SYNTHESIS OF THE ANTI-OXIDANT EUGENOL BENZOATE USING *Candida rugosa* LIPASE IMMOBILIZED ONTO MULTI WALLED CARBON NANOTUBES

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This dissertation is dedicated to my parents, my husband and my lovely daughter for their endless support and encouragement.

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ABSTRACT

Multi walled carbon nanotube (MWCNTs) having high surface area was used for the immobilization of lipase from *Candida rugosa* (CRL) by physical adsorption. The ability of the MWCNT immobilized CRL was compared against the free CRL in the esterification of benzoic acid with eugenol to produce the antioxidant eugenol benzoate, a potent lipoxygenase inhibitor. In this study, the effect of four parameters; reaction time, temperature, molecular sieves and enzyme loading were evaluated. The maximum yield of ester of reactions catalyzed by immobilized CRL and free lipase were found to be 31.62 and 27.2%, respectively. The study revealed that the optimal conditions for the highest yield of ester attained for both free CRL and immobilized CRL were similar. The attained optimum conditions were 5 mg/mL enzyme, 0.5:1.5 molar ratio of benzoic acid/eugenol dissolved in 38.5 mL chloroform, 500 mg of molecular sieves with 50 mL total volume of reaction mixture, stirred under 200 rpm at 40^{o}C for 4 h. It was observed the MWCNT immobilized CRL afforded slightly higher yield of ester in all parameters investigated as compared to the free CRL. The outcome suggests that the MWCNT immobilized CRL could be used to synthesize eugenol benzoate. Furthermore, treatment of the esterification process is now easier due to simpler methods of separation of the end product and the biocatalyst could be recycled.

ABSTRAK

Tiub nano berdinding lapis (MWCNT) mempunyai luas permukaan yang besar telah digunakan untuk immobilisasi lapis daripada *Candida rugosa* (CRL) secara penjerapan fizikal. Kebolehan CRL yang terimmobilisasi atas tiub nano berdinding lapis telah dibandingkan dengan CRL bebas di dalam pemangkinan pengesteran asid benzoik dengan eugenol untuk menghasilkan antioksidan eugenol benzoat, suatu perencat lipoksigenase. Di dalam kajian ini kesan empat parameter; masa tindakbalas, suhu, ayak molekul dan muatan enzim telah dikaji. Hasil maksimum ester untuk tindakbalas yang dimangkinkan oleh CRL terimmobilisasi dan CRL bebas didapati masing-masing adalah 31.62 dan 27.2%. Kajian mendapati keadaan optimum untuk pembentukan ester yang paling banyak yang dicapai oleh kedua-dua lapis bebas dan terimmobilisasi adalah serupa. Keadaan optimum yang tercapai ialah 5 mg/mL enzim, 0.5:1.5 nisbah molar asid benzoik/eugenol yang dilarutkan dalam 38.5 mL klorofom, 500 mg ayak molekul dengan jumlah isipadu campuran tindakbalas 50 mL, dikacau pada 200 rpm pada 40^{o}C selama 4 jam. Didapati CRL yang terimmobilisasi ke atas MWCNT membentuk hasil ester yang sedikit tinggi di dalam kesemua parameter yang dikaji berbanding CRL bebas. Hasil kajian menunjukkan bahawa CRL yang terimmobilisasi ke atas MWCNT boleh digunakan untuk sintesis eugenol benzoat. Manakala, proses pengesteran kini lebih mudah hasil dari kaedah pemisahan produk akhir yang lebih mudah dan mangkin biologi yang boleh dikitar semula.

