

UNIVERSITI PUTRA MALAYSIA

ENZYMATIC ESTERIFICATION OF KOJIC ACID AND PALMITIC ACID BY IMMOBILIZED LIPASE FOR SYNTHESIS OF KOJIC ACID PALMITATE

NURAZWA BINTI ISHAK

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By

NURAZWA BINTI ISHAK

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

April 2015

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrone) is an organic acid produced from various carbon sources in an aerobic fermentation by many species of *Aspergillus*, *Penicillium* and *Acetobacter*. The importance of kojic acid (KA) is recently focused on its role as whitening agent in cosmetic formulation. Kojic acid is water soluble and has low stability towards light exposure. KA has also been criticized for weak depigmenting effect and unstable for long storage. The hydrophilic property of KA has restricted its application in cosmetic, oily food and pharmaceutical products. In order to improve the chemical and biological activities of KA, its derivatives with new and improved chemical properties and biological activities needs to be developed. Various KA derivatives such as KA esters have been synthesized at industrial scale. KA esters are normally produced via chemical process where strong acid or alkali is used. This chemical process is not environmentally friendly and also produces complex mixtures that make the product purification difficult and high cost.

The possibility of using lipase, lipozyme RMIM, in the esterification of KA with palmitic acid (PA) in acetone to synthesize KA palmitate (KAP) was investigated in this study. Preliminary, the effects of organic solvent, substrate ratio, enzyme loading, temperature and reaction time on the yield of KAP were evaluated. The appropriate ranges for each variable were subsequently used for optimization using response surface methodology (RSM). The optimal reaction condition for ester production was then applied in 500 mL stirred tank reactor (STR) using two types of impeller [Rushton turbine (RT) and Pitch blade disc turbine (PBDT)] to investigate the effect of agitation speed on the esterification performance. Among the organic solvent tested for esterification to synthesize KAP, acetone was the preferred solvent. Optimal conditions for esterification as suggested by RSM were as follows: PA to KA ratio, 6.74; enzyme loading, 0.59 g; reaction temperature, 45.9°C and reaction time, 20 h, which gave the percentage of esterification of 64.47%. For the esterification in STR, the percentage yield of KAP was significantly higher for RT than PBDT at all agitation speeds tested (150 to 450 rpm). The highest yield of KAP (82.14%) was obtained in STR with RT agitated at 250 rpm. Results from this study have demonstrated that substantially high yield of KA esters could be produced by enzymatic esterification using lipase. This alternative

method has potential to be used industrially. Since the use of hazardous chemical can be minimized, enzymatic synthesis of KA esters is more natural and appears to be more appealing to the customers than the chemical process.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia Sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PENGESTERAN BERENZIM ASID KOJIK DAN ASID PALMITIK MENGGUNAKAN LIPASE TERSEKAT GERAK UNTUK SINTESIS ASID KOJIK PALMITIK

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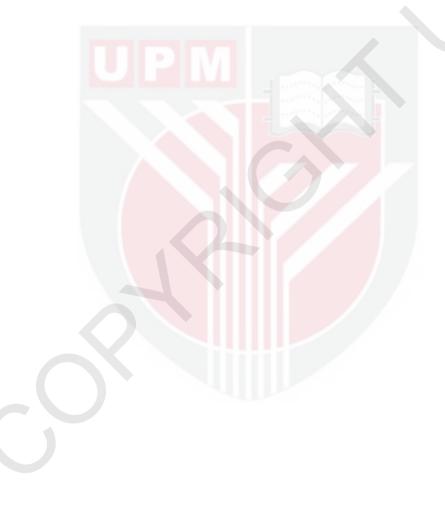
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Asid kojik (5-hydroxy-2-hydroxymethyl-4-pyrone) adalah asid organik yang dihasilkan daripada pelbagai sumber karbon menggunakan fermentasi aerobik oleh spesies *Aspergillus, Penicillium* dan *Acetobacter*. Kepentingan asid kojik (KA) kini tertumpu kepada fungsinya sebagai agen pencerah dalam formulasi kosmetik. KA adalah larut air dan tidak stabil terhadap pendedahan kepada cahaya matahari. KA mempunyai kesan depigmentasi yang lemah dan tidak stabil untuk simpanan jangka lama. Sifat kesukaannya kepada air menjadikan kegunaannya dalam kosmetik, makanan berasaskan minyak dan farmaseutikal terhad. Bagi meningkatkan aktiviti biokimia KA, terbitan KA dengan sifat biokimia yang lebih baik harus dibangunkan. Pelbagai terbitan KA seperti ester KA dihasilkan pada skala industri. Ester KA biasanya dihasilkan melalui proses kimia dalam kehadiran asid atau alkali kuat. Proses kimia ini tidak mesra alam dan menghasilkan campuran kompleks yang sukar ditulenkan dengan kos yang tinggi.

