SYNTHESIS OF EUGENYL BENZOATE CATALYZED BY *RHIZOMUCOR MIEHEI* LIPASE IMMOBILIZED ON REINFORCED CHITOSAN-CHITIN NANOWHISKERS SUPPORT

FATIN MYRA BINTI ABD MANAN

A thesis submitted in fulfilment of the

requirements for the award of the degree of

Master of Philosophy

Faculty of Science

Universiti Teknologi Malaysia

SEPTEMBER 2017

DEDICATION

To my parents,

Abd Manan and Fatimah,

And all of my friends,

Without whom none of my success would be possible

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful

Alhamdulillah, all praises to Allah swt for all the strengths and His blessing in completing this thesis, I would never have been able to finish this thesis without the guidance of my suprevisor, help from friends and support from family.

Foremost, I would like to express my sincerest gratitude to my advisor, Dr Roswanira Ab Wahab, for the continuous support of my study, for her patience, motivation, enthusiasm, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my study. One simply could not wish for a better and supervisor. Also, I would like to offer my special thanks to my co-supervisors, Dr Nursyafreena and Dr Zainoha whom have made enormous contribution in giving insightful comments and suggestions which has improved my studies.

My sincere thanks also goes to my fellow labmates in Enzyme Technology and Green Synthesis Lab: Haziqah, Syafikah, Anith, Emmanuel, Elham and Kalaivani for their friendship and support, and for creating an enjoyable working environment. My deepest appreciation goes to Ida Nurhazwani, who have been a very good companion throughout these two years. Her keen interest and enccouragement were a great help in completing this work made my time here at UTM a lot more fun. Special thanks also to Amirul Iman for the unyielding support. Also I thank my friends Eleena and Nik Aisah for for their constant love and support.

Finally, my deep and sincere gratitude to my family for their continuous and unparalleled love and support. I am forever indebted to my parents for giving me the opportunities and experiences that have made me who I am. This journey would not have been possible if not for them, and I dedicate this milestone to them. To those who indirectly contributed in this research, your kindness means a lot to me. Thank you very much.

ABSTRACT

The chemical route to produce esters has several drawbacks associated with the utilization of homogeneous acid catalysts that require high reaction temperatures, hence is energy intensive, not reusable, creates dissolved solids and involves laborious separation processes. Hence, a biotechnological route *via* enzymatic esterification has been proposed as an alternative way to synthesize the problematic anti-oxidant, eugenyl benzoate. This is because an enzyme-catalyzed synthesis offers favorable advantages such as the use of a more sustainable reaction process with high yields and purity, as well as the biocatalyst reusability. An ecofriendly support was prepared from chitosan-chitin nanowhiskers (CS-CNWs) for covalent immobilization of *Rhizomucor miehei* lipase (RML) to be used as the biocatalysts. Analyses on the RML-CS/CNWs using Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis-differential thermogravimetry (TGA-DTG), field emission scanning electron microscopy (FESEM), X-ray diffraction (XRD) and fluorescence microscopy affirmed the successful covalent immobilization of RML onto the surface of CS-CNWs. The resultant RML-CS/CNWs biocatalysts were studied for catalyzing synthesis of eugenyl benzoate for various reaction parameters. One-Variable-at-A-Time (OVAT) study revealed that under optimal experimental conditions of 50 °C at 250 rpm with catalyst loading of 3 mg/mL and 3:1 molar ratio of eugenol to benzoic acid, the maximum yield that reached 62.1% was attained after 5 h as compared to free RML (50.7%). The RML-CS-CNWs also demonstrated good operational stability, whereby the biocatalysts retaining 50% of its initial activity for up to eight successive esterification cycles. The present work also reports a response surface methodology (RSM) with Box-Behnken design (BBD) optimization process to synthesize eugenyl benzoate. The effects of four reaction parameters: reaction time, temperature, the substrate molar ratio of eugenol to benzoic acid and enzyme loading were assessed based on OVAT findings. Under optimum conditions, a maximum conversion yield of 66.8% was attained at 50 °C in 5 h using 3.75 mg/mL of the RML-CS-CNWs, and substrate molar ratio (eugenol:benzoic acid) of 3:1. Meanwhile, 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} and turnover number (k_{cat}) of 40.39 min^{-1} under an optimized experimental condition. Based on the findings, it can be concluded that the use of the RML-CS-CNWs biocatalysts was promising in affording relatively satisfactory yield of eugenyl benzoate within a reasonably short time. Aside from improving enzymatic operational activity and stability, the immobilization strategy can facilitate rapid and easy removal of the RML-CS-CNWs from the reaction mixture when completed.

ABSTRAK

Laluan kimia untuk menghasilkan ester mempunyai beberapa kelemahan yang dikaitkan dengan penggunaan mangkin asid homogen yang memerlukan suhu tindak balas yang tinggi, maka ianya intensif tenaga, tidak boleh diguna semula, menghasilkan pepejal terlarut dan melibatkan proses pemisahan yang menjerihkan. Maka, laluan bioteknologi melalui pengesteran berenzim telah dicadangkan sebagai suatu laluan alternatif untuk mensintesis anti-oksida yang bermasalah, eugenil benzoat. Ini kerana sintesis bermangkinkan enzim menawarkan kelebihan menguntungkan iaitu penggunaan proses tindak balas yang lebih lestari dengan hasil yang tinggi dan tulen, serta kebolehgunaan semula biomangkin tersebut. Suatu penyokong mesra alam telah disediakan daripada nanomisai kitin-kitosan (CS-CNWs) untuk pemegunan kovalen lipase *Rhizomucor miehei* (RML) untuk digunakan sebagai biomangkin. Analisis terhadap RML-CS-CNWs menggunakan spektroskopi inframerah transformasi Fourier (FTIR), analisis termogravimetri–termogravimetri pembezaan (TGA-DTG), mikroskopi imbasan elektron-medan pelepasan (FESEM), pembelauan sinar-X (XRD) dan mikroskopi pendarfluor membuktikan kejayaan pemegunan kovalen RML ke atas permukaan CS/CNWs. Biomangkin RML-CS-CNWs yang terhasil telah dikaji untuk memangkinkan sintesis eugenil benzoat untuk pelbagai parameter tindak balas. Kajian satu-pembolehubah-pada-satu-masa (OVAT) mendedahkan bahawa dalam keadaan optimum eksperimen iaitu 50 °C pada 250 rpm dengan muatan mangkin sebanyak 3 mg/mL dan nisbah molar eugenol kepada asid benzoik 3:1, hasil maksimum yang menjangkau 62.1% telah dicapai selepas 5 jam berbanding dengan RML bebas (50.7%). RML-CS-CNWs tersebut juga menunjukkan kestabilan operasi yang baik, di mana biomangkin mengekalkan 50% daripada aktiviti awal sehingga lapan kitaran pengesteran berturut-turut. Kajian ini turut melaporkan proses pengoptimuman menggunakan kaedah gerak balas permukaan (RSM) dengan reka bentuk Box-Behnken (BBD) untuk mensintesis eugenil benzoat. Kesan empat parameter tindak balas: masa tindak balas, suhu, nisbah molar substrat eugenol kepada asid benzoik dan muatan enzim dinilai berdasarkan keputusan OVAT. Dalam keadaan optimum, hasil penukaran maksimum sebanyak 66.8 % telah dicapai pada 50 °C dalam waktu 5 jam menggunakan 3.75 mg/mL RML-CS-CNWs, dan nisbah molar substrat (eugenol:asid benzoik) sebanyak 3:1. Sementara itu, penilaian kinetik mendedahkan RML-CS-CNWs memangkin tindak balas melalui mekanisma Ping-pong Bi Bi dengan perencatan eugenol, dicirikan oleh V_{max} 3.83 mM min^{-1} dan nombor perolehan (k_{cat}) sebanyak 40.39 min^{-1} di bawah keadaan eksperimen yang optimum. Berdasarkan keputusan kajian, dapat disimpulkan bahawa penggunaan biomangkin RML-CS-CNWs menjanjikan hasil eugenil benzoat yang relatif memuaskan di dalam jangka masa yang agak singkat. Selain daripada meningkatkan aktiviti operasi enzim dan kestabilan, strategi pemegunan membolehkan pengeluaran RML-CS-CNWs yang pantas dan mudah daripada campuran tindak balas setelah selesai.

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of Variance
BBD	-	Box-Behnken design
CNWs	-	Chitin nanowhiskers
CS	-	Chitosan
EDAC	-	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
MES	-	2-(N-morpholino)ethanesulfonic acid
NHS	-	N-hydroxysuccinimide sodium salt
OVAT	-	One Variable at-a-Time
RML	-	*Rhizomucor miehei* lipase
RSM	-	Response surface methodology

LIST OF SYMBOLS

hr	-	hour
g	-	gram
M	-	molar (concentration)
mg	-	miligram
min	-	minute
mL	-	mililitre
mM	-	millimolar (concentration)
rpm	-	rotation per minute
s	-	second

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Eugenol derived from *Eugenia caryophyllata* is the principal chemical component of clove oil (76.8%) and the compound is popularly known for its excellent analgesic, anti-inflammatory and antibacterial effects (Moon *et al.*, 2011). Eugenol is a pale-yellow oil with a warm, pungent yet pleasing aroma and smell of bay leaves and clove. It is a familiar fragrance in dentistry as it is often mixed into a paste and used as a local antiseptic, dental cement, filler and restorative material (Devi *et al.*, 2010). Eugenol is usually used in its dimeric forms (i.e. eugenol esters) for increased bioactivity (Horchani *et al.*, 2010; Sadeghian *et al.*, 2008) while reducing allergic reactions and inflammatory effects when in contact with cells due to liberation of phenoxyl radicals as well as quinone intermediates (Horchani *et al.*, 2010; Yadav and Yadav, 2012). In terms usage safety, the use of eugenol has been deemed safe by the Food and Drug Administration (Hemaiswarya and Doble, 2009).