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LIST OF ABBREVIATIONS

AChE	-	Acetylcholinesterase
Ca-Alg	-	Calcium alginate
CALB	-	*Candida antarctica* lipase B
CRL	-	*Candida rugosa* lipase
CRL-MWCNT	-	*Candida rugosa* lipase immobilized onto multi-walled carbon nanotubes
CPW	-	Carboxypeptidase II
CNT	-	Carbon nanotubes
CCVD	-	Catalytic chemical vapour decomposition
CVD	-	Chemical vapour deposition
CMC	-	Critical micellular concentration
DLH	-	Dienlacton hydrolase
EDX	-	Energy Dispersive X-ray Spectroscopy
EC	-	Enzyme Commission
FESEM	-	Field Emission Scanning Electron Microscope
FT-IR	-	Fourier Transform Infrared
MWCNT-COOH	-	Functionalized multi-walled carbon nanotubes
GLP	-	*Geotrichum Candidum* lipase
GA	-	Glutaraldehyde
HAL	-	Haloalkane dehalogenase
HMDI	-	Hexamethylene diisocyanate
PI	-	Isoelectric point
MWNT	-	Multi-walled carbon nanotubes
NSAIDs	-	Nonsteroidal Anti-Inflammatory drugs
PEI	-	Polyethylenimine
PUFA	-	Polyunsaturated fatty acids
SEM	-	Scanning Electron Microscope

SWNT	-	Single walled carbon nanotubes
STDEV	-	Standard deviation
TBME	-	Tert-Butyl methyl ether
TEM	-	Transmission Electron Microscopy

LIST OF SYMBOLS

Å	-	Angstrom
Cm	-	Centimeter
^{o}C	-	Degree Celecius
G	-	Gram
g/cm^{3}	-	Gram per cubic centimeter
h	-	Hour
L	-	Liter
Mg	-	Microgram
Mg	-	Milligram
mg/mL	-	Milligram per milliliter
mL	-	Milliliter
Mm	-	Millimeter
mM	-	Millimolar
Nm	-	Nanometer
N	-	Normality
%	-	Percent
psig	-	Pounds per square inch gauge
rpm	-	Unit of agitation speed
U/mg	-	Unit per milligram
v/v	-	Volume per volume

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Enzymes are protein compounds that have wide range of compositions along with structural complexities. The employment of enzyme as catalysts in biotransformation has acquired an escalating interest in the past several years due to their excessive catalytic activities alongside with special unique substrate specification in organic synthesis. The two key properties of enzymes are usually satisfactory to minimize energy and process consumption cost (Palmer, 1985; Perez *et al*., 2007). Hydrolase are enzymes that acquire great attention because of their high possible commercial application. Lipases are one of several hydrolase enzymes which may have increased resistance to deactivation in non-aqueous mass media. Due to excessive stableness, they can be utilized regularly for many reactions with no significant loss of their activity (Stamatis *et al*., 1993).

Reactions that are catalyzed by enzymes are usually considered more productive and efficient when compared to inorganic catalyzed reactions, due to ultimate decrease in the reactions activation energy which is why enzyme-catalyzed reactions are carried out in typically mild conditions such as; lower temperature, low atmospheric pressure and physiological pH values in comparison to the inorganic catalyst. The lipase-catalyzed reactions are mostly in different medium which include biphasic organic-aqueous, reversed micelles, organic and supercritical CO_2 system

(Martinek *et al*., 1981; Steytler *et al*., 1991; Yang *et al*., 1997). Because many hydrophobic substrates are insoluble in aqueous media, a new method of utilizing enzyme in organic media have been proposed which leads to increase in use of lipase enzyme mainly because numerous hydrophobic substrates usually are soluble within this media (Okahata and Mori, 1997).

Esterification reactions are generally omnipresent inside fine compounds, more advanced along with petrochemical industries. Besides hydrolysis involving triglyceride, lipases can also be employed for esterification between fatty acid and alcohol. Numerous researches have been carried out in the facet of controlling normal water activity as a way to promote the reaction towards synthesis direction. The product isolation and excess substrate concentrations have been evaluated and removed by different method and the products are obtained in high yield (Bloomer *et al*., 1992; Yadav *et al*., 2012).

The use of nanobiocatalyst is a rapid growing research area which refers to the use of enzymes that have been immobilized on nanomaterials. Among the used nanobiocatalyst, the carbon-based nanomaterial such as; carbon nanotubes (CNTs) and graphene have greatly attracted scientific interest (Kim *et al*., 2008). The use of CNTs was first initiated by Endo in the late 19^{th} century with a carbon tubular structure (also called carbon filaments in the sixties) that has a diameter of less than 100 mm (Cisseli, 2007). The multi-walled carbon nanotube which consist of two or more concentric cylindrical shells of graphene sheets that are arranged around a central hollow was the first CNT to be observed *via* Transmission Electron Microscopy (TEM) analysis and is currently the most attractive nanomaterial (Ajayan *et al*., 2006).