Kajian ini mengkaji kebolehan enzim lipase, Lipozyme RMIM dalam pengesteran KA dan asid palmitik (PA) dalam aseton untuk menghasilkan asid kojik palmitik (KAP). Eksperimen pengesteran pada permulaannya dilakukan untuk menilai kesan pelarut yang sesuai, nisbah substrat, jumlah enzim, suhu dan masa tindak balas pada hasil KAP. Julat daripada setiap pembolehubah kemudiannya digunakan untuk pengoptimuman menggunakan kaedah tindak balas permukaan (RSM). Keadaan optimum tindak balas penghasilan ester ini digunakan dalam pengesteran menggunakan 500 mL reaktor tangki teraduk (STR) untuk mengkaji kesan kelajuan pengadukan bagi dua jenis pengaduk [Rushton turbine (RT) dan Pitch blade disc turbine (PBDT)] terhadap prestasi pengesteran. Aseton didapati merupakan pelarut pilihan berbanding pelarut lain. Keadaan optimum pengesteran yang dicadangkan oleh RSM adalah seperti berikut: nisbah PA kepada KA, 6:74; jumlah enzim, 0.59 g; suhu, 45.9°C; dan masa tindak balas, 20 jam, yang memberikan peratusan pengesteran sebanyak 64.47%. Peratusan hasil asid kojic palmitik adalah lebih tinggi bagi RT berbanding PBDT pada semua kelajuan pengadukan (150-450 rpm) yang diuji bagi pengesteran dalam STR. Hasil tertinggi KAP (82.14%) diperoleh dalam STR menggunakan RT apabila diaduk pada kelajuan 250 rpm. Hasil kajian ini menunjukkan jumlah KAP yang tinggi boleh dihasilkan oleh pengesteran berenzim menggunakan lipase. Kaedah alternatif ini berpotensi digunakan dalam perindustrian. Oleh kerana penggunaan bahan kimia berbahaya dapat dikurangkan, sintesis enzim ester KA adalah lebih asli dan kelihatan lebih menarik kepada pelanggan.



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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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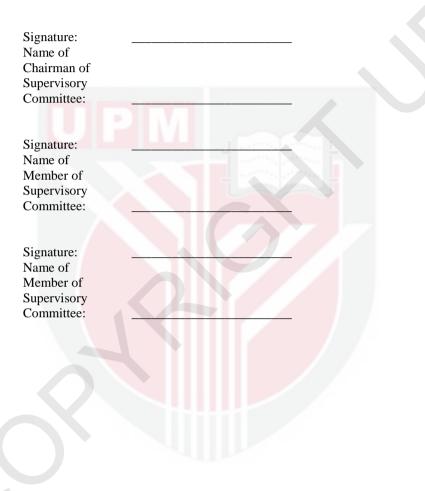


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LIST OF ABBREVIATIONS

ANN	Artificial Neural Network
ANOVA	
	Analysis of variance
CCRD	Central composite rotatable design
Di	Impeller diameter
Dt	Tank diameter
FBR	Fluidized Bed Reactor
FT-IR	Fourier Transform-Infrared Spectroscopy
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HL	Fluid depth
KA	Kojic acid
KAP	Kojic acid palmitate
L	Tank height
MPOB	Malaysian Palm Oil Board
NMR	Nuclear Magnetic Resonance
PBDT	Pitch Blade Disc Turbine
PBR	Packed Bed Reactor
R2	Coefficient of determination
Rf	Retention factor
Rt	Retention time
rpm	Rotation per minute
RSM	Response Surface Methodology
RT	Rushton Turbine
STR	Stirred Tank Reactor
TAG	Triacylglycerol
TLC	Thin Layer Chromatography
UV	Ultraviolet
v/v	Volume per volume
W	Ribbon width
**	NIUUUI widui



CHAPTER 1

INTRODUCTION

Kojic acid (2-hydroxymethyl-5-hydroxy- γ -pyrone), an organic acid, is normally produced in an aerobic fermentation by fungi such as *Aspergillus* and *Penicillium* or bacteria such as *Acetobacter*. Kojic acid has diverse application in medical, food, agricultural, chemistry and cosmetic industry. In medicine, it is used as an anti-inflammatory drug and painkiller (Kayahara *et al.*, 1990). Kojic acid is widely used as a precursor for flavor enhancer and as an anti-browning agent to prevent agricultural products such as crustacean, meat and fresh vegetables from blackening (Le Blanch and Akers, 1989). Kojic acid is recognized as important intermediates in the production of the chemicals and pharmaceutics.