The current chemical approach to synthesizing eugenol esters has several inherent drawbacks, among them is the use of strong acid catalysts that incurs tedious downstream processing which increases the overall costs of manufacturing process (Yadav and Yadav, 2012). To overcome such drawbacks, the biotechnological approach of employing enzymes in non-aqueous media may prove advantageous since all reactions are carried out under mild conditions (Horchani *et al.*, 2010). The feasibility of such method in employing enzyme as biocatalyst was previously reported by several researchers (Chiaradia *et al.*, 2012; Horchani *et al.*, 2010; Mohamad *et al.*, 2015b). Herein, employment of lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) to catalyze the synthesis of eugenol esters i.e. eugenyl benzoate is proposed.

Currently, lipase from *Rhizomucor miehei* (RML) has gained considerable attention and successfully employed in various synthesis reactions, especially esterification. Such preference of the scientific and manufacturing community towards RML may be attributable to numerous reports on its catalytic potential at different conditions of temperature, pressure, water content and substrates (Lorenzoni *et al.*, 2012; Skoronski *et al.*, 2014). Similar to other free forms of lipases, RML has a high tendency of deactivation in prolonged contact with high temperature and extreme pH, low activity in organic solvents and, hence insufficiently stable to withstand tough industrial processing conditions (Rodrigues and Fernandez-Lafuente, 2010). In this context, immobilization of RML onto a suitable solid support is one of the possible solutions that offers easy recovery and reusability of the biocatalysts for better productivity (Zou *et al.*, 2010), as well as improved stability and activity of enzymes (Mateo *et al.*, 2007; Zhao *et al.*, 2015).

In the present work, the organic polymers of chitosan (CS) and chitin nanowhiskers (CNWs) are chosen as the matrices for immobilization due to their excellent benefits. CS is a natural cationic biopolymer with the benefits of biocompatibility, non-toxicity as well as high mechanical strength, whereby the material can be easily fabricated into high quality films, fibres or bead forms. The versatility of this biomaterial is also due to the presence of amino and hydroxyl groups in its structure that facilitate attachment of enzymes *via* covalent bonding or crosslinking (Solanki *et al.*, 2009). Moreover, the performance of CS as the support for enzyme immobilization can be enhanced by introducing certain nanomaterials such as CNWs as filler. The use of CNWs has become increasingly popular in recent years due to their exceptional mechanical properties, large specific surface area, high aspect ratio, environmental benefits and low cost (Qin *et al.*, 2016). According to a recent report, the combination a polymer i.e. CS with the nano-filler CNWs to afford the CS/CNWs hybrid matrix can be regarded as a single polymer (polysaccharide) composite (Šimkovic, 2013) whose mechanical parameters are strongly influenced by the origin of the components. The observably enhanced strength and stiffness of the CS/CNWs composite has been suggested to be a consequence of the high modulus of CNWs and additional interactions that occurred between both components (Kelnar *et al.*, 2015).

The process parameters for the RML-CS/CNWs-catalyzed esterification to synthesize eugenyl benzoate was 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 (Marzuki *et al.*, 2015a). RSM merges the experimental designs with interpolation by first or second-order polynomial equations in a sequential testing procedure (Pandiyan *et al.*, 2014) 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-catalyze esterification of eugenol and benzoic acid using a three-level-four-factor Box-Behnken design (BBD).

This present study was aimed at investigating the optimized reaction parameters for the application of RML-CS/CNWs as potential sustainable biocatalysts. The developed RML-CS/CNWs beads was used as biocatalysts for the preparation of eugenyl benzoate from eugenol and benzoic acid. The esterification process was optimized using the OVAT and RSM by Box-Behnken Design (BBD) for relevant parameters that include incubation time, temperature, molar ratio of acid to alcohol and enzyme loading.

1.2 Statement of Problem

Currently, eugenyl benzoate is chemically synthesized as one of the important inhibitors of lipoxygenase, which is responsible in avoiding major cause of inflammation in asthma and allergic rhinitis (Sadeghian *et al.*, 2008). However, the prevailing chemical route to produce eugenyl benzoate may harm the environment as well as requiring a lot of energy and time (Chaibakhsh *et al.*, 2012), development of new methods that are sustainable and cost effective to overcome such drawbacks may prove pertinent and timely. So far, attempts to produce eugenyl benzoate *via* the biotechnological route (Horchani *et al.*, 2010) remains lacking and the potential of such method to produce the ester has yet to be fully exploited. Although there are studies employing lipases as a biocatalyst in the synthesis of eugenyl benzoate , the outcome has been unsatisfactory due to the low conversion yield or the use of lipase derived from the pathogenic *Staphylococcus aureus*, in which the latter may pose adverse implications towards human health (Bartolomeu *et al.*, 2016; Hu *et al.*, 2012).

In this study, covalently bound RML onto CNWs reinforced chitosan (RML-CS/CNWs) will be used as biocatalysts for the lab scale synthesis of eugenyl benzoate. It was previously described that CS reinforced with CNWs using the biodegradable tannic acid as the crosslinker favorably altered the stability and the mechanical properties of the resultant CS/CNWs carrier support (Rubentheren *et al.*, 2015). It is hypothesized that the covalent attachment of RML onto CS/CNWs may confer certain benefits of biocompatibility of both supports to RML and additional structural integrity to the RML protein structure, thereby increasing operational stability of the nanobioconjugates for improved yield of eugenyl benzoate.

1.3 Objectives of the Study

The objectives of this study are:

i. To characterize the morphology, physicochemical properties and stability of the RML-CS/CNWs.
ii. To optimize the RML-CS/CNWs catalyzed synthesis of eugenyl benzoate.
iii. To develop kinetic model for the RML-CS/CNWs catalyzed synthesis of eugenyl benzoate.

1.4 Scope of Study

The scopes of this project involve the preparation of chitin nanowhiskers using acid hydrolysis reaction in which the supports of CS/CNWs bead are prepared for the immobilization of the RML. The study subsequently assessed the morphological characteristics of the CS/CNWs and RML-CS/CNWs beads by:

a) Fourier transform infrared (FTIR)
b) Field emission scanning electron microscopy (FESEM)
c) Thermal gravimetric analysis (TGA)
d) X-ray diffraction (XRD)
e) Fluorescence optical microscopy

Next, the study of the characterization of the physicochemical properties and optimize the RML-CS/CNWs assisted esterification of eugenol and benzoic acid to afford eugenyl benzoate was carried out using the method of OVAT for parameters temperature, amount of enzyme, incubation time, substrate molar ratio (alcohol:acid), stirring rate, reusability and thermal stability. The ANOVA on the results of the OVAT study is crucial to identify the four relevant parameters to be investigated in the subsequent RSM study.

The following part of the study is the optimization of the RML-CS/CNWs assisted synthesis of eugenyl benzoate using the method of RSM for four relevant parameters as the following: temperature, incubation time, amount of enzyme and molar ratio of acid to alcohol, according to the proposed conditions by the Design Expert 7.1.6 software utilizing the BBD method. The response of the reaction is determined in terms of the percentage yield of eugenyl benzoate. Lastly, the kinetic study for the RML-CS/CNWs catalyzed esterification of eugenol and benzoic acid based on different concentrations of the substrates were carried out to ascertain the mechanism that the developed biocatalysts and to identify the kinetic parameters of the lipase *viz.* V_{max}, K_m, K_{cat} and K_{eff}.

1.5 Significance of Study

The RML-CS/CNWs developed in this study offers a one-pot synthesis that promotes the use of the Green Chemistry philosophy in producing eugenyl benzoate while promoting lesser use of environmentally unfriendly chemicals and hazardous acids. In addition, the modification of the CS/CNWs polymer matrix by introducing tannic acid as the crosslinker is beneficial owing to its biodegradability, non-cytotoxicity and less expensive to produce (Rubentheren *et al.*, 2015). Moreover, an enzyme-assisted reaction is noteworthily carried out under mild conditions and the biocatalyst can be reused for several cycles of reaction, hence is prospectively cost-saving. Moreover, application of RML-CS/CNWs as the biocatalyst would make a significant cost reduction as the amount of enzyme utilized is low.

REFERENCES

Abdulmalek, E., Hamidon, N. F., and Rahman, M. B. A. (2016). Optimization and Characterization of Lipase Catalysed Synthesis of Xylose Caproate Ester in Organic Solvents. *Journal of Molecular Catalysis B: Enzymatic, 132*, 1-4.

Aelenei, N., Popa, M. I., Novac, O., Lisa, G., and Balaita, L. (2009). Tannic Acid Incorporation in Chitosan-Based Microparticles and in Vitro Controlled Release. *J Mater Sci Mater Med, 20*(5), 1095-1102.

