Rhizomucor miehei LIPASE SUPPORTED ON CHITOSAN-GRAPHENE OXIDE BEADS FOR THE PRODUCTION OF GERANIOL PROPIONATE

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To my beloved wife, Fatima Lawan

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ABSTRACT

The biotechnological route to manufacturing geraniol propionate may present a feasible solution to drawbacks associated with the production of such ester by the chemical synthesis or extraction from plants. The use of such technique can be advantageous considering the ever increasing demands for such products while reducing waste production and simplifying the manufacturing process. The properties and morphology of the developed *Rhizomucor miehei* lipase (RML) immobilized onto activated chitosan-graphene oxide (CS/GO) support were characterized using field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA). The morphological evaluations strongly indicated successful covalent attachment of the RML on the support. It was evident from the thermogram of TGA that 13.5% of RML was successfully immobilized onto the CS/GO matrix. The approach of response surface methodology (RSM) employing the Box-Behnken design (BBD) based on four parameters (incubation time, temperature, substrate molar ratio, and enzyme loading) were used to seek the optimized experimental conditions for the RML-CS/GO catalyzed synthesis of geraniol propionate. The study illustrated that the predicted and actual responses were well correlated, suggesting adequacy of the generated model for predicting the yield of the ester, as well as the factor of reaction time being most impacting in the RML-CS/GO catalyzed synthesis of geraniol propionate. Under optimized conditions, the highest yield of geraniol propionate (49.46%) was obtained at 17.98 h, 37.67 ℃, 100.70 rpm, and molar ratio of acid:alcohol of 1:3.28 in the solvent free esterification of propionic acid and geraniol. The investigation demonstrated that the developed RML-CS/GO was a promising alternative to overcome drawbacks associated with solvent assisted enzymatic reactions. Therefore, the RML-CS/GO biocatalysts developed here appear to be a promising substitute and yet environmentally practical biocatalyst for the production of geraniol propionate.

ABSTRAK

Laluan bioteknologi untuk penghasilan geraniol propionat mungkin merupakan penyelesaian kepada kelemahan yang dikaitkan dengan penghasilan ester tersebut dengan menggunakan sintesis kimia atau pengekstrakan daripada tumbuh-tumbuhan. Penggunaan teknik sedemikian adalah berfaedah memandangkan peningkatan permintaan yang berterusan terhadap produk tersebut di samping mengurangkan penghasilan bahan buangan dan memudahkan proses pembuatan. Sifat dan morfologi *Rhizomucor miehei* lipase (RML) terpegun pada penyokong kitosan teraktif-grafin oksida (RML-CS/GO) yang dibangunkan telah dicirikan menggunakan mikroskopi pengimbasan elektron pancaran medan (FESEM), spektroskopi inframerah transformasi Fourier (FTIR) dan analisis termogravimetri (TGA). Penilaian morfologi menunjukkan secara jelas kejayaan pengikatan RML secara kovalen dengan penyokong. Ini terbukti daripada termogram TGA bahawa 13.5% daripada RML telah berjaya dipegunkan ke atas matriks CS/GO. Pendekatan kaedah permukaan respons (RSM) menggunakan reka bentuk Box-Behnken (BBD) berasaskan empat pembolehubah (masa pengeraman, suhu, kadar pengacauan, dan nisbah molar substrat) telah digunakan untuk mendapatkan keadaan eksperimen yang optimum dalam sintesis geraniol propionat bermangkinkan RML-CS/GO. Kajian ini menjelaskan bahawa respons yang diramal dan sebenar berhubungkait dengan baik, mencadangkan model yang dihasilkan adalah memuaskan untuk meramalkan penghasilan ester, di samping juga faktor masa tindak balas yang paling memberi impak kepada sintesis geraniol propionat bermangkinkan RML/CS/GO. Pada keadaan optimum, penghasilan tertinggi geraniol propionat (49.46 %) telah diperoleh pada 17.98 h, 37.67 ℃, 100.70 rpm, dan nisbah molar asid:alkohol 1:3.28 dalam pengesteran bebas pelarut bagi asid propionik dan geraniol. Kajian ini menunjukkan bahawa biomangkin RML-CS/GO yang dibangunkan adalah alternatif yang berpotensi untuk mengatasi kelemahan yang dikaitkan dengan tindak balas enzim berbantukan pelarut. Oleh itu, biomangkin RML-CS/GO yang dibangunkan ini merupakan pengganti dan biopemangkin yang praktikal alam sekitar untuk penghasilan geraniol propionat.

