SYNTHESIS OF METHYL ACETATE BY LIPASE CATALYZED ESTERIFICATION

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LIST OF SYMBOLS

Symbol	Description	Unit
C_{Ac}	Concentration of acid	Mol/l
C_{Al}	Concentration of alcohol	Mol/l
K_{mAc}	Michaelis constant for acid	Mol/l
K_{mAl}	Michaelis constant for alcohol	Mol/l
K_{iAl}	Inhibition constant for alcohol	Mol/l
K_{iAc}	Inhibition constant for acid	Mol/l
$-r_{Ac}$	Rate of disappearance of acid	Mol/l/g/min
$(-r_{Ac})_{max}^{f}$	Maximum rate of disappearance of acid	Mol/l/g/min
V	Difference in titer values between blank and sample	ml
W	Weight of lipase	mg
x	Na_2HPO_4 concentration	
y	$NaH_2PO_4.H_2O$ concentration	

LIST OF ABBREVIATIONS

CAGR	Compound Annual Growth Rate
CS-MNPs	Chitosan coated
CRL	*Candida rugosa* lipase
FFD	Fractional Factorial Design
HAP	Hydroxyapatite
HPLC	High Performance Liquid Chromatography
MCO	Movement Control Order
MeOAc	Methyl acetate
NAOH	Sodium Hydroxide
OFAT	One-Factor-at-A-Time
RSM	Response Surface Methodology

PENGESTERAN ASETAT ACID DAN METANOL OLEH *CANDIDA RUGOSA* LIPASE TERSEKATGERAK UNTUK SINTESIS METIL ASETATE: PENGOPTIMUMAN PROSES

ABSTRAK

Permulaan proses esterifikasi kebanyakannya adalah dengan menggunakan sintesis kimia dengan bantuan pemangkin kimia yang sering melibatkan campuran azeotropik binari atau tersier yang menyebabkan kesukaran dalam menghasilkan produk yang tinggi kualitinya dan masalah persekitaran yang tinggi. Oleh itu, esterifikasi dengan bantuan biopemangkin yang telah diperkenalkan bahkan terbukti mempunyai potensi yang lebih baik terutama lipase untuk menghasilkan ester melalui esterifikasi enzimatik. Dalam kajian ini, metil asetat yang mempunyai permintaan pasaran yang tinggi kerana kegunaan akhirnya telah disintesis dengan tindakbalas esterifikasi langsung yang bermangkinkan oleh lipase tersekatgerak dari *Candida rugosa* dengan menggunakan n-heksana sebagai pelarut organik dalam sistem kelompok. Celite-545 digunakan sebagai bahan sokongan pada lipase *Candida* rugosa yang tersekatgerak dengan aktiviti pemangkin 0.6833 U / mg.

Kaedah satu faktor pada satu masa (OFAT) dikaji untuk mengenal pasti kesan parameter pengoptimuman termasuk masa tindakbalas, pemuatan enzim, kelajuan pengadukan dan nisbah molar substrat asid asetik dan metanol. Penukaran asid optimum untuk metil asetat adalah 58% yang diperoleh pada masa inkubasi 2 jam dengan 10 U/ml pemuatan enzim pada suhu 45℃ dan 150 rpm kelajuan pengadukan dengan nisbah molar substrat 1:3 (asid asetik kepada metanol). Perbandingan antara model kinetik esterifikasi pemangkin enzim dengan data sekunder daripada artikel tersebut juga dikaji dengan menggunakan analisis regresi tidak linear. Parameter model yang diperolehi adalah $(-r_{Ac})^{f}_{max} = 2.972\ mmol/min$.l.g,

$K_{mAc} = 0.2488\ mol/l$, $K_{mAl} = 0.2609\ mol/l$, $K_{iAl} = 0.953 \times 10^{-3}\ mol/l$, $K_{iAc} = 1.03 \times 10^{-3}\ mol/l$.