Ajmal, M., Fieg, G., and Keil, F. (2016). Analysis of Process Intensification in Enzyme Catalyzed Reactions Using Ultrasound. *Chemical Engineering and Processing: Process Intensification, 110*, 106-113.

Al Mossawi, L. H. M., Mahdi, N. Z., and Zwain, L. A. H. (2016). Higher Production of Lipase Enzyme from Different Microorganisms Grown in Local Natural Culture Media. *Int. J. Adv. Res. Biol. Sci, 3*(5), 232-239.

Alves, J. S., Garcia-Galan, C., Danelli, D., Paludo, N., Barbosa, O., Rodrigues, R. C., and Fernandez-Lafuente, R. (2015). Use of Lecitase-Ultra Immobilized on Styrene-Divinylbenzene Beads as Catalyst of Esterification Reactions: Effects of Ultrasounds. *Catalysis Today, 255*, 27-32.

Ansari, S. A., and Husain, Q. (2012). Potential Applications of Enzymes Immobilized on/in Nano Materials: A Review. *Biotechnology advances, 30*(3), 512-523.

Araki, J., and Mishima, S. (2015). Steric Stabilization of "Charge-Free" Cellulose Nanowhiskers by Grafting of Poly(Ethylene Glycol). *Molecules, 20*(1), 169-184.

Arung, E. T., Matsubara, E., Kusuma, I. W., Sukaton, E., Shimizu, K., and Kondo, R. (2011). Inhibitory Components from the Buds of Clove (Syzygium Aromaticum) on Melanin Formation in B16 Melanoma Cells. *Fitoterapia, 82*(2), 198-202.

Asfaram, A., Ghaedi, M., Hajati, S., Rezaeinejad, M., Goudarzi, A., and Purkait, M. K. (2015). Rapid Removal of Auramine-O and Methylene Blue by Zns: Cu Nanoparticles Loaded on Activated Carbon: A Response Surface Methodology Approach. *Journal of the Taiwan Institute of Chemical Engineers, 53*, 80-91.

Babaki, M., Yousefi, M., Habibi, Z., Mohammadi, M., Yousefi, P., Mohammadi, J., and Brask, J. (2016). Enzymatic Production of Biodiesel Using Lipases Immobilized on Silica Nanoparticles as Highly Reusable Biocatalysts: Effect of Water, T-Butanol and Blue Silica Gel Contents. *Renewable Energy, 91*, 196-206.

Badgujar, K. C., and Bhanage, B. M. (2014). Application of Lipase Immobilized on the Biocompatible Ternary Blend Polymer Matrix for Synthesis of Citronellyl Acetate in Non-Aqueous Media: Kinetic Modelling Study. *Enzyme and microbial technology, 57*, 16-25.

Badgujar, K. C., Dhake, K. P., and Bhanage, B. M. (2013). Immobilization of Candida Cylindracea Lipase on Poly Lactic Acid, Polyvinyl Alcohol and Chitosan Based Ternary Blend Film: Characterization, Activity, Stability and

Its Application for N-Acylation Reactions. *Process Biochemistry, 48*(9), 1335-1347.

Barbosa, J. D., Silva, V. B., Alves, P. B., Gumina, G., Santos, R. L., Sousa, D. P., and Cavalcanti, S. C. (2012). Structure–Activity Relationships of Eugenol Derivatives against Aedes Aegypti (Diptera: Culicidae) Larvae. *Pest management science, 68*(11), 1478-1483.

Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R. C., and Fernandez-Lafuente, R. (2014). Glutaraldehyde in Bio-Catalysts Design: A Useful Crosslinker and a Versatile Tool in Enzyme Immobilization. *Rsc Advances, 4*(4), 1583-1600.

Bartolomeu, M., Rocha, S., Cunha, Â., Neves, M., Faustino, M. A., and Almeida, A. (2016). Effect of Photodynamic Therapy on the Virulence Factors of Staphylococcus Aureus. *Frontiers in microbiology, 7.*

Baş, D., and Boyacı, İ. H. (2007). Modeling and Optimization I: Usability of Response Surface Methodology. *Journal of food engineering, 78*(3), 836-845.

Basri, M., Kassim, M. A., Mohamad, R., and Ariff, A. B. (2013). Optimization and Kinetic Study on the Synthesis of Palm Oil Ester Using Lipozyme Tl Im. *Journal of Molecular Catalysis B: Enzymatic, 85*, 214-219.

Bassegoda, A., Cesarini, S., and Diaz, P. (2012). Lipase Improvement: Goals and Strategies. *Computational and structural biotechnology journal, 2*(3), 1-8.

Bayramo lu, G. (2017). Methacrylated Chitosan Based Uv Curable Support for Enzyme Immobilization. *Materials Research*, 0-0.

Ben Akacha, N., and Gargouri, M. (2015). Microbial and Enzymatic Technologies Used for the Production of Natural Aroma Compounds: Synthesis, Recovery Modeling, and Bioprocesses. *Food and Bioproducts Processing, 94*, 675-706.

Berg, J., Tymoczko, J., and Stryer, L. (2002). The Michaelis-Menten Model Accounts for the Kinetic Properties of Many Enzymes. *Biochemistry*, 319-330.

Bezbradica, D., Stojanović, M., Veličković, D., Dimitrijević, A., Carević, M., Mihailović, M., and Milosavić, N. (2013). Kinetic Model of Lipase-Catalyzed Conversion of Ascorbic Acid and Oleic Acid to Liposoluble Vitamin C Ester. *Biochemical engineering journal, 71*, 89-96.

Bhardwaj, K. K., Saun, N. K., and Gupta, R. (2017). Immobilization of Lipase from Geobacillus Sp. And Its Application in Synthesis of Methyl Salicylate. *Journal of Oleo Science, 66*(4), 391-398.

Bolivar, J. M., Eisl, I., and Nidetzky, B. (2016). Advanced Characterization of Immobilized Enzymes as Heterogeneous Biocatalysts. *Catalysis Today, 259*, 66-80.

Box, G. E., and Behnken, D. W. (1960). Some New Three Level Designs for the Study of Quantitative Variables. *Technometrics, 2*(4), 455-475.

Božič, M., Gorgieva, S., and Kokol, V. (2012). Homogeneous and Heterogeneous Methods for Laccase-Mediated Functionalization of Chitosan by Tannic Acid and Quercetin. *Carbohydrate polymers, 89*(3), 854-864.

Brena, B., González-Pombo, P., and Batista-Viera, F. (2013). Immobilization of Enzymes: A Literature Survey. *Immobilization of Enzymes and Cells: Third Edition*, 15-31.

Cantone, S., Ferrario, V., Corici, L., Ebert, C., Fattor, D., Spizzo, P., and Gardossi, L. (2013). Efficient Immobilisation of Industrial Biocatalysts: Criteria and Constraints for the Selection of Organic Polymeric Carriers and Immobilisation Methods. *Chemical Society Reviews, 42*(15), 6262-6276.

Cao, S. L., Huang, Y. M., Li, X. H., Xu, P., Wu, H., Li, N., Lou, W. Y., and Zong, M. H. (2016). Preparation and Characterization of Immobilized Lipase from Pseudomonas Cepacia onto Magnetic Cellulose Nanocrystals. *Sci Rep, 6*, 20420.

Carneiro, L. A. B. C., Costa-Silva, T. A., Souza, C. R. F., Bachmann, L., Oliveira, W. P., and Said, S. (2014). Immobilization of Lipases Produced by the Endophytic Fungus Cercospora Kikuchii on Chitosan Microparticles. *Brazilian Archives of Biology and Technology, 57*(4), 578-586.

Chaibakhsh, N., Basri, M., Mohamed Anuar, S. H., Abdul Rahman, M. B., and Rezayee, M. (2012). Optimization of Enzymatic Synthesis of Eugenol Ester Using Statistical Approaches. *Biocatalysis and Agricultural Biotechnology, 1*(3), 226-231.

Chaibakhsh, N., Chiet, E. C., and Basri, M. (2014). Optimization of Biocatalytic Synthesis of a Nutraceutical Ester. *Optimization, 1*(10).

Che Marzuki, N. H., Mahat, N. A., Huyop, F., Buang, N. A., and Wahab, R. A. (2015). Candida Rugosa Lipase Immobilized onto Acid-Functionalized Multi-Walled Carbon Nanotubes for Sustainable Production of Methyl Oleate. *Appl Biochem Biotechnol, 177*(4), 967-984.

Chiaradia, V., Paroul, N., Cansian, R. L., Junior, C. V., Detofol, M. R., Lerin, L. A., Oliveira, J. V., and Oliveira, D. (2012). Synthesis of Eugenol Esters by Lipase-Catalyzed Reaction in Solvent-Free System. *Appl Biochem Biotechnol, 168*(4), 742-751.

Choi, J.-M., Han, S.-S., and Kim, H.-S. (2015). Industrial Applications of Enzyme Biocatalysis: Current Status and Future Aspects. *Biotechnology advances, 33*(7), 1443-1454.

Chowdhury, A., and Mitra, D. (2015). A Kinetic Study on the Novozyme 435-Catalyzed Esterification of Free Fatty Acids with Octanol to Produce Octyl Esters. *Biotechnology progress, 31*(6), 1494-1499.

