of fit	2.97	10	0.29	0.5	0.8285
	Pure error	2.37	4	0.59		
	Corr total	2014.11	28			

** Indicates that the effect is highly significant.

3.3. Influence of experimental factors on conversion yield of eugenyl benzoate

The following quadratic polynomial regression model for the conversion percentage of eugenyl benzoate is obtained as follows (Equation (5)):

$$\text{Conversion}\ (\%) = +65.08 - 0.45\ A - 0.1\ B - 0.94C + 7.81\ D - 1.8\ AB - 4.5\ AC - 2.8\ AD - 0.05\ BD + 0.28\ CD - 6.76\ A^2 - 7.19\ B^2 - 9.05\ C^2 - 9.52\ D^2 \quad (5)$$

where A is the reaction time (h), B is the temperature (°C), C is the substrate molar ratio of eugenol to benzoic acid and D is the enzyme loading (mg/mL).

The linear term for enzyme loading (D), interaction terms AB (time vs. temperature), AC (time vs. substrate molar ratio) and AD (time vs. enzyme loading) as well as the quadratic terms (A^2, B^2, C^2, D^2) are highly significant (P value < 0.0001) to affect the percentage conversion of eugenyl benzoate (Table 4). Linear terms A (time) and C (substrate molar ratio) are significant (P value < 0.05) while the factor of temperature (B) was not (P value > 0.05). Remarkably, the linear effect of B (temperature) is not significant within the assessed ranges in this study, possibly indicating the logic range of values for the reaction temperature is sufficient to support production of high yields of the ester. The interactive effect, BC (temperature vs. substrate molar ratio) has an insignificant influence on the yield of eugenyl benzoate as the term is not included in the regression equation (Equation (5)). The positive sign in front of the terms, D (linear) and CD (interaction) reflects a synergistic effect, whereas the negative sign for the remaining terms is indicative of antagonistic effects on the response. As the constructed plots show presence of a saddle point, the ridge maximum and canonical analyses are used to identify the critical levels of the design variables that maximize percent conversion of eugenyl benzoate.

b illustrates the perturbation plot which describes the interactions between the percent conversion of eugenyl benzoate and variables, reaction time (A), temperature (B), substrate molar ratio of eugenol to benzoic acid and (C) and enzyme loading (D). The percent conversion of eugenyl benzoate in relevance to A (time) appears to increase steadily over longer reaction duration for up to 5 h. Similarly, percent

Table 4
ANOVA for the quadratic model and coefficient values for enzymatic synthesis of eugenyl benzoate.

Source	Degree of freedom	F value	P value
Conversion (%)			
Linear			
A	1	6.37	0.0243*
B	1	0.31	0.5836
C	1	27.91	0.0001*
D	1	1919.18	< 0.0001**
Interaction			
AB	1	33.99	< 0.0001**
AC	1	212.47	< 0.0001**
AD	1	82.26	< 0.0001**
BC	1	0	1.0000
BD	1	0.03	0.8737
CD	1	0.79	0.3881
Quadratic			
A^2	1	777.73	< 0.0001**
B^2	1	878.58	< 0.0001**
C^2	1	1393.04	< 0.0001**
D^2	1	1543.14	< 0.0001**

** Indicates that the effect is highly significant.
* Indicates that the effect is significant. No asterisk indicates that the effect is not significant.

conversion for eugenyl benzoate increased until the midpoint of the plot for the effect of temperature (B). It was clear the presence of surplus eugenol and enzyme loading beyond 4 mg/mL and molar ratio 3:1 (alcohol/acid) (C), respectively, are counterproductive for the enzymatic reaction.

3.4. Mutual interaction of process variables on the percentage yield of eugenyl benzoate

3.4.1. Effect of time and reaction temperature

Using the response surface and contour plots of the quadratic polynomial model, the associations between the variables and the percent conversion of eugenyl benzoate can be better understood by holding two variables, molar ratio (alcohol: acid) and enzyme loading constant at their mid values: 3:1 and 3 mg/mL, respectively, and observing the interaction between the variables, time and temperature. Both process parameters are among the few deciding factors known to affect reaction yields of the ester product, as the activity and stability of the lipase catalyzing the reaction can be considerably affected [34].