ESTERIFICATION OF ACETIC ACID AND METHANOL FOR SYNTHESIS OF METHYL ACETATE BY IMMOBILIZED OF *CADIDA RUGOSA* LIPASE: PROCESS OPTIMIZATION

ABSTRACT

The beginning of esterification process is mostly by using chemical synthesis with the help of chemical catalyst which often involved binary or tertiary azeotropic mixture which lead to difficulty in producing high purity of product and environmental issues. Hence, esterification with the aid of biocatalyst was introduced which is proven to have a better potential especially with lipase in producing esters through enzymatic esterification. In this study, methyl acetate which has high market demand due to its end uses was synthesise by direct esterification reaction catalysed by immobilized lipase from *Candida rugosa* by using n-hexane as organic solvent in batch system. Celite-545 was used as a support material on immobilized *Candida rugosa* lipase with catalytic activity of 0.6833 U/mg.

One-Factor-at-One-Time (OFAT) method was studied in order to identify the effect of the optimizing parameters including reaction time, enzyme loading, agitation speed and substrate molar ratio of acetic acid and methanol. The optimal acid conversion for methyl acetate was 58% obtained at 2 hours incubation time with 10 U/ml of enzyme loading at 45℃ and 150 rpm of agitation speed with a substrate molar ratio of 1:3 (acetic acid to methanol). The comparison between kinetic models of enzyme catalysed esterification with the secondary data from the article was also performed by using non-linear regression analysis. The model parameters obtained was $(-r_{Ac})^{f}_{max} = 2.972\ mmol/min$.l.g, $K_{mAc} = 0.2488\ mol/l$, $K_{mAl} = 0.2609\ mol/l$, $K_{iAl} = 0.953 \times 10^{-3}\ mol/l$, $K_{iAc} = 1.03 \times 10^{-3}\ mol/l$.

CHAPTER 1

INTRODUCTION

1.1 Background

Methyl acetate is widely known with many names for example methyl ethanoate, acetic acid methyl ester and mostly used MeOAc as a short form for methyl acetate. It has molecular formula of $C_3H_6O_2$ which is a carboxylate ester. The characteristic of methyl acetate is a colourless compound with less toxicity and strong dissolving ability (Hongchao, 2020), making it really compatible to act as cellosolve and spray solvent. Methyl cellosolve is known as an organic compound that act as a solvent to dissolve a variety of different types of chemical compounds. However, methyl acetate is a very flammable compound due to lower flashpoint which is $-10℃$ and is categorized as class 3 flammability rating. From the website on market research, (Expert market research, 2016) the global demand on the methyl acetate is estimated to increase at 6.5% Compound Annual Growth Rate (CAGR) for the next five years from 2020-2025. The flexographic printing ink is the product which triggers the global demand of methyl acetate to use as photoresistor strippers. The increasing flexographic ink in printing industry is because of its advantage which can dry quickly during printing process.