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LIST OF SYMBOLS

°C	-	Degree Celsius
g	-	Gram
h	-	Hour
L	-	Liter
mg	-	Milligram
mL	-	Milliliter
mg/mL	-	Milligram per milliliter
M	-	Molar
rpm	-	Rotation per minutes
v/v	-	Volume per volume
w/v	-	Weight per volume
w/w	-	Weight per weight
%	-	Percentage

LIST OF ABBREVIATIONS

CS	-	Chitosan
GO	-	Graphene Oxide
BBD	-	Box-Behnken Design
FT-IR	-	Fourier Transform Infrared
FESEM	-	Field Emission Scanning Electron Microscope
TEM	-	Transmission Electron Microscope
TGA	-	Thermal Gravimetric Analysis
RSM	-	Response Surface Methodology
EDAC	-	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
NHS	-	*N*-hydroxysuccinimide

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Terpene esters of alcohol are economically important fragrance and flavor compounds used in food, pharmaceutical, beverage and cosmetic industries; geraniol, citronellol and linalool being the principal components in essential oils. These esters are traditionally obtained by chemical synthesis, physical extraction from plants and by fermentation of natural precursors (You *et al*., 2011). However, natural flavor esters extracted from plant sources are too expensive or scarce for commercial purposes, while the production of terpene esters using chemical synthesis incurs undesirable use of strong acids. The drawbacks of such method are formation of by-products which have an effect on the odor of terpene esters. Also, the products produced by the method are considered as not natural, therefore, are marketed with less value than esters obtained from natural sources (Stamatis *et al*., 1998; You *et al*., 2011). Hence, the biotechnological route to producing fragrances and flavors using natural raw materials may prove useful for industrial processes in view of the ever increasing demands for such products (Paroul *et al*., 2010).

Biotechnology as an emerging science promotes the development of better industrial processes such as manufacturing of new aromas and production of high purity compounds for use in food industries. Presently, biocatalysis is a feasible alternative to chemical synthesis method, especially when regioselectivity or stereoselectivity of the resulting end product is pertinent. Among the important widely used industrial enzymes are carbohydrases, proteases and lipases. The major benefits of using these biocatalysts in synthetic reactions such as esterification and transesterification reactions refers to their high activity of enzymes in organic solvents and water and also, their ability to convert large amounts of substrate with high stereospecificity (Ferraz *et al*., 2015). In this context, lipases (triacylglycerol hydrolase E.C 3.1.1.3) can catalyze esterification reactions under ambient conditions with good activity and selectivity. The mesophilic *Rhizomucor miehei* lipase (RML) is the frequent biocatalyst of choice due to its specificity, versatility, and its suitability for ester synthesis under different conditions of temperature, pressure, water content, and substrates (Skoronsi *et al*., 2014). The lipase is commercially obtainable both in soluble and immobilized form (Rodrigues and Fernandez-Lafuente, 2010).

Utilization of RML to catalyze esterification reactions in anhydrous media is advantageous as compared to other lipases that preferred transesterification reactions (Rodrigues and Fernandez-Lafuente, 2010). However, use of the free form RML has drawbacks of being unstable and prematurely denatured under extensive reaction time, susceptible to high temperature, extreme pH and organic solvents. In addition, the homogeneity of RML with the reaction mixture results in difficult recovery of the enzyme in reactive mixtures. Considering the numerous shortcomings associated with the free RML, immobilization of the lipase onto appropriate solid support may prove feasible to improve the catalytic characteristics of RML, facilitate enzyme recovery (Zou et al., 2010) and allow reusability of the enzyme (Mohamad *et al*., 2015a). According to review of literature (Palla and Carrin, 2014), the *R. miehei* lipase immobilized onto modified chitosan microspheres can stabilize the open conformation of the lipase and promote their hyperactivation after immobilization.

To date, many techniques such as biological, physical and chemical methods have been used by biochemists to improve the performance of the lipase in order to meet the target goal (Zhang *et al*., 2008). Enzyme immobilization offers a multitude of benefits in terms of improved structural stability and enzyme activity, ease of recovery of the biocatalyst (Masakapalli *et al*., 2014; Palla *et al*., 2011; Park *et al*., 2015; Sharma *et al*., 2001), longer life of enzymes, specificity and selectivity as well as minimizing product contamination (Palla *et al*., 2011; Tang *et al*., 2014;

Worzakowska, 2014). Among the challenges in this field is the well known slight loss in catalytic activity of the enzyme upon immobilization (Palla *et al*., 2011).