Fig. 2a depicts the effect of time (A) and temperature (B) to affect the percent conversion of eugenyl benzoate catalyzed by the RML − CS/CNWs. Under an optimum condition, it was clear the maximum percent yield of eugenyl benzoate, as high as 65% is favored when both the time and temperature of the reaction are set close to their mid values. The relatively sharp curvature of the surface plot that (Fig. 2a(i)) represents the surface confined within the smallest ellipse in the contour plot (Fig. 2a(ii)) clearly shows a maximum yield is attainable at 50 °C after 5 h (Fig. 2a). The ANOVA for the linear factors of time and temperature shows the effect of reaction time, A (F value = 6.37) has a larger influence over temperature, B (F value = 0.31) (Table 4) to maximize the ester yield. However, their interaction is antagonistic (−1.8 AB) (Equation (5)), implying simultaneous increase of both variables beyond their mid points can render the RML − CS/CNWs catalyzing lower yields of the eugenyl benzoate.

It is apparent that the reaction temperature has a positive effect on the enzymatic reaction around the midpoint of the assessed variables. Hence, lower yields of the ester are expected when the temperature is either at its lowest or highest values. The initial increase in conversion and the initial rate of reaction seen here are because at elevated temperatures, viscosity of the mixture is reduced. This improves solubility and consequently facilitate mass transfer of the components in the reactions [34]. The elevated kinetic energy within the system also promotes effective collisions between the substrates and the RML − CS/CNWs. The positive effects of temperature up to this point is due to the enzymes on the surface of the supports gradually becoming less rigid and unfolding into their active form [34,35], concomitant with the elevation in percentage conversion of eugenyl benzoate (Fig. 2a). In contrary, reaction temperatures > 50 °C clearly exceeded the optimal threshold of the biocatalysts. These results in the excessive unraveling of the RMLs on the surface of the CS/CNWs support and the lipases are progressively inactivated. Beyond their optimum temperature, excessive breaking of the intramolecular hydrogen bonds, van der Waals forces, ionic and hydrophobic interactions that stabilizes the enzyme tertiary structure is enhanced. This induces structural over-flexibility and consequently disrupts the active tertiary structure of the enzyme [36,37], hence lowering the reaction yield.

The study also observed an increasing trend in the percent conversion of the ester with increasing reaction time. The reason for this kind of behavior is because a longer contact time increases probability of effective collisions [38,39] between the substrates and RML-CS/CNWs. But further prolonging the reaction time beyond 5 h was ineffective to improve reaction yield, presumably due to the increased presence of water as the by-product of esterification [40]. As the yield of the ester increases over time, so does the amount of liberated water that inadvertently favors the reverse counterproductive hydrolysis [32] of the produced ester. Similar observations have been reported for several other lipase-catalyzed esterification studies [20,33,41,42].

3.4.2. Effect of time and substrate molar ratio

The contribution of substrate molar ratio is also another key factor in any enzymatic reactions. To assert a forward direction in the reaction equilibrium requires increasing the nucleophile concentration while the effect of acid on the lipase is closely monitored. The mutual interaction, for the effects of time (A) and substrate molar ratio (eugenol: benzoic acid) (C) is assessed at fixed midpoints for enzyme loading and temperature at 3 mg/mL and 50 °C, respectively (Fig. 2b). The effect of molar ratio for alcohol: acid (F value = 27.91) is seen to be greater than the reaction time (F value = 6.37), suggesting the former has a more dominant effect on the response (Table 4). The corresponding surface (Fig. 2b(i)) and contour (Fig. 2b(ii)) plots at the lower and upper levels for effect of molar ratio of alcohol: acid is seen to influence the response in a quadratic manner, similar to that observed in Section 3.4.1. Percent of eugenyl benzoate gradually increased and reached a maximum of ~65% when the reaction time and molar ratio of eugenol: benzoic acid is increased from 3 to 5 h and 2:1 to 3:1, respectively. Beyond these values, a general decline in the yields of the ester is observed. Correspondingly, the negative coefficient of AC (−4.5AC) (Equation (5)) indicates an antagonistic interaction between both factors, implying that higher yields of the ester is possible when the conditions of each variable are inversed.

The significant rise in the product conversion in the initial stages can be described by the thermodynamics of the reaction whereby the forward reaction is favored when the starting material is present in excess, more so for a reversible reaction i.e. esterification [16]. For a favorable outcome, the study deliberately used a surplus amount of eugenol to improve the ester yield, aside from reducing the toxicity of benzoic acid that could destabilize the structure of the RML-CS/CNWs. Previous studies also reported enhanced product yields when higher concentrations of the alcohol are used [33,34,43,44]. However, the noteworthy lower yields of eugenyl benzoate beyond the 3:1 critical molar ratio of eugenol: benzoic acid indicates greater inhibitory effects of eugenol on the RML-CS/CNWs. It is also symptomatic of a slower diffusion rate of eugenol molecules into the active site of RML. The same trend is also reported in literature [45]. Also, an excess of the highly polar alcohol molecules tend to enhance the over-stripping of essential water surrounding the enzyme structure [33,46] causing the enzymes to partially lose their activity. Moreover, the competitive