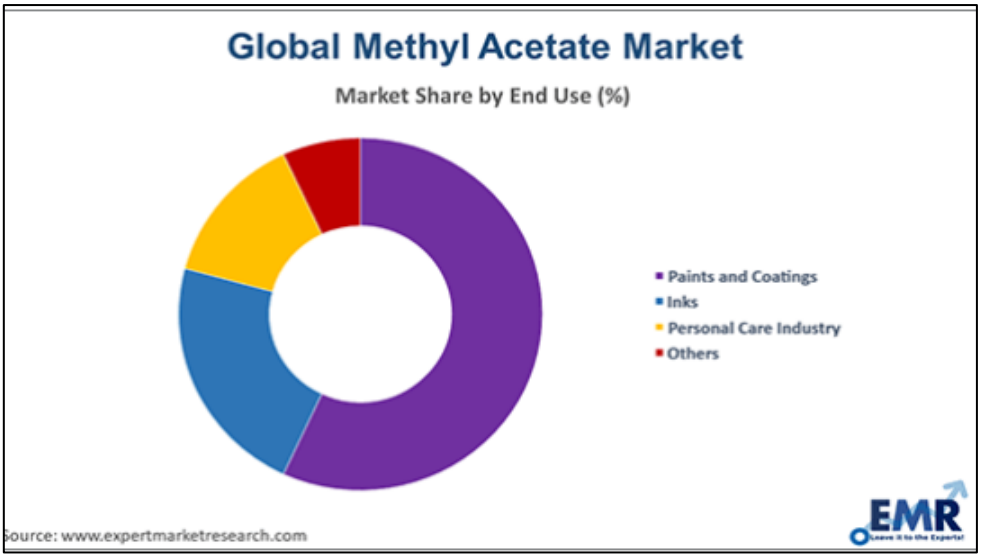

Figure 1: The global methyl acetate market by end use **(Expert market research, 2016)**

Based on Figure 1, methyl acetate production can be categorized by several end-uses according to the global methyl acetate market. The main end-uses for the production of methyl acetate are paints and coatings, inks personal care industry and others. From the expert market research, paints and coatings has the highest global demand for the end-use of methyl acetate which is more than 50%. As mentioned in the beginning, the growth of the methyl acetate market is because of global demand in flexographic ink due to its application as a printing material in a packaging industry. Not to mention, the nation such as China, India, Germany and US are rapidly increasing in the packaging industry because of the internet trade and e-commerce popularity (Expert market research, 2016).

The production of methyl acetate is based on the reaction between acetic acid and methanol with the help of solid catalyst to give more efficient conversion. The presence of solid catalyst also required low equipment corrosion since catalyst usually has more than three years' service life (Methyl Acetate Plant).

There are other techniques to produce methyl acetate with a better performance and high conversion yield for example production of methyl acetate by using enzyme immobilisation. “Biocatalyst has high conversion rates than chemical catalyst” is a common statement. This statement can be proven due to enzymes properties where enzymes are known as natural catalyst which has high specificity to certain substrates. Not to mention, enzyme is operated under mild conditions where temperature, pressure and pH are not too severe but can produce high conversion rates (Danielle Gonçalves Filho, 2019). The most beneficial thing is biocatalyst can increase the speed of reaction without affecting its thermodynamics properties.

In industrial scale, by using biocatalyst it can reduce the use of protecting group, minimized side reaction and the most important thing is environmental problem can be reduced. However, there are still lacking aspects that can be encountered if using biocatalyst. This is the reason why researchers are still exploring the potential of biocatalyst. Substrate inhibition, product inhibition and limited operating regions are one of the biocatalyst drawbacks which need to be overcome (Atul Dev, 2018). From the research that has been conducted, the key for the lacking area in biocatalyst can be overcome by using enzyme immobilisation technology. Enzyme immobilisation is where enzyme is imprisoned in a distinct or matrix where it allows the exchange of medium so that it can retain full or most of its activity. This technology has become a powerful tool especially to reduce the production costs (Maximiliano L.Cacicedo, 2019). Nowadays, the immobilized enzymes not only can be reused as enzymes are costly but it also can improve the enzyme catalyst function such as stability and selectivity (V.Chatzikonstantinou, 2018).

In the immobilisation technology, there are several techniques which include adsoption, covalent binding, entrapment, capsulation and cross-linking. These various types of interaction were due to the different types of support materials that have been used. However, the conditions of the catalytic process and enzyme that is used are the main factor for the selection of the support materials. Generally, the support materials can be divided into three categories which are organic, inorganic and hybrid or composition. The main functions of support material are to protect the enzyme and help it to retain high catalytic activity (Jakub Zdarta, 2018).