Clark, J. H., Farmer, T. J., Macquarrie, D. J., and Sherwood, J. (2013). Using Metrics and Sustainability Considerations to Evaluate the Use of Bio-Based and Non-Renewable Brønsted Acidic Ionic Liquids to Catalyse Fischer Esterification Reactions. *Sustainable Chemical Processes, 1*(1), 23.

Cleland, W. W. (1973). Derivation of Rate Equations for Multisite Ping-Pong Mechanisms with Ping-Pong Reactions at One or More Sites. *Journal of Biological Chemistry, 248*(24), 8353-8355.

Collins, S. E., Lassalle, V., and Ferreira, M. L. (2011). Ftir-Atr Characterization of Free Rhizomucor Meihei Lipase (Rml), Lipozyme Rm Im and Chitosan-Immobilized Rml. *Journal of Molecular Catalysis B: Enzymatic, 72*(3-4), 220-228.

Datta, S., Christena, L. R., and Rajaram, Y. R. S. (2013). Enzyme Immobilization: An Overview on Techniques and Support Materials. *3 Biotech, 3*(1), 1-9.

Demitri, C., De Benedictis, V. M., Madaghiele, M., Corcione, C. E., and Maffezzoli, A. (2016). Nanostructured Active Chitosan-Based Films for Food Packaging Applications: Effect of Graphene Stacks on Mechanical Properties. *Measurement, 90*, 418-423.

Derewenda, Z. S., Derewenda, U., and Dodson, G. G. (1992). The Crystal and Molecular Structure of the Rhizomucor Miehei Triacylglyceride Lipase at 1.9 Å Resolution. *Journal of molecular biology, 227*(3), 818-839.

Devi, K. P., Nisha, S. A., Sakthivel, R., and Pandian, S. K. (2010). Eugenol (an Essential Oil of Clove) Acts as an Antibacterial Agent against Salmonella

Typhi by Disrupting the Cellular Membrane. *J Ethnopharmacol, 130*(1), 107-115.

Diaconu, M., Litescu, S. C., and Radu, G. L. (2010). Laccase–Mwcnt–Chitosan Biosensor—a New Tool for Total Polyphenolic Content Evaluation from in Vitro Cultivated Plants. *Sensors and Actuators B: Chemical, 145*(2), 800-806.

Dwevedi, A. (2016). Basics of Enzyme Immobilization. In *Enzyme Immobilization* (pp. 21-44): Springer.

Edet, E., Ntekpe, M., and Omereji, S. (2013). Current Trend in Enzyme Immobilization: A Review. *Int J Mod Biochem, 2*, 31-49.

Elsabee, M. Z., and Abdou, E. S. (2013). Chitosan Based Edible Films and Coatings: A Review. *Materials Science and Engineering: C, 33*(4), 1819-1841.

Fernandez-Lopez, L., Pedrero, S. G., Lopez-Carrobles, N., Gorines, B. C., Virgen-Ortíz, J. J., and Fernandez-Lafuente, R. (2016). Effect of Protein Load on Stability of Immobilized Enzymes. *Enzyme and Microbial Technology.*

Fernandez, J. G., and Ingber, D. E. (2013). Bioinspired Chitinous Material Solutions for Environmental Sustainability and Medicine. *Advanced Functional Materials, 23*(36), 4454-4466.

Ferreira, S. C., Bruns, R., Ferreira, H., Matos, G., David, J., Brandao, G., da Silva, E. P., Portugal, L., Dos Reis, P., and Souza, A. (2007). Box-Behnken Design: An Alternative for the Optimization of Analytical Methods. *Analytica chimica acta, 597*(2), 179-186.

Garlapati, V. K., and Banerjee, R. (2013). Solvent-Free Synthesis of Flavour Esters through Immobilized Lipase Mediated Transesterification. *Enzyme Res, 2013*, 367410.

Gilani, S. L., Najafpour, G. D., Moghadamnia, A., and Kamaruddin, A. H. (2016). Stability of Immobilized Porcine Pancreas Lipase on Mesoporous Chitosan Beads: A Comparative Study. *Journal of Molecular Catalysis B: Enzymatic, 133*, 144-153.

Gofferje, G., Stäbler, A., Herfellner, T., Schweiggert-Weisz, U., and Flöter, E. (2014). Kinetics of Enzymatic Esterification of Glycerol and Free Fatty Acids in Crude Jatropha Oil by Immobilized Lipase from Rhizomucor Miehei. *Journal of Molecular Catalysis B: Enzymatic, 107*, 1-7.

Gopalan Nair, K., and Dufresne, A. (2003). Crab Shell Chitin Whisker Reinforced Natural Rubber Nanocomposites. 1. Processing and Swelling Behavior. *Biomacromolecules, 4*(3), 657-665.

Gopi, S., Balakrishnan, P., Pius, A., and Thomas, S. (2017). Chitin Nanowhisker (Chnw)-Functionalized Electrospun Pvdf Membrane for Enhanced Removal of Indigo Carmine. *Carbohydrate Polymers, 165*, 115-122.

Hajar, M., and Vahabzadeh, F. (2016). Production of a Biodiesel Additive in a Stirred Basket Reactor Using Immobilized Lipase: Kinetic and Mass Transfer Analysis. *Korean Journal of Chemical Engineering, 33*(4), 1220-1231.

Hamed, I., Özogul, F., and Regenstein, J. M. (2016). Industrial Applications of Crustacean by-Products (Chitin, Chitosan, and Chitooligosaccharides): A Review. *Trends in Food Science & Technology, 48*, 40-50.

Harshvardhan, K., Suri, M., Goswami, A., and Goswami, T. (2017). Biological Approach for the Production of Vanillin from Lignocellulosic Biomass (Bambusa Tulda). *Journal of Cleaner Production, 149*, 485-490.

He, L., Yao, L., Yang, D., Cheng, Q., Sun, J., Song, R., and Hao, Y. (2011). Preparation and Characterization of Chitosan-Blended Multiwalled Carbon Nanotubes. *Journal of Macromolecular Science, Part B, 50*(12), 2454-2463.

Hemaiswarya, S., and Doble, M. (2009). Synergistic Interaction of Eugenol with Antibiotics against Gram Negative Bacteria. *Phytomedicine, 16*(11), 997-1005.

Horchani, H., Ben Salem, N., Zarai, Z., Sayari, A., Gargouri, Y., and Chaabouni, M. (2010). Enzymatic Synthesis of Eugenol Benzoate by Immobilized Staphylococcus Aureus Lipase: Optimization Using Response Surface Methodology and Determination of Antioxidant Activity. *Bioresour Technol, 101*(8), 2809-2817.

Hossain, K. M. Z., Jasmani, L., Ahmed, I., Parsons, A. J., Scotchford, C. A., Thielemans, W., and Rudd, C. D. (2012). High Cellulose Nanowhisker Content Composites through Cellosize Bonding. *Soft Matter, 8*(48), 12099-12110.

Hu, C., Xiong, N., Zhang, Y., Rayner, S., and Chen, S. (2012). Functional Characterization of Lipase in the Pathogenesis of Staphylococcus Aureus. *Biochemical and biophysical research communications, 419*(4), 617-620.

Huang, B., Bates, M., and Zhuang, X. (2009). Super-Resolution Fluorescence Microscopy. *Annual review of biochemistry, 78*, 993-1016.

Huang, J., Xia, J., Yang, Z., Guan, F., Cui, D., Guan, G., Jiang, W., and Li, Y. (2014). Improved Production of a Recombinant Rhizomucor Miehei Lipase Expressed in Pichia Pastoris and Its Application for Conversion of Microalgae Oil to Biodiesel. *Biotechnology for biofuels, 7*(1), 111.

Ifuku, S. (2014). Chitin and Chitosan Nanofibers: Preparation and Chemical Modifications. *Molecules, 19*(11), 18367-18380.

Isah, A. A., Mahat, N. A., Jamalis, J., Attan, N., Zakaria, I. I., Huyop, F., and Wahab, R. A. (2017). Synthesis of Geranyl Propionate in a Solvent-Free Medium Using Rhizomucor Miehei Lipase Covalently Immobilized on Chitosan–Graphene Oxide Beads. *Preparative Biochemistry and Biotechnology, 47*(2), 199-210.

Jadhav, S. H., and Gogate, P. R. (2014). Ultrasound Assisted Enzymatic Conversion of Non Edible Oil to Methyl Esters. *Ultrasonics sonochemistry, 21*(4), 1374-1381.

Jain, A., Ong, V., Jayaraman, S., Balasubramanian, R., and Srinivasan, M. (2016). Supercritical Fluid Immobilization of Horseradish Peroxidase on High Surface Area Mesoporous Activated Carbon. *The Journal of Supercritical Fluids, 107*, 513-518.

Jensen, E. C. (2012). Types of Imaging, Part 2: An Overview of Fluorescence Microscopy. *The Anatomical Record, 295*(10), 1621-1627.

Jiang, S., Qin, Y., Yang, J., Li, M., Xiong, L., and Sun, Q. (2017). Enhanced Antibacterial Activity of Lysozyme Immobilized on Chitin Nanowhiskers. *Food chemistry, 221*, 1507-1513.