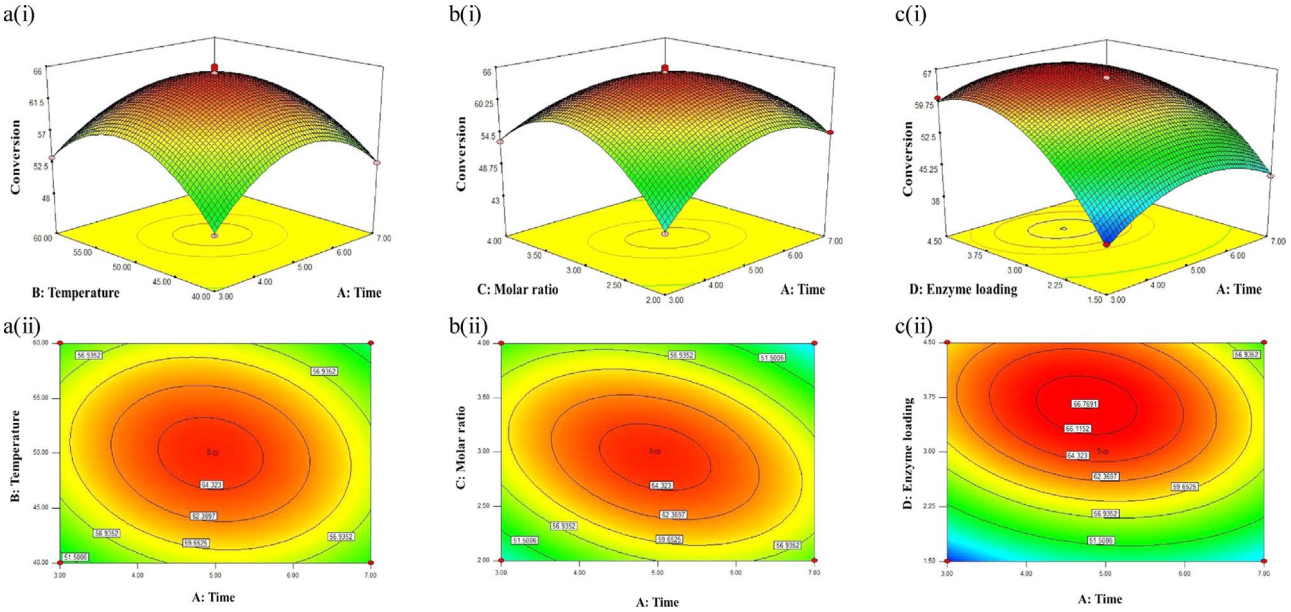


Fig. 2. The response (i) surface and (ii) contour plots showing the interactive effects for (a) A: time and B: temperature on the yield of eugenyl benzoate at constant substrate molar ratio of 3:1 (eugenol: benzoic acid), (b) A: time and C: substrate molar ratio (eugenol: benzoic acid) and (c) A: time and D: enzyme loading to affect the percent conversion of eugenyl benzoate catalyzed by the RML-CS/CNWs.

binding of alcohol (eugenol) molecules to the enzymes' active site in the first step of the catalytic mechanism, instead of the acid molecule, further lowers the rate of the reaction. The phenomena described above are consistent with the observed declining in percent conversions of eugenyl benzoate at greater ratio of eugenol over benzoic acid (Fig. 2b). Nonetheless, such trend seen here is not a definite proof of an alcohol-induced inhibition on the RML-CS/CNWs. Further experimental assessments using a kinetic model fitting to study the actual inhibitory effect of both substrates on this reaction is further discussed in Section 3.6.

3.4.3. Effect of time and enzyme loading

An optimum use of enzyme loading that favors cost-effective reaction and high esterification yield is essential for any given enzymatic reaction. Focusing on maximizing the reaction yield and economic feasibility of the reaction to produce eugenyl benzoate, assessment on the interactive effect of time (A) and enzyme loading (D) was carried out. The variables, temperature and molar ratio of alcohol: acid are kept constant at their mid values. Fig. 2c illustrates the interactive effect for time versus enzyme loading on the percent conversion of eugenyl benzoate.