1.2 Problem Statement

Methyl acetate can be produced through the process of esterification of acetic acid (electrophile) with methanol to act as acyl acceptor (nucleophile) with the presence of the hydrochloric acid as a chemical catalyst. The earlier experiment that has been conducted by Rolfe and Hinsshelwood (1934) on kinetics reaction of esterification of methyl acetate which is being investigated in both alcoholic and non-hydroxylic media. Homogeneous catalyst such as sulphuric acid is one of the chemical catalysts that are usually being introduced in the production of methyl acetate. However, there are several disadvantages if methyl acetate production is performed with the traditional process where there are one reactor and nine distillation columns. This is because; methyl acetate, methanol and water will initially form binary and tertiary azeotropic mixtures making it more difficult to produce high purity of product. Thus to counter this problem, Reactive-Distillation Process has been developed where reaction and separation undergo simultaneously in a reactive section (Mekala Mallaiah, 2016).

As an alternative enzyme has been introduced since it has wide range of biotransformation not to mention it has great potential as being environmentally friendly (Guang Yang, 2014). There are several industries that already used enzymes as alternative catalyst to undergo processes such as production of pharmaceuticals, pesticides and agrochemicals. Compared to chemical catalyst, the number of advantages introduced by enzyme as biocatalyst are already exceeding the number of advantages from chemical catalyst. For example, biocatalyst provides high efficiency, high degree of selectivity, environmental friendly and others (Weng Lin Tang, 2009).

Due to great achievement of biochemical catalyst that has been shown in recent years, interest in biocatalyst especially in industrial field has been greatly increased. This is because the use of biocatalyst instead of chemical catalyst can solve many global challenges such as climate change, fossil substitution and feeding a growing population (L. Lange, 2017). Enzyme is one of the biocatalyst that comes from several of sources. For the industrial scale, biocatalyst can reduce the energy used since it can perform under mild condition and make industrial processes more sustainable. However, the use of enzyme or free enzyme as a catalyst without further development such as enzymes immobilization can lead to several problems such as substrate inhibition, product inhibition and limited operating regions (Atul Dev, 2018).

Even though free enzymes can be considered much cheaper than immobilized enzyme, in terms of enzyme activity enzyme immobilization is much superior. Immobilisation of the enzyme especially lipase are highly in demand since it plays an important role in a large-scale industrial in producing several of applications such as food, flavour agent, cleaning product and cosmetic. There are many advantages that can be included by conducting this method since enzyme can be reused in multiple reaction cycle

and contamination of the final product can be avoided during recovery of enzyme. However, this current technology still can be improved to get a better performance.

In this study, Celite-545 will be used as a support material for lipase immobilisation. The immobilized lipase will catalyse the esterification reaction of acetic acid and methanol to produce methyl acetate.

1.3 Objectives

I. To compare the hydrolytic activities of free *Candida rugosa* lipase (CRL), CRL immobilized on celite-545 and chitosan. The effects of pH and temperature on hydrolytic activities are also compared.

II. To study the operating parameter of esterification of methyl acetate : reaction time, enzyme loading, substrate molar ratio and agitation speed by using One-Factor-At-A-Time (OFAT) method.

III. To apply and compare between kinetic models of enzyme catalyzed esterification by correlating experimental findings from reported research study.

CHAPTER 2

LITERATURE REVIEW

2.1 Enzyme as biological catalyst

Enzyme was one of the important substances that were involved in the real life processes such as in a digestion process where energy was being produce from digested food. In this type of process, there are many chemical reactions which were related to the biological functions where enzymes were needed to catalyse the reaction. That's why biocatalyst was also known as natural substance which can speed up the chemical reactions (Yokoyama, 2010). There were some benefits that can be obtained by using biocatalyst instead of chemical catalyst. One of them was ability of biocatalyst to remove toxic by-product of chemical catalyst and hence make it cleaner. The operating condition of biocatalyst can be operated in mild condition where substrate and newly formed molecules will not require any protection when the reaction occurs. Biocatalyst was also much bigger than chemical catalyst means the contact surface between substrate and enzyme was increased.