João, C. F., Echeverria, C., Velhinho, A., Silva, J. C., Godinho, M. H., and Borges, J. P. (2017). Bio-Inspired Production of Chitosan/Chitin Films from Liquid Crystalline Suspensions. *Carbohydrate Polymers, 155*, 372-381.

Jumbri, K., Rozy, M. F. A.-H., Ashari, S. E., Mohamad, R., Basri, M., and Masoumi, H. R. F. (2015). Optimisation and Characterisation of Lipase-Catalysed Synthesis of a Kojic Monooleate Ester in a Solvent-Free System by Response Surface Methodology. *PloS one, 10*(12), e0144664.

Juneidi, I., Hayyan, M., Hashim, M. A., and Hayyan, A. (2017). Pure and Aqueous Deep Eutectic Solvents for a Lipase-Catalysed Hydrolysis Reaction. *Biochemical Engineering Journal, 117*, 129-138.

Kadokawa, J.-i., Endo, R., Tanaka, K., Ohta, K., and Yamamoto, K. (2015). Fabrication of Porous Chitin with Continuous Substructure by Regeneration from Gel with Cabr 2· 2h 2 O/Methanol. *International journal of biological macromolecules, 78*, 313-317.

Kamatou, G. P., Vermaak, I., and Viljoen, A. M. (2012). Eugenol—from the Remote Maluku Islands to the International Market Place: A Review of a Remarkable and Versatile Molecule. *Molecules, 17*(6), 6953-6981.

Kartal, F. (2016). Enhanced Esterification Activity through Interfacial Activation and Cross-Linked Immobilization Mechanism of Rhizopus Oryzae Lipase in a Nonaqueous Medium. *Biotechnology progress, 32*(4), 899-904.

Kelnar, I., Kovářová, J., Tishchenko, G., Kaprálková, L., Pavlová, E., Carezzi, F., and Morganti, P. (2015). Chitosan/Chitin Nanowhiskers Composites: Effect of Plasticisers on the Mechanical Behaviour. *Journal of Polymer Research, 22*(2).

Khan, N., Jadhav, S., and Rathod, V. K. (2016). Enzymatic Synthesis of N-Butyl Palmitate in a Solvent-Free System: Rsm Optimization and Kinetic Studies. *Biocatalysis and Biotransformation, 34*(3), 99-109.

Khudsange, C. R., and Wasewar, K. L. (2017). Process Intensification of Esterification Reaction for the Production of Propyl Butyrate by Pervaporation. *Resource-Efficient Technologies, 3*(1), 88-93.

Kiechel, M. A., and Schauer, C. L. (2013). Non-Covalent Crosslinkers for Electrospun Chitosan Fibers. *Carbohydrate polymers, 95*(1), 123-133.

Kiran, K., Krishna, S. H., Babu, C. S., Karanth, N., and Divakar, S. (2000). An Esterification Method for Determination of Lipase Activity. *Biotechnology Letters, 22*(19), 1511-1514.

Kirk, J. F., Ritter, G., Finger, I., Sankar, D., Reddy, J. D., Talton, J. D., Nataraj, C., Narisawa, S., Millán, J. L., and Cobb, R. R. (2013). Mechanical and Biocompatible Characterization of a Cross-Linked Collagen-Hyaluronic Acid Wound Dressing. *Biomatter, 3*(4), e25633.

Kuo, C.-H., Chen, G.-J., Chen, C.-I., Liu, Y.-C., and Shieh, C.-J. (2014). Kinetics and Optimization of Lipase-Catalyzed Synthesis of Rose Fragrance 2-Phenylethyl Acetate through Transesterification. *Process Biochemistry, 49*(3), 437-444.

Kuo, C. H., Chen, H. H., Chen, J. H., Liu, Y. C., and Shieh, C. J. (2012). High Yield of Wax Ester Synthesized from Cetyl Alcohol and Octanoic Acid by Lipozyme Rmim and Novozym 435. *Int J Mol Sci, 13*(9), 11694-11704.

Kuperkar, V. V., Lade, V. G., Prakash, A., and Rathod, V. K. (2014). Synthesis of Isobutyl Propionate Using Immobilized Lipase in a Solvent Free System: Optimization and Kinetic Studies. *Journal of Molecular Catalysis B: Enzymatic, 99*, 143-149.

Lau, S. C., Lim, H. N., Basri, M., Fard Masoumi, H. R., Ahmad Tajudin, A., Huang, N. M., Pandikumar, A., Chia, C. H., and Andou, Y. (2014). Enhanced Biocatalytic Esterification with Lipase-Immobilized Chitosan/Graphene Oxide Beads. *PLoS One, 9*(8), e104695.

Li, W., Wu, H., Liu, B., Hou, X., Wan, D., Lou, W., and Zhao, J. (2015). Highly Efficient and Regioselective Synthesis of Dihydromyricetin Esters by Immobilized Lipase. *Journal of biotechnology, 199*, 31-37.

Liu, L., Wang, R., Yu, J., Jiang, J., Zheng, K., Hu, L., Wang, Z., and Fan, Y. (2016a). Robust Self-Standing Chitin Nanofiber/Nanowhisker Hydrogels with Designed Surface Charges and Ultralow Mass Content Via Gas Phase Coagulation. *Biomacromolecules, 17*(11), 3773-3781.

Liu, M., Zheng, H., Chen, J., Li, S., Huang, J., and Zhou, C. (2016b). Chitosan-Chitin Nanocrystal Composite Scaffolds for Tissue Engineering. *Carbohydrate polymers, 152*, 832-840.

Lopresto, C. G., Calabrò, V., Woodley, J. M., and Tufvesson, P. (2014). Kinetic Study on the Enzymatic Esterification of Octanoic Acid and Hexanol by Immobilized Candida Antarctica Lipase B. *Journal of Molecular Catalysis B: Enzymatic, 110*, 64-71.

Lorenzoni, A. S., Graebin, N. G., Martins, A. B., Fernandez-Lafuente, R., Ayub, Z., Marco, A., and Rodrigues, R. C. (2012). Optimization of Pineapple Flavour Synthesis by Esterification Catalysed by Immobilized Lipase from Rhizomucor Miehei. *Flavour and Fragrance Journal, 27*(2), 196-200.

M. M. Elnashar, M. (2010). Review Article: Immobilized Molecules Using Biomaterials and Nanobiotechnology. *Journal of Biomaterials and Nanobiotechnology, 01*(01), 61-77.

Ma, B., Qin, A., Li, X., Zhao, X., and He, C. (2014). Bioinspired Design and Chitin Whisker Reinforced Chitosan Membrane. *Materials Letters, 120*, 82-85.

Ma, N., Liu, X.-W., Yang, Y.-J., Li, J.-Y., Mohamed, I., Liu, G.-R., and Zhang, J.-Y. (2015). Preventive Effect of Aspirin Eugenol Ester on Thrombosis in K-Carrageenan-Induced Rat Tail Thrombosis Model. *PloS one, 10*(7), e0133125.

Manan, F. M. A., Rahman, I. N. A., Marzuki, N. H. C., Mahat, N. A., Huyop, F., and Wahab, R. A. (2016). Statistical Modelling of Eugenol Benzoate Synthesis Using Rhizomucor Miehei Lipase Reinforced Nanobioconjugates. *Process Biochemistry, 51*(2), 249-262.

Martins, A. B., da Silva, A. M., Schein, M. F., Garcia-Galan, C., Ayub, M. A. Z., Fernandez-Lafuente, R., and Rodrigues, R. C. (2014). Comparison of the Performance of Commercial Immobilized Lipases in the Synthesis of Different Flavor Esters. *Journal of Molecular Catalysis B: Enzymatic, 105*, 18-25.

Martins, A. B., Friedrich, J. L., Cavalheiro, J. C., Garcia-Galan, C., Barbosa, O., Ayub, M. A., Fernandez-Lafuente, R., and Rodrigues, R. C. (2013). Improved Production of Butyl Butyrate with Lipase from Thermomyces Lanuginosus Immobilized on Styrene–Divinylbenzene Beads. *Bioresource technology, 134*, 417-422.

Martins, A. B., Graebin, N. G., Lorenzoni, A. S., Fernandez-Lafuente, R., Ayub, M. A., and Rodrigues, R. C. (2011). Rapid and High Yields of Synthesis of Butyl Acetate Catalyzed by Novozym 435: Reaction Optimization by Response Surface Methodology. *Process Biochemistry, 46*(12), 2311-2316.

Marzuki, N. H. C., Huyop, F., Aboul-Enein, H. Y., Mahat, N. A., and Wahab, R. A. (2015a). Modelling and Optimization Ofcandida Rugosananobioconjugates Catalysed Synthesis of Methyl Oleate by Response Surface Methodology. *Biotechnology & Biotechnological Equipment, 29*(6), 1113-1127.

Marzuki, N. H. C., Mahat, N. A., Huyop, F., Buang, N. A., and Wahab, R. A. (2015b). Candida Rugosa Lipase Immobilized onto Acid-Functionalized Multi-Walled Carbon Nanotubes for Sustainable Production of Methyl Oleate. *Applied biochemistry and biotechnology, 177*(4), 967-984.

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., and Fernandez-Lafuente, R. (2007). Improvement of Enzyme Activity, Stability and

Selectivity Via Immobilization Techniques. *Enzyme and microbial technology, 40*(6), 1451-1463.