It is clear that the loading of the RML-CS/CNWs was central in ensuring high yields of the ester, mirrored in its very large F value (1919.18) as compared to time (6.37) (Table 4). A maximum yield of eugenyl benzoate at 66.8% is possible when the reaction uses an enzyme load of ~3.75 mg/mL and is progressed up to 4.75 h. The relatively small area in the center of the obtained elliptical contour plot (c (ii)) implies that their interactive effect is significant, however, their interaction is antagonistic (− 2.8AD) (Equation (5)). In this regard, the model specifies that the values for the reaction time should be inversed to that of enzyme loading to ensure a favorable response in this study.

We can see that increasing in the enzyme loading beyond 3.75 mg/mL is ineffective in improving the response. This behavior can be attributed to the phenomenon of agglomeration of surplus RML-CS/CNWs [3,33,45] or steric hindrance of the RML-CS/CNWs at higher enzyme levels that impede access of substrates into the active sites. Although a higher enzyme loading may offer more active sites for interaction with substrates to produce higher product conversion [47], it is also the limiting factor for any given esterification reaction. When a saturation level of the enzymatic reaction has been reached (all enzymes are bound to the substrates) the rate of reaction will no longer increase and equilibrium has been attained [46,48]. Under such circumstances, only the RML-CS/CNWs located on the outermost layer of the immobilized enzyme aggregates are available and effectively catalyzing the reaction. The aforementioned phenomena agree well with our observations, as the reaction mixtures appear more viscous when the enzyme levels are elevated beyond 4 mg/mL. For these reasons, an inhomogeneous dispersion as well as an inclusive decline in mass transfer have been described in literature [32]. Our results also show the use of lower enzyme levels cause an insignificant decline in percent conversion. The produced eugenyl benzoate remains relatively high at ~ 63% when using 2.75 mg/mL of the RML-CS/CNWs and the reaction time was left to advance between 3 − 5 h. We firmly believe that a high yield of eugenyl benzoate at lower RML-CS/CNWs loading is cost-effective and can be translated into potential savings.

3.5. Attaining optimum condition and model verification for enzymatic synthesis of eugenyl benzoate

The study proceeded for validation of the generated RSM model to ensure the model is accurate and reliable. The software (Design Expert 7.1.6) proposed several experimental conditions to find the optimum point and maximize the percent conversion of eugenyl benzoate under the following preferred conditions: (1) all reaction factors within the model range, (2) minimum enzyme loading and (3) minimum reaction temperature. The validation experiments were carried out within the design space and the reactions were monitored every hour, and the results are illustrated in Fig. 3. The validation results show the actual values are in good agreement with those of the predicted (Table 5). The ester yield declines after 5 h (Fig. 3), hence echoing the trend seen in the earlier optimization experiments (Section 3.4). The highest yield of eugenyl benzoate at 64.80% is reached when the validation variables are in range. Percent conversion of eugenyl benzoate is relatively lower at the lowest enzyme loading and temperature to afford a 57.30 and 57.80% of the ester, respectively. The deviations between the actual and predicted values are found to be very small (1.13 − 3.1%), hence further affirms the reliability and accuracy of the RSM model to predict the optimum conditions.

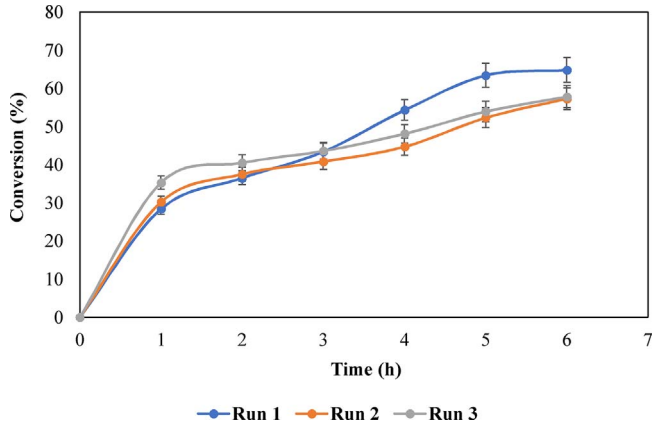


Fig. 3. Determination of the optimum conditions for the highest percent conversion of eugenyl benzoate catalyzed the RML-CS/CNWs.