2.1.1 Advantage of lipase as biocatalyst

Lipase was the most common catalyst and has been widely used especially in industrial and pharmaceutical field. This was because of its natural reaction in fat hydrolysis. That's why there were abundance of reactions that lipases can be catalyse for example, esterification, amidation and transesterification either ester or organic carbonates (Neena N. Gandhi, 2000). These were the reasons why lipases were known as versatile catalyst. The ability to maintain its regioselectivity and stereoselectivity when accepting a variety type of substrates was also one of the lipase specialities. Not to mention, under unfavourable condition such as high temperature and organic solvent lipase are still highly stable. In the

industrial field, lipase biocatalyst undergoes several types of reactions which occur either in aqueous medium or non-aqueous medium (Ashok Kumar, 2016). In a global state, lipase was highly in demand especially by organic chemists due to its ability to substrate tolerance, easy to handle, high stability under unfavourable condition and availability commercially. Organic solvent was a medium that was most compatible for synthetic reactions especially on industrial scale due to non-polar compounds which were much easier to dissolve. However, medium like organic solvent were not very suitable to the living cells such as enzymes due to its ability to bind with membrane cells which later can affect its integrity and stability. However, lipase can also be used in nearly anhydrous organic solvents as a biocatalyst. Furthermore, it also offered other new possibilities such as enable the use of hydrophobic substrates, improved thermal stability of the enzyme and reduce the contamination.

Since most of the enzyme can easily be denatured and inactive with the presence of organic solvent, many researchers have developed to improve the characteristics of these enzymes in the presence of organic solvent (Adlercreutz, 2013). With the modern technology, physical and chemical methods such as immobilisation, modification and entrapment were being developed. With the biocatalytic potential of microbial lipase either in aqueous or non-aqueous media, many industries have shifted towards this enzyme. High value-added fatty acid esters that have been produced by lipase lead to many other advantages rather than chemical synthesis between alcohols and carboxylic acid with the use of mineral acid as a catalyst (Kumar A, 2011). Even though lipases contribute significant role in enhancing the quality of live while protecting the environment, they still need greater effort to complete the understanding of different type of lipases. Developing this lipase will surely help the improvement of biotechnology especially in industrial field. Performing biocatalysis in organic solvent especially lipase, provide many advantages such as increased solubility of hydrophobic substrates, elimination of microbial contamination, water dependent suppression

for side reaction, provide variety of chemical reaction which are not practical in an aqueous media and others (Ashok Kumar, 2016).

2.1.2 Methyl acetate and its application

As mentioned earlier, methyl acetate was known as a volatile solvent where it can be used as a photoresistor strippers which are very beneficial in a printing industry. However, methyl acetate also has other applications in a commercial use for example in food industry, cosmetic product, industrial coating and others (World Class Chemical solutions, 2018). In this study, the production of methyl acetate will be focused more in the food industry as flavouring agents in food additives for rum, brandy and whisky. The production of methyl acetate will be conducted by using immobilisation of enzyme specifically using *Candida rugosa* lipase (CRL) as a biocatalyst for a better performance. Lipase mostly can be found from many sources such as animal, vegetable and microbiological and lipases are the most common enzyme in industrial purpose that has very wide application for example in pharmaceutical, cosmetic and food industries. This was because, immobilisation of lipase can be reusable and easy to recover making it more beneficial rather than using chemical catalyst (Abdallah R.Ismailab, 2020).

2.1.3 Immobilization techniques of lipase

Other reasons why immobilisation of lipase was being introduced rather than soluble lipase were due to cost of the process that can be reduced significantly and increase their stability. In the immobilisation of lipase, support materials are playing important role for this process to achieve a better performance. This was because; support material is responsible for

the techniques such as adsoption, covalent binding, entrapment, capsulation and cross-linking can be achieved according to the types of support materials that have been used. However, properties of the enzyme and its support material were also important before the preparation of this process (Dr. Sikander Ali, 2017). For example, the affinity between functional groups between enzyme and support material was one of the properties that need to focus which later can create the formation of enzyme-matrix interaction.

