FABRICATION OF *Rhizomucormiehei*LIPASE REINFORCED NANOBIOCONJUGATES AS BIOCATALYSTS FOR A STATISTICALLY OPTIMIZED PRODUCTION OF EUGENOL BENZOATE

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Abstract

The chemical synthesis ofeugenol benzoate is associated with numerous environmentally unfavorable practices *viz.* the use toxic chemicals, tedious separation process and liberation of harmful by-products. On this standpoint, an alternative approach utilizing *Rhizomucormiehei*lipase (RML) immobilized onto activated chitosan-multiwalled carbon nanotubes (RML/CS/MWCNTs) is suggested. The properties of the biocatalyst were characterized using Fourier-Transform Infrared Spectroscopy (FTIR), Field Emission Scanning Electron Microscopy (FESEM) and thermogravimetric analysis (TGA). Response surface methodology employing central composite design (CCD) based on four relevant parameters (incubation time, temperature, substrate molar ratio, and enzyme loading) was used to optimize the experimental conditions for the enzymatic synthesis to produce eugenol benzoate. The study found the high conversion of eugenol benzoate is greatly affected by temperature and incubation time. The highest yield of eugenol benzoate was 56.13 %, under optimized conditions of 60 °C, incubation time of 6 hr, enzyme loading of 150 IU and molar ratio of eugenol/benzoic acid of 4:1. The findings suggest that the RML/CS/MWCNTs biocatalyst developed here is a promising alternative to overcome shortcomings associated with the chemically produced esters.

Keywords: esterification, eugenol benzoate, Rhizomucormieheilipase, nanobioconjugates, Response Surface Methodology.

INTRODUCTION

Eugenol (4-allyl-2-methoxyphenol) is a member of phenylpropanoids, and it is commonly found in some essential oils especially in clove leaf oil, cinnamon oil, nutmeg, bay and basil leaf (Chaibakhsh*et al.,* 2012). Eugenol exists as a clear to pale yellow oily liquid and is commonly used in cosmetics and food products as a flavoring agent.Conventionally, esters of eugenol such as eugenol benzoate are acquired through the chemical route of the esterification process which is concomitant with a myriad of shortcomings (Radzi*et al.,* 2011). Such approach to synthesize the eugenol esters incurs the use of strong acidcatalysts and hazardous chemical such as halogenated metals. Apart from low conversion and extensive reaction time, the chemical route has been associated with tedious separation process and emancipation of harmful unwanted by-products (Charpe and Rathod., 2011). These problems can be overcome by opting for the biotechnological route utilizing specific biocatalysts such as lipases. Lipases (triacylglycerol ester hydrolysis EC 3.1.1.3) are enzymes that catalyze esterification reactions under ambient conditions and do not require conditions of high temperature and pressure. The biotechnological route is relatively straightforward when compared with the chemical synthesis (Charpe and Rathod., 2011).

In this context, *Rhizomucormiehei* lipase (RML) is the biocatalyst of choice owing to its versatility, strong specificity, and its suitability for ester synthesis has been evaluated under different conditions of temperature, pressure, water content, and substrates (Skoronski*et al.,* 2014). In order to improve the properties of the lipase, RML was immobilized onto a suitable solid support; in this case an activated chitosan/multiwalled carbon nanotube and used as catalyst. The immobilized enzymeallows reuse of the biocatalysts for better productivity (Zou*et al.,* 2010), improve stability and activity of enzymes (Cesar *et al.,* 2007). Currently, many materials have been explored for purposes of immobilizing lipases such as polymer resins, silica, chitin, chitosan, carbon nanotubes, and microspheres. Among these polymers, chitosan and carbon nanotubes are often favored by researchers. Chitosan (CS) is a natural cationic biopolymer that offers the benefits of biocompatibility, non-toxicity, and has high mechanical strength. This is due to the presence of amino and hydroxyl groups in CS that facilitates attachment of enzymes via covalent bonding or crosslinking (Solanki*et al.,* 2009). Likewise, carbon nanotubes (CNTs) have extraordinary mechanical strength, good electrical and thermal properties (Mubarak *et al.,* 2014). Once the surfaces of the CNTs are functionalized, the functional groups afford good sites of linkage with enzyme proteins.