3.6. Kinetic study for esterification of eugenol with benzoic acid catalyzed by RML-CS/CNWs

3.6.1. Effect of substrate concentrations on reaction rates

The kinetics of the RML-CS/CNWs-catalyzed (3 mg/mL) synthesis of eugenyl benzoate was investigated by studying the effect of the concentrations of both eugenol (100–350 mM) and benzoic acid (100–225 mM) on the initial rates of the esterification. Fig. 4a(i) represents the relationship between the initial rate of esterification using different concentrations of benzoic acid with the concentration eugenol fixed at 100–300 mM. It is evident the initial rates of reaction for both depictions resembled the classic Michaelis–Menten-shaped curve [49], in which the rates of reaction are elevated with the increase in benzoic acid concentration (Fig. 4a(i)). The general increase in the rates of reaction at higher acid concentration suggests the activity of RML-CS/CNWs is not adversely influenced by the range of benzoic acid concentrations used in this study. In contrary, higher concentrations of eugenol starting from 200 mM is notably counterproductive, as seen in the apparent reduced rate of reaction (Fig. 4a(i)). Although the effects are relatively minor, the outcome seen here suggests the RML-CS/CNWs-catalyzed synthesis of eugenyl benzoate is more inhibited by eugenol than benzoic acid. Earlier works by Gofferje et al. [50], Mathpati et al. [47], Raita et al. [26] and Yu et al. [51] also observed similar alcohol inhibition for various lipase-catalyzed esterification reactions.

The effect of varying eugenol concentrations at a fixed concentration of benzoic acid (50–100 mM) is illustrated in Fig. 4b(i). Elevating the concentration of benzoic acid contributed to higher reaction rates, which rules out the inhibitory effect of benzoic acid on the RML-CS/CNWs-catalyzed synthesis of eugenyl benzoate. The plateau observed for benzoic acid concentrations, 50 and 75 mM is concomitant with mass transfer limitation, commonplace for reactions carried out at low substrate concentrations [50]. The relatively low reaction rate when the concentration of eugenol is increased in relevance to the concentration of benzoic acid is presumed to be caused by the inactivation of RML-CS/CNWs by the excess eugenol. However, this finding alone is inadequate to prove the inhibitory effect of eugenol on the RML-CS/CNWs. To ascertain the inhibitory effect of eugenol on the lipase, the corresponding double reciprocal plots Lineweaver–Burk were constructed for better comprehension of the reaction kinetics [42].

3.6.2. Lineweaver–Burk plots for the RML-CS/CNWs-catalyzed esterification of eugenol and benzoic acid

Fig. 4a(ii) and b(ii) depict the double reciprocal Lineweaver–Burk plots for both set of experiments, in which the concentration of benzoic acid and eugenol were varied, respectively. As seen in Fig. 4a(ii), formation of steeper lines suggests increasing reaction rates when the concentrations of benzoic acid and eugenol are elevated. The two sets of parallel lines present on the Lineweaver–Burk plots (Fig. 4a(ii) and b(ii)) are both characteristic of a ping-pong bi–bi mechanism. This confirms the RML-CS/CNWs-catalyzed synthesis of eugenyl benzoate followed such mechanism, which agrees well with earlier reports for enzymatic esterification carried out by other researchers [50,52,53].

The lower reaction rates at higher concentrations of eugenol (Fig. 4b(ii)) are affirmative of the inhibitory effects of eugenol. Such effect was also prevalent in earlier optimization experiments (Section 3.3.2) when the influence of the substrate molar ratio on the percent yield of eugenyl benzoate was assessed. At higher concentrations of eugenol, three phenomena that essentially inactivate catalysis by the RML-CS/CNWs may occur; i) eugenol becomes increasingly immiscible at the catalytic site [26] of the RML which consequently deactivates the lipase, ii) complex interaction between the hydrophobic pockets of the RML and eugenol which leads to larger conformational mobility and finally iii) formation of dead-end inhibition complex [47,54], all of which obstruct the proper interactions at the active sites of the lipase and reduce reaction rates.

Using the ping-pong bi–bi model with eugenol inhibition (b), the mechanism of esterification for eugenol with benzoic acid depicted in Cleland's notation, is proposed (Fig. 5). The first step of the mechanism involves the binding of benzoic acid, (A) to the RML, (E) to form a non-covalent enzyme-benzoic acid complex (EA). The EA isomerizes to produce the first product, water (P) and acylated-RML (E*) are liberated. The following step sees the second substrate, eugenol (B) interacts with E* to produce the acylated-RML-eugenol complex (E*B), which is subsequently transformed to the acylated RML-benzoic-eugenol ester complex (EQ) before yielding the product, eugenyl benzoate ester (Q) and free RML (E). The irreversible binding of eugenol to the enzyme (E) and to the eugenol-enzyme complex also occurs simultaneously to produce dead-end complexes (EB). Fitting of the reaction rate for the esterification reaction to Equation (4) produces the kinetic parameters and the results are summarized in Table 6. The apparent Michaelis-Menten constants highlights the fact that the RML-CS/CNWs have higher affinity for benzoic acid ($K_{m,A}$ = 34.04 mM) than eugenol ($K_{m,B}$ = 138.28 mM). For most enzyme-catalyzed reactions, the Michaelis-Menten constant usually falls between 10^{-1}–10^{-7} M [34], well within the range of the kinetic constant in this study. The inhibition constant for eugenol, $K_{i,B}$ which measures the sensitivity of the RML-CS/CNWs-catalyzed esterification to inhibition by the substance, reveals the inhibitory behavior of eugenol to be at $K_{i,B}$ = 438.6 mM. The decline in the esterification rate beyond this point is due the increased propensity of irreversible binding the eugenol molecules to form the inactive dead-end complex. A matter of fact, a similar phenomenon has been reported by several studies [50,55]. Hence, we successfully demonstrated the obtained inhibition constant directly correlates with the inhibitory effect of eugenol on the esterification reaction.