Mathpati, A. C., Badgujar, K. C., and Bhanage, B. M. (2016). Kinetic Modeling and Docking Study of Immobilized Lipase Catalyzed Synthesis of Furfuryl Acetate. *Enzyme and microbial technology, 84*, 1-10.

Mekhzoum, M. M., Essassi, E., Qaiss, A., and Bouhfid, R. (2016). Fluorescent Bio-Nanocomposites Based on Chitosan Reinforced Hemicyanine Dye-Modified Montmorillonite. *RSC Advances, 6*(112), 111472-111481.

Melgosa, R., Sanz, M. T., Solaesa, Á. G., Bucio, S. L., and Beltrán, S. (2015). Enzymatic Activity and Conformational and Morphological Studies of Four Commercial Lipases Treated with Supercritical Carbon Dioxide. *The Journal of Supercritical Fluids, 97*, 51-62.

Mendes, A. A., Freitas, L., de Carvalho, A. K., de Oliveira, P. C., and de Castro, H. F. (2011). Immobilization of a Commercial Lipase from Penicillium Camembertii (Lipase G) by Different Strategies. *Enzyme Res, 2011*, 967239.

Michaux, F., Zoumpanioti, M., Papamentzelopoulou, M., Stébé, M. J., Blin, J. L., and Xenakis, A. (2010). Immobilization and Activity of Rhizomucor Miehei Lipase. Effect of the Matrix Properties Prepared from Nonionic Fluorinated Surfactants. *Process Biochemistry, 45*(1), 39-46.

Mincea, M., Negrulescu, A., and Ostafe, V. (2012). Preparation, Modification, and Applications of Chitin Nanowhiskers: A Review. *Rev Adv Mater Sci, 30*(3), 225-242.

Mohamad, N., Huyop, F., Aboul-Enein, H. Y., Mahat, N. A., and Wahab, R. A. (2015a). Response Surface Methodological Approach for Optimizing Production of Geranyl Propionate Catalysed by Carbon Nanotubes Nanobioconjugates. *Biotechnology & Biotechnological Equipment, 29*(4), 732-739.

Mohamad, N. R., Buang, N. A., Mahat, N. A., Lok, Y. Y., Huyop, F., Aboul-Enein, H. Y., and Abdul Wahab, R. (2015b). A Facile Enzymatic Synthesis of Geranyl Propionate by Physically Adsorbed Candida Rugosa Lipase onto Multi-Walled Carbon Nanotubes. *Enzyme Microb Technol, 72*, 49-55.

Mohamad, N. R., Marzuki, N. H., Buang, N. A., Huyop, F., and Wahab, R. A. (2015c). An Overview of Technologies for Immobilization of Enzymes and Surface Analysis Techniques for Immobilized Enzymes. *Biotechnol Biotechnol Equip, 29*(2), 205-220.

Mohammadi, M., Ashjari, M., Dezvarei, S., Yousefi, M., Babaki, M., and Mohammadi, J. (2015). Rapid and High-Density Covalent Immobilization of Rhizomucor Miehei Lipase Using a Multi Component Reaction: Application in Biodiesel Production. *RSC Adv., 5*(41), 32698-32705.

Mohammadi, M., Habibi, Z., Dezvarei, S., Yousefi, M., Samadi, S., and Ashjari, M. (2014). Improvement of the Stability and Selectivity of Rhizomucor Miehei Lipase Immobilized on Silica Nanoparticles: Selective Hydrolysis of Fish Oil Using Immobilized Preparations. *Process Biochemistry, 49*(8), 1314-1323.

Moon, S. E., Kim, H. Y., and Cha, J. D. (2011). Synergistic Effect between Clove Oil and Its Major Compounds and Antibiotics against Oral Bacteria. *Arch Oral Biol, 56*(9), 907-916.

Mubarak, N., Wong, J., Tan, K., Sahu, J., Abdullah, E., Jayakumar, N., and Ganesan, P. (2014). Immobilization of Cellulase Enzyme on Functionalized Multiwall Carbon Nanotubes. *Journal of Molecular Catalysis B: Enzymatic, 107*, 124-131.

Muhamad, S. K., Radzi, S. M., Othman, S. S., Rahman, M. B. A., and Noor, H. M. (2014). Optimization of Lipase-Catalyzed Synthesis of Flavour Esters in Solvent Free System. *Malaysian Journal of Fundamental and Applied Sciences, 6*(1).

Muzzarelli, R., Aiba, S., Fujiwara, Y., Hideshima, T., Hwang, C., Kakizaki, M., Izume, M., Minoura, N., Rha, C., and Shouij, T. (1986). Filmogenic Properties of Chitin/Chitosan. In *Chitin in Nature and Technology* (pp. 389-402): Springer.

Nardini, M., and Dijkstra, B. W. (1999). A/B Hydrolase Fold Enzymes: The Family Keeps Growing. *Current opinion in structural biology, 9*(6), 732-737.

Nawaz, M. A., Karim, A., Bibi, Z., Rehman, H. U., Aman, A., Hussain, D., Ullah, M., and Qader, S. A. U. (2016). Maltase Entrapment Approach as an Efficient Alternative to Increase the Stability and Recycling Efficiency of Free Enzyme within Agarose Matrix. *Journal of the Taiwan Institute of Chemical Engineers, 64*, 31-38.

Nguyen, T. Y., Cai, C. M., Kumar, R., and Wyman, C. E. (2015). Co-Solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass. *ChemSusChem, 8*(10), 1716-1725.

Nie, K., Wang, M., Zhang, X., Hu, W., Liu, L., Wang, F., Deng, L., and Tan, T. (2015). Additives Improve the Enzymatic Synthesis of Biodiesel from Waste Oil in a Solvent Free System. *Fuel, 146*, 13-19.

Ozyilmaz, G., and Gezer, E. (2010). Production of Aroma Esters by Immobilized Candida Rugosa and Porcine Pancreatic Lipase into Calcium Alginate Gel. *Journal of Molecular Catalysis B: Enzymatic, 64*(3-4), 140-145.

Paës, G. (2014). Fluorescent Probes for Exploring Plant Cell Wall Deconstruction: A Review. *Molecules, 19*(7), 9380-9402.

Paiva, A. L., Balcao, V. M., and Malcata, F. X. (2000). Kinetics and Mechanisms of Reactions Catalyzed by Immobilized Lipases☆. *Enzyme and microbial technology, 27*(3), 187-204.

Paludo, N., Alves, J. S., Altmann, C., Ayub, M. A., Fernandez-Lafuente, R., and Rodrigues, R. C. (2015). The Combined Use of Ultrasound and Molecular Sieves Improves the Synthesis of Ethyl Butyrate Catalyzed by Immobilized Thermomyces Lanuginosus Lipase. *Ultrasonics sonochemistry, 22*, 89-94.

Pandiyan, K., Tiwari, R., Singh, S., Nain, P. K., Rana, S., Arora, A., Singh, S. B., and Nain, L. (2014). Optimization of Enzymatic Saccharification of Alkali Pretreated Parthenium Sp. Using Response Surface Methodology. *Enzyme research, 2014*.

Pasma, S. A., Daik, R., Maskat, M. Y., and Hassan, O. (2013). Application of Box-Behnken Design in Optimization of Glucose Production from Oil Palm Empty Fruit Bunch Cellulose. *International Journal of Polymer Science, 2013*.

Patel, V., Gajera, H., Gupta, A., Manocha, L., and Madamwar, D. (2015). Synthesis of Ethyl Caprylate in Organic Media Using Candida Rugosa Lipase Immobilized on Exfoliated Graphene Oxide: Process Parameters and Reusability Studies. *Biochemical Engineering Journal, 95*, 62-70.

Patel, V., Shah, C., Deshpande, M., and Madamwar, D. (2016). Zinc Oxide Nanoparticles Supported Lipase Immobilization for Biotransformation in Organic Solvents: A Facile Synthesis of Geranyl Acetate, Effect of Operative

Variables and Kinetic Study. *Applied biochemistry and biotechnology, 178*(8), 1630-1651.

Patil, H. M., Sawant, D. K., Bhavsar, D. S., Patil, J. H., and Girase, K. D. (2011). Ftir and Thermal Studies on Gel Grown Neodymium Tartrate Crystals. *Journal of Thermal Analysis and Calorimetry, 107*(3), 1031-1037.

Pereira, A. G., Muniz, E. C., and Hsieh, Y. L. (2014). Chitosan-Sheath and Chitin-Core Nanowhiskers. *Carbohydr Polym, 107*, 158-166.

Poppe, J. K., Garcia-Galan, C., Matte, C. R., Fernandez-Lafuente, R., Rodrigues, R. C., and Ayub, M. A. Z. (2013). Optimization of Synthesis of Fatty Acid Methyl Esters Catalyzed by Lipase B from Candida Antarctica Immobilized on Hydrophobic Supports. *Journal of Molecular Catalysis B: Enzymatic, 94*, 51-56.

Qin, Y., Zhang, S., Yu, J., Yang, J., Xiong, L., and Sun, Q. (2016). Effects of Chitin Nano-Whiskers on the Antibacterial and Physicochemical Properties of Maize Starch Films. *Carbohydrate polymers, 147*, 372-378.