The structure (shape and size) of the support material has an influence on the immobilized enzyme (Palla *et al*., 2011). Among the various supports available for enzyme immobilization, chitosan (CS), graphene oxide (GO) and multi-walled carbon nanotubes (MWCNTs) are gaining considerable popularity as the support of choice. CS is chosen due to its ability to exhibit many properties such as availability of reactive functional groups for chemical modification, biocompatibility, regenerability, mechanical stability, and easy to prepare in different geometrical configurations suitable for a particular biotransformation. Furthermore, chitosan is a cheap material making it possible to prepare cheap carriers for large scale applications (Popiskova et al., 2013). Chitosan beads embedded with graphene oxide has an open cell foam structure, ultrafine pores and a cell size that results to a large surface area. Graphene oxide can enhance the physical strength of chitosan due to its low thermal conductivity and superior mechanical integrity. In addition, the epoxy and hydroxyl functional groups of GO can provide additional active sites for the immobilization of lipase (Lau *et al*., 2014).

Apart from enhancing the robustness of enzymes for use in synthetic reactions, the conditions employed in the reactions may also affect the yield of the product. This is due to the fact that enzymes are biological entities whose catalytic activity is sensitive to conditions of the surroundings (Mohamad *et al.,* 2015a) such as temperature, duration of catalysis, stirring rate, molar ratio of reaction substrates and etc. Considering the multiple complexities associated with enzyme assisted reaction, predictions of the optimum conditions to improve efficiency of its bioprocesses is almost infeasible. This is attributable to the nonlinearity and complicated structure of many biotechnological practices. To expedite prediction of best reaction conditions of processes or products, utilization of response surface methodology may prove valuable (Wahab *et al*., 2014). The method facilitates rapid and less expensive empirical investigation to establish the optimum conditions of reactions as compared to that of the conventional one-variable-at-a-time or full factorial experiment (Wahab *et al*., 2014; Mohamad *et al*., 2015b).

1.2 Problem Statement

Considering that geraniol propionate obtained from plants or by chemical synthesis faces drawbacks such as production in low yield and produced at high cost which may not meet the high commercial demand for the ester. Therefore, development of an alternative green method to produce high yield of the ester, preferably at a low cost would be of considerable advantage. Furthermore, the use of of green nanobioconjugates of RML-CS-GO for the preparation of geraniol propionate is yet to be explored and the the feasibility of such biocatalysts for such purpose remains unknown.

1.3 Objectives

The objectives of the research are as follows:

1. To prepare the chitosan beads reinforced with graphene oxide (CS-GO) and immobilize the *R. miehei* lipase onto the CS-GO (RML-CS/GO) beads.
2. To characterize the CS, CS/GO and RML-CS/GO beads.
3. To optimize the RML-CS/GO catalyzed solventless synthesis of geraniol propionate from geraniol and propionic acid.

1.4 Scope of Study

1. To covalently immobilize the prepared CS-GO beads with free RML in order to obtain the RML-CS/GO beads.

2. To characterize the CS, CS/GO and RML-CS/GO beads by using Fourier transform infrared spectroscopy (FT-IR), Field emission scanning electron microscope (FESEM), Transmission electron spectroscopy (TEM), and thermal gravimetric analysis (TGA).
3. To perform RML-CS/GO assisted esterification of geraniol and propionic acid to afford geraniol propionate by response surface methodology (RSM) according to the proposed conditions of the Design Expert 7.1.6 software utilizing the Box- Behnken design (BBD) method.

1.5 Hypothesis

Covalent immobilization of RML onto CS-GO beads may increase rigidity of the RML protein structure through additional covalent bonds between the matrix and RML, and subsequently improve stability of RML to catalyze prolong esterification reactions. To improve the yield of enzyme assisted esterification of geraniol propionate, the statistical multivariate guided experiments can be used to establish the optimal parameters i.e temperature, time, molar ratio and stirring rate to attain high yield of the ester.

1.6 Significance of Study

Herein, immobilizing the free RML onto a modified matrix promotes solventless bioproduction of geraniol propionate which adheres to the philosophy of green chemistry, hence a step towards green and sustainable means of producing important commercial esters. The employment of green chemistry in synthetic reactions may bring long term benefits as such include being environmentally friendly, production of less wastes as well as economically desirable. In this context, covalently immobilizing RML onto CS-GO beads may possibly bring about three benefits: i) improve activity of RML, ii) potentially cost saving due to utilization of small amount of the enzyme, and iii) increase the yield of geraniol propionate due to the well-known enzyme enhancing properties of CS and GO. In addition, immobilizing RML onto the CS-GO matrix effectively insolubilizes the RML and supports cost saving practices as it permits easy recovery, reusability of the enzyme and prolong the reaction life of RML for use in subsequent reactions.

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