The turnover number (k_{cat}) was calculated to be 40.39 min^{-1} and

Table 5
The attained optimum conditions for the RML-CS/CNWs catalyzed synthesis of eugenyl benzoate.

Run No.	Parameters	Time (h)	Temperature (°C)	Molar ratio	Enzyme loading (mg/mL)	Predicted yield (%)	Actual yield (%)	Deviation (%)
1	In range	6	49.29	2.83	3.5	65.54	64.80 ± 0.33	1.13
2	Low enzyme loading	6	49.33	2.82	2.3	58.73	57.30 ± 1.02	2.43
3	Low temperature	6	41.04	2.83	3.5	59.65	57.80 ± 0.41	3.1

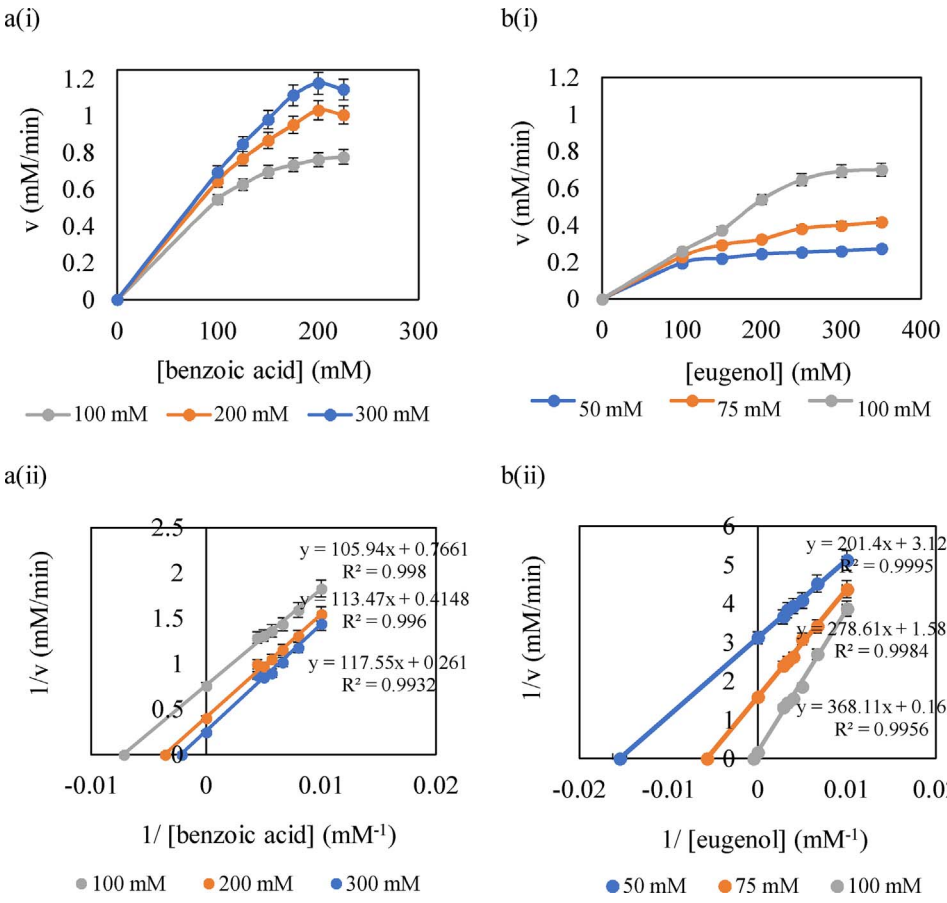


Fig. 4. The reaction rate for the RML-CS/CNWs-catalyzed esterification to synthesize eugenyl benzoate as a function of: a(i) benzoic acid content at varying eugenol concentration (100–300 mM) and b(i) eugenol concentration at varying benzoic acid concentration (50–100 mM). The corresponding Lineweaver–Burk double reciprocal plots a(ii) benzoic acid concentration at varying eugenol concentration (100–300 mM) and b(ii) eugenol concentration at varying benzoic acid concentration (50–100 mM).