2.1.4 Types of support materials

Support materials can be divided into two main groups which were Classic and New material. Classic was the oldest method to sort out the support materials according to the criteria which were having high stability and affinity to the enzyme and have low price. These criteria will lead to the most effective support materials for biocatalyst. There were two classes in this group which are organic and inorganic. Even though in this recent years classic material become less important, it was still one of the important group of support material which was needed in the immobilisation process (Jakub Zdarta, 2018). Due to rapid growth in the immobilisation technology, many researchers have discovered new possibilities for the immobilisation technology. This was why new support materials were being recommended rather than classic materials due to the good mechanical properties. Not to mention, various morphological shapes that has been produced from this group with the nanoparticle size which was really suitable with the enzymes (Vandana Singh, 2016). In this new support material, there were three classes which are organic, inorganic and hybrid where they can greatly improve the catalytic efficiency, purity and quality of the product compared to classic materials

To enhance the performance on methyl acetate production from lipase immobilisation technology, several type of lipases species have been used with different types of support materials. Mina Memarpoor-Yazdi, 2017 reported that lipase from *Rhodothermus marinus sp.* (RD) was being used as a biocatalyst for the methyl acetate production through the immobilisation technique. They used, chitosan coated Fe_3O_4-nanoparticles (CS-MNPs) as a support material with the *Rhodothermus marinus* lipase (RDL) and the reaction yield that can be obtained between free and immobilised are 22% and 67% respectively.

2.2 Reaction system in immobilisation of *Candida* rugosa Lipase

In the industrial field, methyl acetate plays a significant role especially in a printing industry due to its capability to act as photoresistor strippers. However in this study, methyl acetate will be focused more in the food industry application as flavouring agents (World Class Chemical solutions, 2018). The synthesis of esterification between glycerol and caprylic acid were being conducted by using immobilisation of CRL with Celite 545, Chitosan, and Sephadex as support materials. The physical adsoption techniques was being applied by investigating the reaction parameters such as effect of pH and temperature on hydrolytic activity, esterification reaction and residual activity that exhibited by immobilized lipase (Bhagya Sri Kaja, 2018).

2.2.1 Hydrolytic Activity

Bhagya Sri Kaja, (2018), immobilized CRL by using Celite-545 as support material having high hydrolytic activities if compared to the free CRL. In their study, several other support materials were also being used such as chitosan and sephadex which were also resulted in lower hydrolytic activity compared to Celite-545. The suitable support materials that has been used in the immobilisation techniques can no longer be argued since it not only

increased the enzyme stability but also improve the catalytic efficiency (Patrick, 2013). In this immobilisation method, acetone adsoption and alcohol adsorption techniques were used in order to provide high specific activities. From this experiment, acetone adsorption shows a higher specific activities. This was because polarity of acetone was much lower compared to the alcohol (J. C. Wu, 2007) resulting in highest activities among other solvents.

The enzyme activity can be influenced by the surrounding microenvironment. In order to achieve the optimum amount of fatty acids, pH is one of the factors that can play an important role during hydrolysis reaction. This is because, among the R group of amino acids there are hydrogen bonding which can controlled the structure of tertiary protein. The change of pH value can give a great impact on the ionization of these R groups which later will result in the loss of enzymatic activity due to the disruption of its original native conformation. The increase in product inhibition will cause the decrease in the reaction rate. That is the reason why, pH value is the most crucial factor in order to preserve the native structural conformation and its activity. Hence, most of the enzyme has its own range for pH value (D. Goswami, 2009).

a) Effect of pH on Hydrolytic Activity

The enzyme activity can be changed due to the microenvironment surrounding. In order to achieve the optimum amount of fatty acids during hydrolysis reaction, the pH solution plays an important role. This was because interaction between R groups of amino acids and hydrogen bonding was very crucial in determining the structure of tertiary protein. The significant change on the pH value can alter the ionization of R groups resulting to decrease of enzymatic activity. The decrease of reaction rate also will eventually occur due to significant change in the product inhibition. It can be concluded that all enzymes have their