Among of the aforesaid applications, a desire to maintain a youthful appearance has propelled the recent surge of kojic acid in cosmetic industry. The cosmetic formulation with the presence of kojic acid is intended not only to improve visual appearance of the skin but also to offer long lasting effect. In this case, kojic acid acts as a whitening agent and a protective against ultraviolet (Noh *et al.*, 2009; Masse *et al.*, 2001). Kojic acid not only effective to treat melasma, but it also has the ability to suppress hyperpigmentation in human skin by restraining the formation of melanin through inhibition of tyrosinase activity. Convincing evidence which shows that kojic acid is effective in inhibiting melanin synthesis as tested either *in vitro* and *in vivo* have been reported (Nohynek *et al.*, 2004).

Despite of its myriad application, kojic acid is water-soluble and unstable at high temperature for long term storage and also sensitive towards exposure to light. These characteristics restricted its application to be directly incorporated in oil based cosmetic product (Masse *et al.*, 2001). Research on the conversion of kojic acid into stable derivatives aims at improving its lipophilic characteristic is of interest of many researchers. This is usually focussed on the modification of the C-5 or C-7 hydroxyl through esterification process which can be achieved via chemical or enzymatic approach (Lajis *et al.*, 2013).

At present, many esters are industrially manufactured by chemical methods because it is claimed as economical process. However, chemical esterification involves high temperature and extreme pressure which limits this method for the esterification of unstable compound. Furthermore, chemical esterification has low reaction rates with simultaneous production of unwanted side products, which make product purification more complicated with increasing cost. Enzymatic esterification appears to be more appealing to the customer since there is a rapidly growing demand for natural, cleaner, alternative technologies that produces less waste and also to avoid the use of toxic reagent. Liu and Shaw (1998) had successfully performed the enzymatic synthesis of kojic acid esters from various sources of acyl donor. Various parameters such as type of solvent, substrate ratio, enzyme loading, reaction time and temperature may affect the enzymatic esterification of kojic acid esters.

Optimization of the process reaction is essential to maximize the production and to reduce the cost. Process optimization via the classical one-variable-at-a-time approach is simple to plan and execute. However, this method is inefficient and fails to detect any interaction amongst the reaction variables. Response surface methodology (RSM), a statistical tool, is commonly used to overcome such drawbacks. RSM has been successfully applied to study and optimize the enzymatic synthesis of kojic acid monolaurate (Chen et al., 2002). For industrial production, large scale reactor shall be used to perform the esterification at optimal conditions to maximize the yield and productivity. Stirred tank reactor (STR), which is the conventional mixing vessel, has advantage of low capital cost and low operating costs. STR has been used in many lipasecatalyzed esterification process for production of various esters (Mat Radzi et al., 2010; Keng et al., 2008). A wide variety of impeller designs are available to produce different flow pattern inside the stirred tank vessel to accommodate the specific requirement of the enzymatic process. Appropriate degree of mixing and flow pattern is required in enzymatic process employing immobilized enzyme to ensure the homogenous particle in suspension is achieved. Excessive shear effect created by the agitated impeller may cause damage to immobilized enzyme particle, which in turn, reduce the activity and reusability of the enzyme. The yield and productivity of the enzymatic esterification process may also be improved using suitable mode of reactor operation. For example, production of lauroyl kojic acid through lipase-catalyzed esterification has been improved in continuous STR (Kobayashi et al., 2001).

Therefore, this particular work was carried out with the following objectives:

- 1. To investigate the possibility of using commercial immobilized lipase, which are known to have specificity towards ester bonds, for the synthesis of kojic acid palmitate by esterification in solvent system.
- 2. To optimize the reaction parameters for the synthesis of kojic acid palmitate in shake flask reaction using response surface methodology (RSM).
- 3. To investigate the effect of agitation speed on the synthesis of kojic acid palmitate in stirred tank reactor employing two different designs of impeller.

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