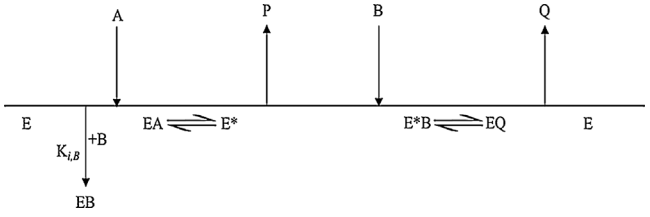


Fig. 5. Schematic representation of the Ping-Pong Bi–Bi mechanism with eugenol inhibition. E, A, P, B, Q, and E* denote RML, benzoic acid, water, eugenol, eugenyl benzoate and acylated-RML, respectively. EB is the dead-end inhibition complex of RML-eugenol.

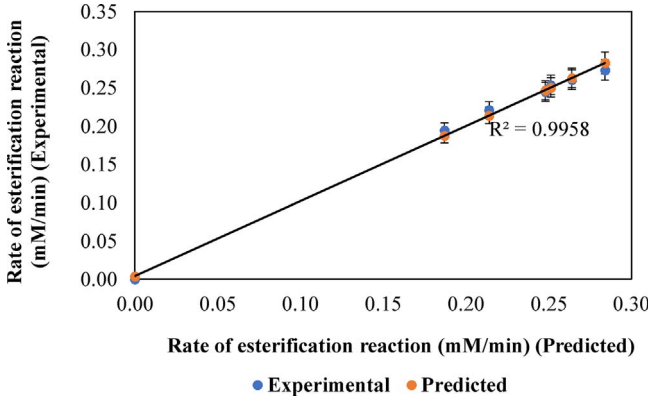


Fig. 6. Parity plot of theoretical reaction rate versus experimental rate.

Table 6
Determined values of the model kinetic constants.

Kinetic parameters	RML-CS/CNWs
v_{max} (mM min^{-1})	3.83 ± 0.21
$K_{m,A}$ (mM) – acid	34.04 ± 0.93
$K_{m,B}$ (mM) – alcohol	138.28 ± 1.21
$K_{i,B}$(mM) – inhibition by alcohol	438.6 ± 5.88
k_{cat} (min^{-1})	40.39 ± 0.61
k_{eff} (min^{-1} mM^{-1})	0.09 ± 0.18

*SSE value for this study (RML-CS/CNWs) = 0.007.

the ratio between k_{cat} and K_M gives the catalytic efficiency (k_{eff}) of the RML-CS/CNWs equals to 0.09 min^{-1} mM^{-1}. These values represent the enzyme's overall ability to convert the substrate into the corresponding product [56]. Correspondingly, a low SSE (0.007) (Table 6) infers the obtained model is well fitted [57] and the highly similar values between the R^2 (0.996) and adjusted R^2 (0.995) also corroborates the goodness of the model. A very high R^2 (0.9958) of the constructed parity plot for the theoretical versus the experimental esterification rate further verifies the validity of the obtained ping-pong bi–bi model with inhibition by eugenol (Fig. 6).

3.7. Reusability and thermal stability

Relative activity of the RML-CS/CNWs was retained up to 50% after 8 successive cycles of esterification (Fig. 7a). The data also indicated that the multi-point stabilization of RML to the surface of CS/CNWs was the contributing stabilizing factor. Essentially, the biopolymer acted as a backbone support to prevent the lipase from undergoing premature inactivation after repeated cycles of esterification. On the contrary, the decline in lipase activity after each reaction cycle can be attributed to lipase deactivation by benzoic acid as well the slow obstruction of RML actives sites by deposits of eugenyl benzoate or the starting materials.