The current conventional method of optimization requires screening of large number of variables, requires a large number of experiments, and involves lots of time and resources (Chaibakhsh*et al.,* 2012). Hence, response surface methodology (RSM) is employed as a fast and economical statistical technique that can be used to determine the optimal conditions of a multivariable system. RSM has been extensively applied for optimization of enzymatic processes (Chaibakhsh*et al.,* 2012). This present study was aimed at investigating the applicationof covalently bonded of RML ontoactivated chitosan/multiwalled carbon nanotube as potential economical biocatalysts. The RML/CS/MWCNTs beads were then used in synthesizingeugenol benzoate. In

addition, the enzymatic synthesis is then optimized by employing RSM, and the parameters investigated are incubation time, temperature, molar ratio of acid to alcohol, and enzyme loading.

EXPERIMENTAL

Materials

Commercial lipase of *Rhizomucor miehei*, activity of ≥20,000 U/g was purchased from Sigma-Aldrich (St. Louis, USA). Eugenol (>99% purity) and benzoic acid (>99% purity) were used as reagents for esterification reactions. Sodium hydroxide (NaOH) pallet, chloroform, glacial acetic acid, and nitric acid (65%), sodium phosphate buffer (pH 5.6), phenolphthalein were also purchased from Sigma-Aldrich. Meanwhile, powdered chitosan and MWCNTs were a gift from Dr. Zainoha and Dr. See Hong Heng, respectively. Other chemicals such as 2-(N-morpholino)ethanesulfonic acid (MES) salt, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC), and *N*-hydroxysuccinimide (NHS) were purchased from Tokyo Chemical Industry (Korea).

Purification and Functionalization of MWCNTs

Commercial MWCNTs (1.5 g) were refluxed with concentrated HNO_3 (4M, 90 mL) at 80˚C for 5 h to completely remove the amorphous carbon phase and to oxidize the raw material. After cooling to room temperature, the mixture was centrifuged at 6000 rpm for 5 mins. The MWCNTs suspension was washed thouroughly with distilled water until the pH is neutral. Then, the suspension was filtered and dried in the oven (60˚C) for 8 h. For the functionalization process, purified MWCNT (1.5 g) was refluxed in 1:3 v/v a mixture of H_2SO_4 (10ml, 95-97%) and HNO_3 (30ml, 65%). Then, the purified MWCNTs were oxidized under reflux at 100˚C for 6 h to introduce functional carboxylate group. After the mixture is completely cooled, the suspension of functionalized MWCNTs (f-MWCNTs) was centrifuged at 6000 rpm for 5 mins and the liquid decanted. The f-MWCNTs were washed for several times with distilled water until the pH 7.0 was attained. The f-MWCNTs was precipitated by centrifugation (6000 rpm) and dried in vacuum oven at 60˚C (Mohamad*et al.,* 2015).

Preparation of MWCNT/Chitosan Beads

MWCNT/chitosan (CS) beads were prepared by mixing 500 mg of powdered CS into 25 mL acetic acid solution (2.0% v/v) and stirred for 10 min at room temperature. The f-MWCNTs (5% w/w) was added into the CS mixture and homogenously stirred to afford the CS-MWCNTs suspension. The CS-MWCNTs mixture was slowly dropped into 1M sodium hydroxide (NaOH) solution using a pipette under continuous stirring.The initial beads were formed in the mixture and stirred for an additional 30 mins. CS-MWCNTs beads were washed several times with distilled water till neutrality. The beads were left to dry at room temperature overnight (Campos Carneiro*et al.,* 2014).

Covalent immobilization of Rhizomucormiehei on CS-MWCNTs beads and its characterization

Attachment of free FRML using EDAC as cross linker was performed according to the method described by Raghavendra*et al*. (2013) with some modifications. MWCNTs/CS beads (400 mg) were suspended in 20 mL 50 mM 2-(N-morpholino)ethanesulfonic acid (MES buffer at pH 6.1) and the mixture was stirred at 150 rpm for 5 mins before addition of NHS (8.0 mL of 50 mg/mL) followed by continuous stirring for another 5 mins. Under fast stirring, 4.2 mL of EDAC (10 mg/mL) was added and the mixture was stirred magnetically at room temperature for 60 min. The CS-MWCNTs beads were washed with 50 mM MES buffer (pH 6.1) to remove excess EDAC, NHS and urea by-product. The activated MWCNTs/CS beads were dispersed in 9.2 mL of 50 mM MES buffer (pH 6.1) containing 0.8 mL (20,000 IU/g) RML. The mixture was stirred at 4°C for 16 h to afford the CRL-CS-MWCNTs beads. The CRL-MWCNTs/CS beads were washed with 50 mM MES buffer (pH 6.1) to remove any unbound protein until no activity was detected in the washings and vacuum dried. The RML-CS-MWCNTs beads were stored at 4 °C until further use.