Candida rugosa lipase (CRL) is one of the widely used industrial lipase that are mostly utilized in various reaction which include hydrolysis and esterification reactions due to its wide substrate specificity and also in pharmaceutical synthesis due to its stereoselectivity and regioselectivity (Takac and Bakkal, 2007). The immobilization of enzyme is a common strategy to obtain a conventional heterogeneous catalyst onto biological catalyst with desirable features such as; enzyme immobilization on CNTs which other specific groups (e.g. organic, polymeric and biological molecules) to be functionalized to the CNTs which further makes it possible for the enzymes to specifically and selectively bind onto the CNTs. The key step in bionanofabrication is the immobilization of CRL on MWCNTs due to its high surface area, mechanical, thermal and electrical properties and biocompatibility (Kuchibhatla *et al*., 2007).

In this study, the immobilization of a cheap and widely available enzyme (CRL) onto the MWCNTs would be carried out *via* adsorption and would further be used as catalyst in the esterification reaction for the synthesis of eugenol benzoate using benzoic acid as acyl donor, eugenol as substrate and chloroform as solvent. The frequent use of substituted esters (both aliphatic and aromatic) as plasticizers, solvents, flavors and precursors to a range of pharmaceuticals have been reported elsewhere (Yadav *et al*., 2012).

1.2 Problem statement of the study

Eugenol esters are chemically synthesized as potential inhibitors of lipoxygenase. The benefits of these compounds can be more enhance with the improvement of an efficient process for their synthesis under mild conditions. The use of lipases in non-aqueous medium has been described by Stamatis *et al*. (2001) for the preparation of phenolics acid ester. Besides, the esterification yield obtained using free lipases was very low. Other disadvantages include; less stable and low activity in organic solvent cannot withstand high temperature and pressure and requires the use of large amount of starting materials, these results in high waste generation in the industry. Hence, immobilization of the enzyme would drastically reduce the high use of starting materials which are quite expensive and environmental unfriendly.

The challenge of using biological element as catalyst is quite increasing due to their expensiveness, complexity as well as low stability. As such, a simple alternative method is required to reduce the above shortcomings without altering scientific interests. Therefore, enzyme immobilization onto a solid support (MWCNTs) is an alternative route because of its successful recycling property and providing mechanical stability to the enzyme three-dimensional structure. The use of enzyme immobilization is greatly important for the synthesis of eugenol benzoate. As such due to its ease of handling, faster purification, thermal and pH stability, high selectivity and substrate specificity, low energy and high yield of product.

1.3 Objectives of the study

The objectives of the study are:

1. To immobilize CRL onto MWCNTs using physical adsorption method.
2. To determine best reaction conditions for the synthesis of eugenol benzoate.
3. To compare the free CRL and CRL-MWCNT to catalyze the synthesis of eugenol benzoate.

1.4 Scope of the study

This study would report a greener alternative to the synthesis of valuable commercial products (eugenol benzoate) by the use of CRL immobilized onto MWCNT. To study how the molecules interacts with the enzyme and how often they affect the enzyme structure and how the enzyme is arranged on the MWCNTs. The reaction conditions to be optimized include; incubation time, temperature, presence of desiccant (molecular sieves) and effect of enzyme loading.

1.5 Significance of the study

CNTs are new nano-size carbon material and they have many potential applications to be exploited which include its use in the polymer composites and bioscience application that generated a remarkable starting point in the field of material science studies. Enzyme immobilization on CNTs presents some advantages over the bulk solid materials such as high surface area which can lead to higher enzyme loading, nanoscale dispersion and ease of surface functionalization (Kim *et al*., 2008).

In general, the use of enzymes economically and efficiently in aqueous as well as in non-aqueous solvents allows the modification of their activity, selectivity and operational stability. Several applications reveals the use of lipase in an immobilized state due to its ease of separation, stability, reusability, better control of reaction and more favorable economical factors (Nasratun *et al*., 2009). The study will determine whether the CRL immobilized onto MWCNTs by physical adsorption, is a viable option for the synthesis of eugenol benzoate by esterification reactions.

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