Raghavendra, T., Basak, A., Manocha, L. M., Shah, A. R., and Madamwar, D. (2013). Robust Nanobioconjugates of Candida Antarctica Lipase B--Multiwalled Carbon Nanotubes: Characterization and Application for Multiple Usages in Non-Aqueous Biocatalysis. *Bioresour Technol, 140*, 103-110.

Rahman, M. M. (2014). Reusable and Mediator-Free Cholesterol Biosensor Based on Cholesterol Oxidase Immobilized onto Tga-Sam Modified Smart Bio-Chips. *PLoS One, 9*(6), e100327.

Raita, M., Kiatkittipong, W., Laosiripojana, N., and Champreda, V. (2015). Kinetic Study on Esterification of Palmitic Acid Catalyzed by Glycine-Based Crosslinked Protein Coated Microcrystalline Lipase. *Chemical Engineering Journal, 278*, 19-23.

Rajan, A., Sudha, J., and Abraham, T. E. (2008). Enzymatic Modification of Cassava Starch by Fungal Lipase. *Industrial crops and products, 27*(1), 50-59.

Rehm, S., Trodler, P., and Pleiss, J. (2010). Solvent-Induced Lid Opening in Lipases: A Molecular Dynamics Study. *Protein Science, 19*(11), 2122-2130.

Rehman, S., Bhatti, H. N., Bilal, M., and Asgher, M. (2016). Cross-Linked Enzyme Aggregates (Cleas) of Pencilluim Notatum Lipase Enzyme with Improved Activity, Stability and Reusability Characteristics. *International journal of biological macromolecules, 91*, 1161-1169.

Rodrigues, R. C., and Fernandez-Lafuente, R. (2010). Lipase from Rhizomucor Miehei as an Industrial Biocatalyst in Chemical Process. *Journal of Molecular Catalysis B: Enzymatic, 64*(1-2), 1-22.

Rubentheren, V., Ward, T. A., Chee, C. Y., and Tang, C. K. (2015). Processing and Analysis of Chitosan Nanocomposites Reinforced with Chitin Whiskers and Tannic Acid as a Crosslinker. *Carbohydr Polym, 115*, 379-387.

Sadeghian, H., Seyedi, S. M., Saberi, M. R., Arghiani, Z., and Riazi, M. (2008). Design and Synthesis of Eugenol Derivatives, as Potent 15-Lipoxygenase Inhibitors. *Bioorg Med Chem, 16*(2), 890-901.

Salaberria, A. M., Labidi, J., and Fernandes, S. C. (2014). Chitin Nanocrystals and Nanofibers as Nano-Sized Fillers into Thermoplastic Starch-Based Biocomposites Processed by Melt-Mixing. *Chemical Engineering Journal, 256*, 356-364.

Sánchez-Ferrero, A., Mata, Á., Mateos-Timoneda, M. A., Rodríguez-Cabello, J. C., Alonso, M., Planell, J., and Engel, E. (2015). Development of Tailored and

Self-Mineralizing Citric Acid-Crosslinked Hydrogels for in Situ Bone Regeneration. *Biomaterials, 68*, 42-53.

Sarve, A., Sonawane, S. S., and Varma, M. N. (2015). Ultrasound Assisted Biodiesel Production from Sesame (Sesamum Indicum L.) Oil Using Barium Hydroxide as a Heterogeneous Catalyst: Comparative Assessment of Prediction Abilities between Response Surface Methodology (Rsm) and Artificial Neural Network (Ann). *Ultrasonics sonochemistry, 26*, 218-228.

Schejbal, J., Řemínek, R., Zeman, L., Mádr, A., and Glatz, Z. (2016). On-Line Coupling of Immobilized Cytochrome P450 Microreactor and Capillary Electrophoresis: A Promising Tool for Drug Development. *Journal of Chromatography A, 1437*, 234-240.

Schuchardt, U., Sercheli, R., and Vargas, R. M. (1998). Transesterification of Vegetable Oils: A Review. *Journal of the Brazilian Chemical Society, 9*(3), 199-210.

Sebatini, A. M., Jain, M., Radha, P., Kiruthika, S., and Tamilarasan, K. (2016). Immobilized Lipase Catalyzing Glucose Stearate Synthesis and Their Surfactant Properties Analysis. *3 Biotech, 6*(2), 184.

Segal, L., Creely, J., Martin Jr, A., and Conrad, C. (1959). An Empirical Method for Estimating the Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. *Textile Research Journal, 29*(10), 786-794.

Shankar, S., Agarwal, M., and Chaurasia, S. (2013). Study of Reaction Parameters and Kinetics of Esterification of Lauric Acid with Butanol by Immobilized Candida Antarctica Lipase.

Sharif, K., Rahman, M., Azmir, J., Mohamed, A., Jahurul, M., Sahena, F., and Zaidul, I. (2014). Experimental Design of Supercritical Fluid Extraction–a Review. *Journal of Food Engineering, 124*, 105-116.

Sharma, R., Chisti, Y., and Banerjee, U. C. (2001). Production, Purification, Characterization, and Applications of Lipases. *Biotechnology advances, 19*(8), 627-662.

Sheldon, R. A., and van Pelt, S. (2013). Enzyme Immobilisation in Biocatalysis: Why, What and How. *Chemical Society Reviews, 42*(15), 6223-6235.

Shen, Y., Liu, X., Yang, Y., Li, J., Ma, N., and Li, B. (2015). In Vivo and in Vitro Metabolism of Aspirin Eugenol Ester in Dog by Liquid Chromatography Tandem Mass Spectrometry. *Biomedical Chromatography, 29*(1), 129-137.

Shinde, S. D., and Yadav, G. D. (2014). Process Intensification of Immobilized Lipase Catalysis by Microwave Irradiation in the Synthesis of 4-Chloro-2-Methylphenoxyacetic Acid (Mcpa) Esters. *Biochemical Engineering Journal, 90*, 96-102.

Shukla, S. K., Mishra, A. K., Arotiba, O. A., and Mamba, B. B. (2013). Chitosan-Based Nanomaterials: A State-of-the-Art Review. *International journal of biological macromolecules, 59*, 46-58.

Silva, N. C., Miranda, J. S., Bolina, I. C., Silva, W. C., Hirata, D. B., de Castro, H. F., and Mendes, A. A. (2014). Immobilization of Porcine Pancreatic Lipase on Poly-Hydroxybutyrate Particles for the Production of Ethyl Esters from Macaw Palm Oils and Pineapple Flavor. *Biochemical Engineering Journal, 82*, 139-149.

Silva, S. M., Braga, C. R., Fook, M. V., Raposo, C. M., Carvalho, L. H., and Canedo, E. L. (2012). Application of Infrared Spectroscopy to Analysis of Chitosan/Clay Nanocomposites. *Infrared Spectroscopy—Materials Science, Engineering and Technology*, 43-62.

Šimkovic, I. (2013). Unexplored Possibilities of All-Polysaccharide Composites. *Carbohydrate polymers, 95*(2), 697-715.
Singh, J., Singh, M. K., Kumar, M., and Thakur, I. S. (2015). Immobilized Lipase from Schizophyllum Commune Istl04 for the Production of Fatty Acids Methyl Esters from Cyanobacterial Oil. *Bioresource technology, 188*, 214-218.
Skoronski, E., Padoin, N., Soares, C., and Furigo Jr, A. (2014). Stability of Immobilized Rhizomucor Miehei Lipase for the Synthesis of Pentyl Octanoate in a Continuous Packed Bed Bioreactor. *Brazilian Journal of Chemical Engineering, 31*(3), 633-641.
Solanki, P. R., Kaushik, A., Ansari, A. A., Tiwari, A., and Malhotra, B. D. (2009). Multi-Walled Carbon Nanotubes/Sol–Gel-Derived Silica/Chitosan Nanobiocomposite for Total Cholesterol Sensor. *Sensors and Actuators B: Chemical, 137*(2), 727-735.
Stergiou, P.-Y., Foukis, A., Filippou, M., Koukouritaki, M., Parapouli, M., Theodorou, L. G., Hatziloukas, E., Afendra, A., Pandey, A., and Papamichael, E. M. (2013). Advances in Lipase-Catalyzed Esterification Reactions. *Biotechnology advances, 31*(8), 1846-1859.
Su, F., Li, G., Fan, Y., and Yan, Y. (2016). Enhanced Performance of Lipase Via Microcapsulation and Its Application in Biodiesel Preparation. *Scientific reports, 6*, 29670.
Subhedar, P. B., and Gogate, P. R. (2016). Ultrasound Assisted Intensification of Biodiesel Production Using Enzymatic Interesterification. *Ultrasonics Sonochemistry, 29*, 67-75.
Takada, A., and Kadokawa, J.-i. (2015). Fabrication and Characterization of Polysaccharide Ion Gels with Ionic Liquids and Their Further Conversion into Value-Added Sustainable Materials. *Biomolecules, 5*(1), 244-262.
Tang, J., Chen, G., Wang, L., Miao, M., Jiang, B., and Feng, B. (2017). Immobilization of Y. Lipolytica Lipase and the Continuous Synthesis of Geranyl Propionate. *Journal of Molecular Catalysis B: Enzymatic*.
Tekindal, M. A., Bayrak, H., Ozkaya, B., and Genç, Y. (2012). Box-Behnken Experimental Design in Factorial Experiments: The Importance of Bread for Nutrition and Health. *Turkish Journal of Field Crops, 17*(2), 115-123.
Todero, L. M., Bassi, J. J., Lage, F. A. P., Corradini, M. C. C., Barboza, J. C. S., Hirata, D. B., and Mendes, A. A. (2015). Enzymatic Synthesis of Isoamyl Butyrate Catalyzed by Immobilized Lipase on Poly-Methacrylate Particles: Optimization, Reusability and Mass Transfer Studies. *Bioprocess and Biosystems Engineering, 38*(8), 1601-1613.
Tosa, T., Mori, T., Fuse, N., and Chibata, I. (1969). Studies on Continuous Enzyme Reactions: Part V. Kinetics and Industrial Application of Aminoacylase Column for Continuous Optical Resolution of Acyl-Dl-Amino Acids Part Vi. Enzymatic Properties of the Deae-Sephadex-Aminoacylase Complex. *Agricultural and Biological Chemistry, 33*(7), 1047-1059.
Ungcharoenwiwat, P., Canyuk, B., and Aran, H. (2016). Synthesis of Jatropha Oil Based Wax Esters Using an Immobilized Lipase from Burkholderia Sp. Eq3 and Lipozyme Rm Im. *Process Biochemistry, 51*(3), 392-398.
Vadgama, R. N., Odaneth, A. A., and Lali, A. M. (2015). Green Synthesis of Isopropyl Myristate in Novel Single Phase Medium Part I: Batch Optimization Studies. *Biotechnology Reports, 8*, 133-137.