Attenuated Total Reflectance (ATR): An aliquot amount of beads is placed onto the small crystal area. By using the Spectrum 100 Series, the vibrational spectrum of RML-CS-MWCNTs beads was recorded. The spectrumwas obtained using model in transmission mode between 400 -4000 cm^{-1}. ***Field emission scanning electron microscope (FESEM):*** A field emission scanning electron microscope (FESEM) (Brand: Hitachi, Model:SU8020) was used to study the morphology RML-CS-MWCNTs beads.

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RML-CS-MWCNTs Catalyzed Synthesis of Eugenol Benzoate

The esterification reactions were performed in 50 mL screw-capped bottles which consisted of varying molar ratios of eugenol and benzoic acid and, stirred in chloroform as solvent. The RML-CS-MWCNTs beads (1 mg/mL) were added to initiate the reaction and stirred in a horizontal paraffin oil bath at 200 rpm at variable temperatures and incubation time (Table 3.1) (Horchani*et al,* 2010).

Experimental Design and Statistical Analysis

A four-factor-five-level central composite rotatable design (CCRD) was employed in this study, requiring 30 experiments. The fractional factorial design consisted of 16 factorial points, eight axial points, and six centre points. The parameters and their levels used for the optimization of RML-CS-MWCNTS catalysed synthesis of eugenol benzoate were reaction time (2-18 hr), temperature (30-70°C), enzyme loading (50-45- IU) and molar ratio of alcohol:acid (1:1-5:1). The experiments were randomized for statistical reasons and each experiment was run in triplicates.A software package, Design Expert Version 7.0 (Stat-Ease, Statistical Made Easy, Minneapolis, MN, USA) was used for designing and analysing the experimental data. The model equation was used to predict the optimum value and subsequently to elucidate the interaction between the factors. The quadratic equation model for predicting the optimal point was expressed as below:

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i + \sum_{i=j}^{3} \sum_{j=i+1}^{4} b_{ij} x_{ij} + e \qquad \text{(Eqn.1)}$$

where y is the dependent variable (% yield) to be modelled; x_i and x_j are the independent variables (factors), b_0, b_i, b_{ii}, and b_{ij} are the regression coefficients of the model and *e* is the error of the model. Next, analysis of variance (ANOVA) was used to determine the adequacy of the constructed model to describe the observed data. R^2 value presents statistical points to the percentage of the variability of the optimization parameters explained by the model. Three-dimensional surface plots were generated to illustrate the main and interactive effects of the independent variables on the dependent ones.

RESULTS AND DISCUSSION

Rationale on covalent immobilization of RML onto activated MWCNTs/CS beads

Surface functionalization of carbon nanotubes plays an essential role for improving the solubility and dispersion of the nanotubes in aqueous solutions and to design new materials (Yudianti*et al.,* 2011). The functional groups (COO^-) grafted onto the MWCNTs (f-MWCNTs) can potentially increase their interactions with CS, thus resulting in significant improvement in mechanical properties of the MWCNTs/chitosan beads.In this study, carbon nanotubes were proposed as fillers within the polymeric biomaterials of CS and serve to strengthen structure of CS and improve mechanical and biological properties compared to neat CS (Kroustalli*et al.*2013). Covalent attachment of RML to the CS/MWCNTs beads provide powerful link between the lipase and its carrier matrix. The nucleophilic groups (COO^-) will interact strongly with electrophiles on the RML protein. The resultant RML-CS/MWCNTs nanobioconjugates are more desirable and beneficial in terms of reusability and robustness.

Characterization of Immobilized Lipase (RML/CS/MWCNTs)

Field emission scanning electron microscope (FESEM)

CS beads were incorporated with MWCNTs to enhance their mechanical structure. The inclusion of MWCNTs changed the morphology of the beads, as shown in **Figure 1(i).** The CS/MWCNTs bead had a more rigid and spherical structure and its FESEM image shows a spiky structure that resulted from the inclusion of MWCNTs nanoparticles, which could further enhance its mechanical strength (Lau *et al.,* 2014). In addition, the change in the roughness of the beads can be observed **Figure 1(ii)** and it is increased upon N-hydroxysuccinimide (NHS)and EDAC treatment and subsequent RML immobilization on the activated surface (Hedge and Veeranki, 2014).It can be seen that the reactivity of beads was increased due to the formation of amide bonds between carboxylic acid groups on the functionalized MWCNTs, and amine groups from the RML. This process was catalyzed by the activation of carboxyl groups by the EDAC coupling reagent, resulting in successful enzyme immobilization (Alkhatib et al., 2012).