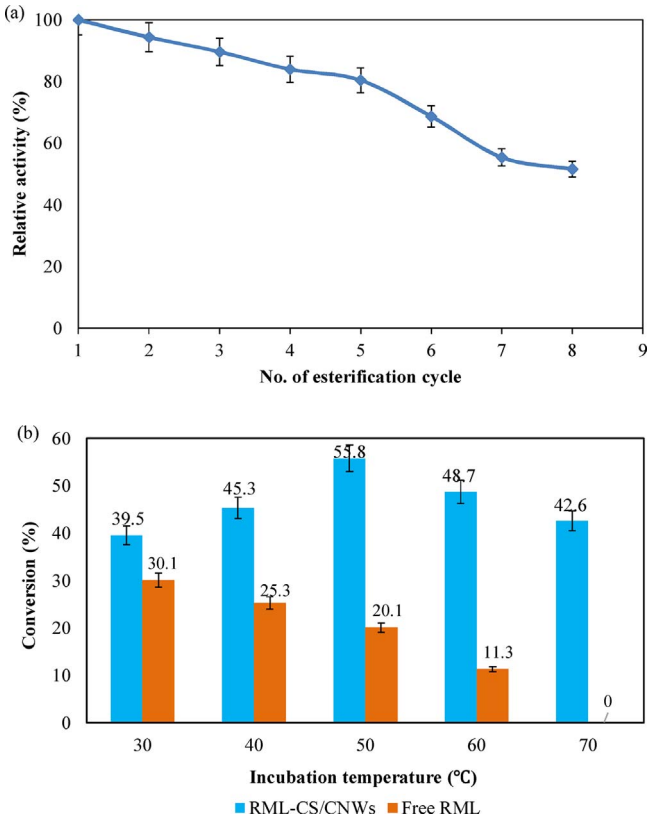


Fig. 7. (a) Reusability of RML-CS/CNWS and (b) thermal stability of RML-CS/CNWs and free RML, respectively.

Earlier esterification studies have also reported a similar outcome [34,58]. Additionally, the data hence affirmed that the RML-CS/CNWs were relatively stable in chloroform as the solvent in the esterification reaction. Reusability of RML-CS/CNWs was slightly better than laccase immobilized on chitosan/poly(vinylalcohol) composite nanofibrous membranes. The biocatalyst was usable up to a successive seven-batch operation [59]. Similarly, various enzyme-catalyzed esterification displayed comparable reusability results; such as immobilized *Thermomyces lanuginosus* lipase on mesoporous poly-hydroxybutyrate particles to produce methyl oleate [60] and *Rhizopus niveus* lipase immobilized onto chitosan coated magnetic nanobiocatalyst for the enzymatic synthesis of glucose oleate ester [61]. Conversely, Lipozyme RM-IM was reusable for only three esterification cycles to synthesize an ester of jatropha oil [62].

Data of the thermal stability study reveal that RML-CS/CNWs consistently afforded higher yields of eugenyl benzoate than free RML for all tested temperatures, affording percentage conversions of the ester between 55.8–39.5% for the RML-CS/CNWs as compared to free RML at 0–30.1%, for assessed temperatures 30–70 °C (Fig. 7b). The dramatic decline in activity of free RML for temperatures > 50 °C signifies the increase propensity of such form of lipases to inactivation as compared to immobilized lipase [63]. The data also imply that the prepared CS/CNWs could improve operational stability by rigidification of the structure of the immobilized RML [64]. Hence, it was shown that the RML-CS/CNWs have higher thermal stability as compared to the free forms of RML.

4. Conclusion

The present study demonstrates the effectiveness of RSM to optimize the esterification of eugenol and benzoic acid catalyzed by RML-CS/CNWs to produce satisfactory yield of eugenyl benzoate. The results indicated that three linear (time, substrate molar ratio and enzyme loading) and interaction effects (time vs. temperature, time vs. substrate molar ratio and time vs. enzyme loading) were significant to improve eugenyl benzoate synthesis. The study found that as much as 66% of eugenyl benzoate could be produced under optimal reaction conditions (at 50 °C, 5 h of reaction time, 3:1 of eugenol to benzoic acid and 3 mg/mL of RML-CS/CNWs). Based on the findings, the application of RSM to optimize the esterification was useful to predict of the optimum conditions that maximizes yield of eugenyl benzoate. Based on the kinetic studies conducted, it was confirmed that the RML-CS/CNWs-catalyzed synthesis of eugenyl benzoate followed the ping-pong bi–bi model. The kinetic system parameters obtained in this study conclusively show the higher preference of the RML-CS/CNWs for benzoic acid and greater inhibitory effect for eugenol. The prepared RML-CS/CNWs consistently demonstrated improved thermal stability over the free RML and could be reused for up to 8 cycles of esterification.

Acknowledgments

This work was supported by the Research University Grants (GUP) of Universiti Teknologi Malaysia, Johor Bahru from the Ministry of Higher Education Malaysia (Q.J130000.2526.09H85 and Q.J130000.2526.13H09). We would also like to acknowledge valuable help and suggestions provided by our colleagues.

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