Vaghari, H., Jafarizadeh-Malmiri, H., Mohammadlou, M., Berenjian, A., Anarjan, N., Jafari, N., and Nasiri, S. (2016). Application of Magnetic Nanoparticles in Smart Enzyme Immobilization. *Biotechnology Letters, 38*(2), 223-233.

van Pouderoyen, G., Eggert, T., Jaeger, K.-E., and Dijkstra, B. W. (2001). The Crystal Structure of Bacillus Subtili Lipase: A Minimal A/B Hydrolase Fold Enzyme. *Journal of molecular biology, 309*(1), 215-226.

Velasco-Lozano, S., López-Gallego, F., Rocha-Martin, J., Guisán, J. M., and Favela-Torres, E. (2016). Improving Enantioselectivity of Lipase from Candida Rugosa by Carrier-Bound and Carrier-Free Immobilization. *Journal of Molecular Catalysis B: Enzymatic, 130*, 32-39.

Verri, F., Diaz, U., Macario, A., Corma, A., and Giordano, G. (2016). Optimized Hybrid Nanospheres Immobilizing Rhizomucor Miehei Lipase for Chiral Biotransformation. *Process Biochemistry, 51*(2), 240-248.

Wahab, R. A., Basri, M., Rahman, R. N. Z. R. A., Salleh, A. B., Rahman, M. B. A., Chaibakhsh, N., and Leow, T. C. (2014). Enzymatic Production of a Solvent-Free Menthyl Butyrate Via Response Surface Methodology Catalyzed by a Novel Thermostable Lipase from Geobacillus Zalihae. *Biotechnology & Biotechnological Equipment, 28*(6), 1065-1072.

Wang, X., Li, D., Qu, M., Durrani, R., Yang, B., and Wang, Y. (2017). Immobilized Mas1 Lipase Showed High Esterification Activity in the Production of Triacylglycerols with N-3 Polyunsaturated Fatty Acids. *Food Chemistry, 216*, 260-267.

Watthanaphanit, A., Supaphol, P., Tamura, H., Tokura, S., and Rujiravanit, R. (2008). Fabrication, Structure, and Properties of Chitin Whisker-Reinforced Alginate Nanocomposite Fibers. *Journal of Applied Polymer Science, 110*(2), 890-899.

Wu, J. C. Y., Hutchings, C. H., Lindsay, M. J., Werner, C. J., and Bundy, B. C. (2015). Enhanced Enzyme Stability through Site-Directed Covalent Immobilization. *Journal of biotechnology, 193*, 83-90.

Xu, F., Weng, B., Gilkerson, R., Materon, L. A., and Lozano, K. (2015). Development of Tannic Acid/Chitosan/Pullulan Composite Nanofibers from Aqueous Solution for Potential Applications as Wound Dressing. *Carbohydrate polymers, 115*, 16-24.

Xu, R., Zhou, Q., Li, F., and Zhang, B. (2013). Laccase Immobilization on Chitosan/Poly (Vinyl Alcohol) Composite Nanofibrous Membranes for 2, 4-Dichlorophenol Removal. *Chemical engineering journal, 222*, 321-329.

Yadav, G. D., and Devendran, S. (2012). Lipase Catalyzed Synthesis of Cinnamyl Acetate Via Transesterification in Non-Aqueous Medium. *Process Biochemistry, 47*(3), 496-502.

Yadav, G. D., and Yadav, A. R. (2012). Insight into Esterification of Eugenol to Eugenol Benzoate Using a Solid Super Acidic Modified Zirconia Catalyst Udcat-5. *Chemical Engineering Journal, 192*, 146-155.

Yan, H.-D., Zhang, Q., and Wang, Z. (2014). Biocatalytic Synthesis of Short-Chain Flavor Esters with High Substrate Loading by a Whole-Cell Lipase from Aspergillus Oryzae. *Catalysis Communications, 45*, 59-62.

Yao, H., and Kimura, K. (2007). Field Emission Scanning Electron Microscopy for Structural Characterization of 3d Gold Nanoparticle Superlattices. *Modern research and educational topics in microscopy. Formatex, Badajoz.*

Ye, N., Xie, Y., Shi, P., Gao, T., and Ma, J. (2014). Synthesis of Magnetite/Graphene Oxide/Chitosan Composite and Its Application for Protein Adsorption. *Materials Science and Engineering: C, 45*, 8-14.

Yu, W. H., Tong, D. S., Fang, M., Shao, P., and Zhou, C. H. (2015). Immobilization of Candida Rugosa Lipase on Msu-H Type Mesoporous Silica for Selective Esterification of Conjugated Linoleic Acid Isomers with Ethanol. *Journal of Molecular Catalysis B: Enzymatic, 111*, 43-50.

Yudianti, R., Onggo, H., Sudirman, Y. S., Iwata, T., and Azuma, J.-i. (2011). Analysis of Functional Group Sited on Multi-Wall Carbon Nanotube Surface. *Open Materials Science Journal, 5*, 242-247.

Zangeneh, N., Azizian, A., Lye, L., and Popescu, R. (2002). *Application of Response Surface Methodology in Numerical Geotechnical Analysis*. Paper presented at the 55th Canadian society for geotechnical conference, Hamilton, Ontario.

Zhang, C., Zhuang, X., Li, X., Wang, W., Cheng, B., Kang, W., Cai, Z., and Li, M. (2016a). Chitin Nanowhisker-Supported Sulfonated Poly (Ether Sulfone) Proton Exchange for Fuel Cell Applications. *Carbohydrate polymers, 140*, 195-201.

Zhang, D.-H., Zhang, J.-Y., Che, W.-C., and Wang, Y. (2016b). A New Approach to Synthesis of Benzyl Cinnamate: Optimization by Response Surface Methodology. *Food chemistry, 206*, 44-49.

Zhang, Z., Zhang, R., Chen, L., and McClements, D. J. (2016c). Encapsulation of Lactase (B-Galactosidase) into K-Carrageenan-Based Hydrogel Beads: Impact of Environmental Conditions on Enzyme Activity. *Food chemistry, 200*, 69-75.

Zhao, X., Qi, F., Yuan, C., Du, W., and Liu, D. (2015). Lipase-Catalyzed Process for Biodiesel Production: Enzyme Immobilization, Process Simulation and Optimization. *Renewable and Sustainable Energy Reviews, 44*, 182-197.

Zheng, M., Zhu, J., Huang, F., Xiang, X., Shi, J., Deng, Q., Ma, F., and Feng, Y. (2015). Enzymatic Deacidification of the Rice Bran Oil and Simultaneous Preparation of Phytosterol Esters-Enriched Functional Oil Catalyzed by Immobilized Lipase Arrays. *RSC Advances, 5*(86), 70073-70079.

Zhou, Z., and Hartmann, M. (2012). Recent Progress in Biocatalysis with Enzymes Immobilized on Mesoporous Hosts. *Topics in Catalysis, 55*(16-18), 1081-1100.

Zou, B., Hu, Y., Yu, D., Xia, J., Tang, S., Liu, W., and Huang, H. (2010). Immobilization of Porcine Pancreatic Lipase onto Ionic Liquid Modified Mesoporous Silica Sba-15. *Biochemical Engineering Journal, 53*(1), 150-153.

Zucca, P., and Sanjust, E. (2014). Inorganic Materials as Supports for Covalent Enzyme Immobilization: Methods and Mechanisms. *Molecules, 19*(9), 14139-14194.

Zuo, P.-P., Feng, H.-F., Xu, Z.-Z., Zhang, L.-F., Zhang, Y.-L., Xia, W., and Zhang, W.-Q. (2013). Fabrication of Biocompatible and Mechanically Reinforced Graphene Oxide-Chitosan Nanocomposite Films. *Chemistry Central Journal, 7*(1), 39.