(i) (ii)

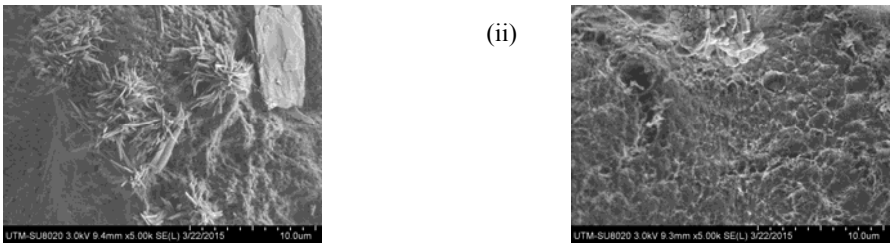

Figure 1: Micrographs of(i) CS-MWCNTs beads and (ii) immobilized RML onto activated CS beads at 5,000 magnifications.

Fourier Transform Infrared Spectroscopy - Attenuated Total Reflectance (ATR)

The spectrum of RML-CS-MWCNTs in **Figure 2**showed numerous peaks in the range of 670-1575 cm^{-1} as a result of activation by NHS and EDAC. Peak presents at 1026.31 cm^{-1} belonged to the secondary amines which indicated the presence of unstable o-acylisourea intermediates formed as the by-product in the reaction of EDAC and –COOH groups (Raghavendra*et al.,* 2013). Emergence of multiple peaks at 1642.98, and 1420.33 cm^{-1}suggesting that immobilized RML retained its native structure (Collins *et. al,* 2011). Slight shifting to higher wavenumber of peaks and increasing of absorption bands indicate a possible covalent interaction between the support and enzyme (Diaconu*et al.,* 2010).

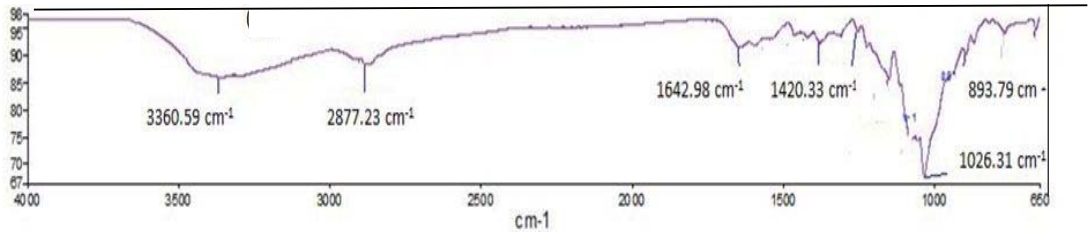

Figure 2: IR spectrum for RMLimmobilized onto activated CS beads.

Thermal Gravimetric Analysis (TGA)

Thermal properties RML/CS/MWCNTs beads were assessed by thermogravimetric analysis and depicted in Figure 3. The thermogram profile of RML/CS/MWCNTs beads revealed a relatively steep mass loss of nearly 30% that occurred at a lower temperature of approximately 250 °C. This observation is due to the enzyme molecules that are proteinaceous in nature that tended to prematurely decompose at lower temperatures as compared to other components in the RML/CS/MWCNTs composite. Based on the thermogram, the study found the covalent immobilization of RML to the surface of the CS/MWCNTs beads as calculated from the final weight percentage of the samples (Raghavendra*et al.,* 2013), was established to be 10.17%.

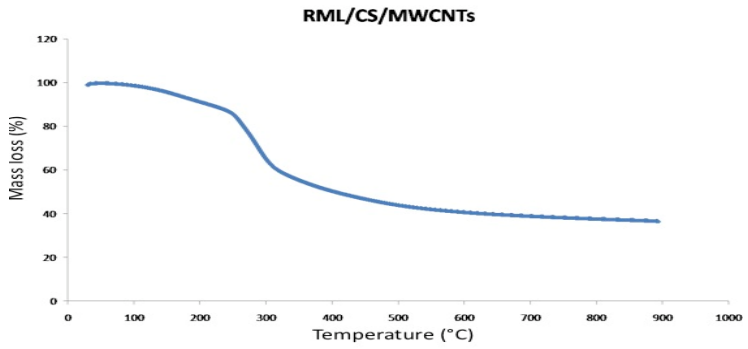

Figure 3: The thermogram for the thermal decomposition of RML/CS/MWCNTs beads

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Model fitting and analysis of variance (ANOVA)

The CCD was the chosen design for the surface optimization of eugenol benzoate catalyzed by the RML-CS-MWCNTs biocatalysts since it is a wide accepted model for optimization studies. Themodel consisted of four factors: time (A), temperature (B), enzyme loading (C) and molar ratio (D) and each factor was studied at five different levels (–2, –1, 0, +1, +2) and required 30 experiments.The experimental conditions design and their employed responses are depicted by the model. The predicted values were acquired with a model fitting technique by using the software Design Expert version 7.1.6 and were observed to be satisfactorily correlated with the observed values.Fitting of the data to various models (linear, two factorial, quadratic and cubic) and their following ANOVA, illustrated that the esterification of eugenol and benzoic acid was most suitably described with a quadratic polynomial model. The polynomial model was regressed for the conversion of benzoic acid and the **Eqn (2)** in terms of coded factors is shown as follows:

$$\text{Conversion (\%)} = + 36.65 - 4.25A + 7.43B - 1.56C + 0.92D + 6.44AB + 2.33AC - 4.91AD - 3.73BC + 3.68BD + 1.63CD \quad \textbf{(Eqn. 2)}$$

Where *A* is the time,*B*- the temperature,*C*- the solvent and *D*- the substrate molar ratio. The positive sign in front of term meant synergistic effect while the negative sign illustrates antagonistic effect, indicating the influence of independent variables on the esterification reaction process. It is agreed that the large *F*-value and smaller the *P*-value, the more significant the corresponding coefficient (Y.C. Wong *et al.,* 2015), hence, the results in this study suggested that the variable with the largest effect was the reaction temperature (**Table 1**).

Table 1: Analysis of variance and model coefficients

Source	Sum of squares	Degree of Freedom	Mean square	*F*-value	*P*-value Prob> F	
Model	3455.26	10	345.53	29.48	< 0.0001	significant
A-time	432.71	1	432.74	36.92	< 0.0001	
B-temperature	1325.66	1	1325.66	113.11	< 0.0001	
C-enzyme loading	58.38	1	58.38	4.98	0.0379	
D-molar ratio	20.44	1	20.44	1.74	0.2023	
AB	664.48	1	664.48	56.69	< 0.0001	
AC	86.72	1	86.72	7.40	0.0136	
AD	385.24	1	385.24	32.87	< 0.0001	
BC	222.83	1	222.83	19.01	0.0003	
BD	216.16	1	216.16	18.44	0.0004	
CD	42.61	1	42.61	3.64	0.0718	
Residual	222.69	19	11.72			
Lack of Fit	171.28	14	12.23	1.19	0.4570	Not significant
Pure Error	51.41	5	10.28			
Cor Total	3677.95	29				

Interactive effects of factors on the RML-CS-MWCNTs catalyzed esterification of eugenol benzoate

Effect of time and temperature

The interactive effect of both variables (**Figure 4a)** were evaluated at hold values of other parameters at molar ratio eugenol: benzoic acid (3:1) and enzyme loading of 250 IU. According to the *F*-value, the effect of the temperature (78.42) is more significant than the incubation time (24.40). The interaction between both parameters was significant because of the very small *P*-value (< 0.0001). It was clearly visible from the figure that the percent conversion of eugenol benzoate increased as the reaction temperature was elevated up to 57-63°C. Yields of eugenol benzoate close to 42% percent can be obtained under short reaction time by setting the reaction temperature to maximum coded levels. Prolonging the reaction time was found to be counterproductive probably due to the build up of water produced by the esterification reaction, favoring the reverse reaction that hydrolyzes (Abdul Rahman*et al.,* 2008) eugenol benzoate. Higher reaction temperature also tended to induce enzyme inactivation due to thermal denaturation of lipase (Chaibakhsh*et al.,*2012) particularly under extensive reaction time.

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Effect of time and substrate molar ratio

The effect of time and molar ratio (acid/alcohol) on percent conversion of eugenol benzoate at a constant temperature of 50°C is depicted in **Figure 4b.** Again the results clearly revealed that the effect of time was more significant than molar ratio. The F-value time (36.92) was higher than that of molar ratio (1.74), highly suggestive that reaction time was more impacting on the RML-CS-MWCNTs catalyzed production of eugenol benzoate (Table 4.2). The ANOVA of factors also indicated that the mutual interaction between both parameters was very significant due to a very small P-value (< 0.0001). It can be seen that the highest percentage conversion can be achieved at short incubation time and at high substrate molar ratio, as the yield of eugenol benzoate as high as 44% was attainable at short reaction time of ~7.0 h by utilizing the substrate molar ratio in the range of 3.5:1 and 4.0:1 (acid: alcohol).On the other hand, the improved yield of the ester when the molar ratio of substrates was set to maximum coded levels may be associated to increased availability of eugenol for the RML-CS-MWCNTs (Chaibaksh*et al.,* 2012). Similarly, the higher ester yield may be the result of better solubility of the solid benzoic acid in higher volumes of eugenol (Abdul Rahman*et al.,* 2008) which elevated the probability of effective substrate-enzyme collisions (Wahab*et al.,* 2014).

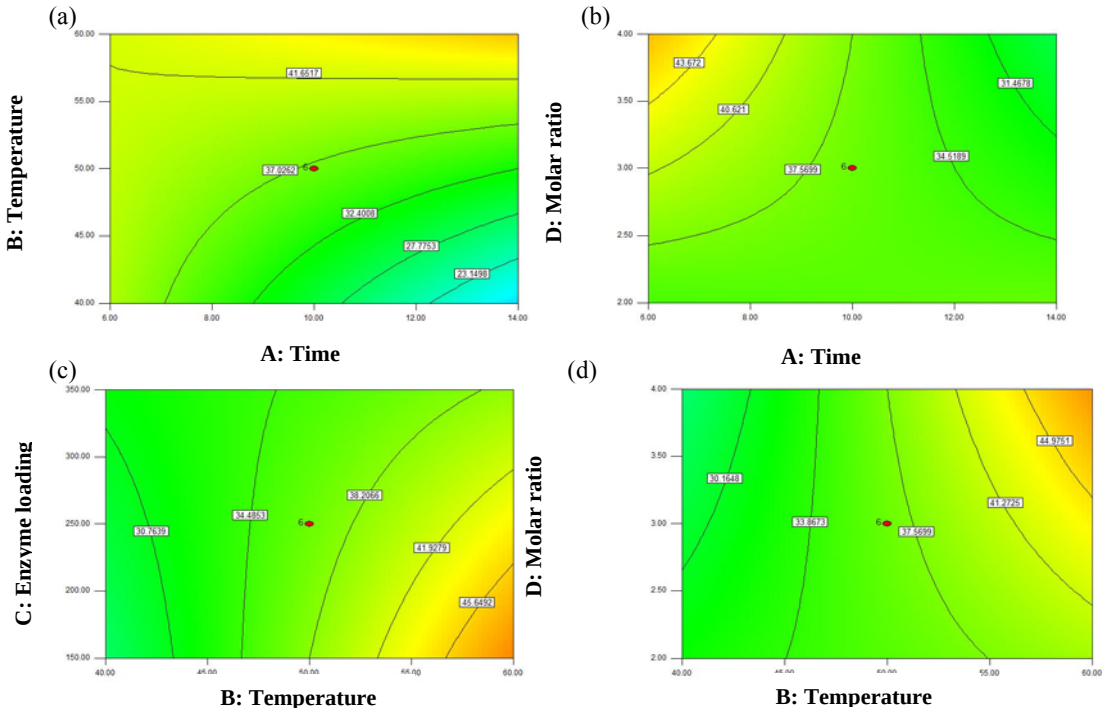

Figure 4: Contour plots between two parameters, (a) time and temperature, (b) time and molar ratio, (c) temperature and enzyme loading, (d) temperature and molar ratio,in the synthesis of eugenol benzoate. The numbers inside the contour plots indicate esterification yield (%) at given reaction conditions.

Effect of temperature and enzyme loading

Figure 3illustrates the contour plot for the interactive effects of temperature versus enzyme loading in the RML-CS-MWCNTs catalyzed synthesis of eugenol benzoate (**Figure 4c**). The interactive effect of both variables were evaluated at hold values of other parameters at molar ratio eugenol: benzoic acid (3:1) and incubation time for 10 h. As indicated by the linear coefficients and *F*-value, the effect of the temperature (1325.66) was substantially more significant than the enzyme loading (4.98). The interaction between both parameters was significant because of the very small *P*-value (0.0003). Generally, minimal use of enzyme for high conversion of product is favored due to the rather high cost of enzymes (Wahab*et al.,* 2014).It was clearly visible that the percent conversion of eugenol benzoate increased as the reaction temperature was elevated to 60°C. The yield of eugenol benzoate can be improved up to 46% percent when the reaction is performed at low enzyme loading at any values between 150-225 IU and temperature in the range of 54 to 60°C.As seen on the contour plot, immobilization of RML onto the CS-MWCNTs hybrid supports has probably improved operational stability of the lipase, reflected in the high yield of the ester when the reaction was increased to

maximum coded levels. Such change can permit enzymes to work in a larger range of environments, in this case, reaction temperature; hence, allowing enzymes to remain active at elevated temperature (Spahn and Minteer, 2008).

Effect of temperature and molar ratio

The interactive effect of temperature versus substrates molar ratio in the RML-CS-MWCNTs catalyzed synthesis of eugenol benzoate was evaluated at hold values of enzyme loading (250 IU) and incubation time of 10 h (**Figure 4d**). As indicated by the linear coefficients and F-value, the effect of the temperature (1325.66) was substantially more significant than the molar ratio (1.74). Their mutual interaction was found to be significant as represented by a very small P-value (0.0004).It was seen that the maximum yield of eugenol benzoate, as high as 45% could be achieved when both variables are set at maximum coded values. By simultaneously elevating the temperature to 55-60 °C and substrates molar ratio at any values between 3.5:1 to 4:1, the yield of eugenol benzoate could be positively enhanced.Apart from improved substrates integration and activation of enzyme activity that may occur at the proposed high reaction temperature, the higher concentrations of eugenol too, may favorably shift the esterification reaction from hydrolysis to ester biosynthesis. Such change can be associated with the re-direction of the reaction's equilibrium towards product formation when the concentration of nucleophile (eugenol) is elevated (Verissimo*et al.,* 2015) in the reaction mixture.

Attaining optimum condition and model validation

For developing efficient industrial processes for the esterification of eugenol that are of environmentally friendly, a statistically assistedoptimized process may provide a convenientsolution to this problem. (Wahab*et al.*, 2014).With regards to this, attaining a high degree of conversion was possible by solving the regression equation (**Eqn 1**) using Design Expert 7.1.6 and determines the optimum point on the response surface (Wahab*et al.,* 2014).The software proposed several experimental conditions in order to find the optimum point that maximizes percentage conversion of eugenol benzoate under a variety of preferred conditions. However, only one set of the predicted conditions suggested by the model were chosen. By evaluating several experimental conditions suggested by the software, a set of the most appropriate esterification condition with desirability value of 1 was chosen for this present study. Utilizing the chose set of conditions using fairly low enzyme loading and short reaction time, the highest percent conversion attained was 55.91% at 6 h of reaction time, 60 °C and substrate molar ratio of 1:4 of benzoic acid: eugenol. This is in comparison to the maximum conversion (56.13 %) for the synthesis of eugenol benzoate was predicted at the same conditions, where the predicted value was 57.34 % with 2.11 % deviation.Since the experimental values were found to be quite close to the predicted of the modelvalues, it was concluded that the proposed model was adequate and its validity confirmed.

CONCLUSION

The chitosan/multiwalled-MWCNTs beads were preparedsuccessfully as seen in the analyses of TGA, FESEM, and FTIR. The study also demonstrated that the RSM technique could be applied effectively to predict the optimized conditions for a reasonableRML/CS/MWCNTs-assistedproduction of eugenol benzoate. The findings showed the RML/CS/MWCNTs biocatalysts afforded a 56.13% yieldof the ester under the preferred optimized conditions of short incubation time and low enzyme loading; 60°C, incubation time of 6 h, enzyme loading of 15 mg and substrate molar ratio of eugenol/benzoic acid 4:1. Since the predicted yield (57.34%) closely agreed with the actual experimental value (56.13%), the suitability of the RSM prediction technique used in this study was justifiably proven. In view of minimizing the impact of conventional synthetic processes on the environment, the RML/CS/MWCNTs developed here as biocatalysts for the production of eugenol benzoate may prove valuable.

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