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*Article*

# Evaluation of Consortium Enzyme towards Kojic Acid Synthesis

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*Abstract*— **Consortium enzyme is a new approach in enzymatic esterification reaction to solve Kojic acid esters' low production. Combining a non-specific enzyme (Novozym 435) and a specific enzyme (Lipozyme RM IM) led to an improvement of enzymatic activity in the enzymatic routes. The findings proved that a significant percentage conversion of ester (29.2 %) was successfully achieved at the enzyme ratio (1:1) of Lipozyme RMIM and Novozym 435, the temperature at 50 ˚C, amount of enzyme 0.12 g and 8 hours of reaction time. Moreover, the enzyme showed high stability against heat at 50$^0$C and can be used repeatedly up to the 5$^{th}$ cycle, as demonstrated by the high percentage conversion of kojic acid ester. This finding confirmed that consortium enzymes have significantly improved the reaction performance by promoting better catalytic efficiency due to the synergistic effect between combined enzymes.**

*Keywords*— **Conventional method, kojic acid, kojic ester, consortium enzyme, Novozym 435, Lipozyme RM IM.**

## I. INTRODUCTION

Kojic acid, 2-hydroxymethyl-5-hydroxy-r-pyrone, is an organic acid produced by fungi and bacteria, such as *Aspergillus sp*., *Acetobacter sp.* and *Penicillium sp.* [1], during the fermentation process of various carbon-containing substrates. It is one of the fungal metabolites used in the fermentation by *Aspergillus sp.* [2]. Kojic acid is also an essential element for skin whitening in cosmetic cream, where it is used to impede pigment development by the profound cells on the skin. The historical background of kojic acid started when the brewers of sake in Japan discovered their hands got fairer after blending the cooked rice with *Aspergillus sp*. by hand in lessening the fermentation heat to homogenize its humidity [3]. Kojic acid solidifies within a variety of colourless, radiant prismatic needles in a vacuum with no changes. The chemical structure of kojic acid is shown in Figure 1.

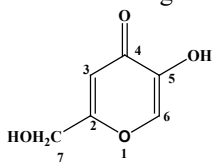


Figure 1. The structure of kojic acid.

Kojic acid essentially works as the primary material for the formulation of skin whitening creams, skin defensive moisturizers, whitening soap, and tooth care products. It also has the ability as the ultraviolet protector, whereby it smothers hyperpigmentation in human skin by controlling the development of melanin through the restraint of tyrosinase formation, the precursor enzyme for skin pigmentation [4]. Besides all the advantages of kojic acid, it, however, faces problems in commercial product applications. Kojic acids dissolve in water and slightly dissolve in oil. This solubility invariably had been a tangle in the cosmetic formulation [5]. Due to the problems, an improvement in the solubility would promote its utilization in diverse fields [6]. Thus, the esterification strategy of hydrophilic kojic acid with lipophilic molecules can be employed to alter its solubility in a hydrophobic medium.

In order to boost the lipophilic properties of kojic acid, esterification with unsaturated fat to produce kojic ester could be an appropriate and appealing technique. Because of that, it has now been discovered that kojic ester can create great impacts in brightening the skin. Besides, the esterification product has superb stability to pH, heat, and light, leading to excellent storability and oil solubility. This causes simple absorption properties into the skin once it is incorporated in various creams [7].

On the other hand, the use of dual enzymes system or consortium enzymes is now receiving much attention due to improved catalytic efficiency. Enzyme mixtures containing two sorts of lipases, both immobilized or a mix of immobilized with non-immobilized lipase, prompted an improvement in enzymatic action, particularly in a batch reactor. According to Andrea *et al.* [8], the higher enzymatic activity of the 1,3-specific lipase concerning the non-specific one must be related not solely to driving catalyst-reagents interactions favoured by the solvent, yet in addition to conceivable distinctive response systems. In 2007, Ibrahim et al. [9] showed that using both Lipozyme RM IM and

Novozym 435 at a specific ratio showed that the reliance of the interactive effects for the blending proportion of two lipases is experimentally repeatable. The fundamental explanation representing the above perceptions are not satisfactory. However, the results seem to recommend that the anergic interaction that existed among two immobilized lipases may relate to the carriers' character of the immobilized enzymes.

It is qualified to note that an essentially synergic impact on enzymatic reaction during the entire time course happened when an equivalent measure of Lipozym RM IM and Novozym 435 was utilized as biocatalyst. In examining the relating single enzyme system, the dual enzyme system indicated a quicker response yet accomplished a higher interesterification degree [9]. On the other hand, using single enzymes as previous work is not economical for industrial application. This is because the cost of enzymes is too expensive, and the use of dual enzymes might reduce the cost and make the process economical. Thus, this work gives a practical explanation of improving the reaction rate by using the dual enzyme system as a biocatalyst.

## II. THE MATERIAL AND METHOD

### *A. Study on The Reaction Synthesis of Producing Kojic Esters Via Lipase-Catalyzed Reaction*

This method was adapted from Kamaruddin *et al*. [10] with some modifications. The reaction system consists of 4.0 mmol of kojic acid, 1 mmol of fatty acids, 2 mL of acetonitrile, and 0.1 g of immobilized lipases (Novozym 435/Lipozyme RMIM at 1:1). They were placed in a screw-capped vial and then incubated in a water bath shaker at 40°C. The agitation speed is then set at 200 rpm and 4 hours reaction time. The reaction was then terminated by adding 7 mL of ethanol:acetone (7:3 v/v). After completion, the response was removed by separating the enzyme from the reaction mixtures. The unreacted kojic acid in the reaction mixture was then determined by titration with 0.01 M of NaOH using an automatic titrator. The lipase activity for each reaction was expressed as a relative percentage conversion of the kojic ester following Eq. (1).

$$\textit{The percentage yield of kojic ester (\%)} = (Vol\ NaOH\ without\ enzyme\ (mL) - Vol\ of\ NaOH\ with\ enzyme\ (mL))/(Vol\ of\ NaOH\ without\ enzyme\ (mL)) \times 100 \quad \text{(Eq. 1)}$$

*Optimization of Reaction Synthesis Via Conventional Study of Varying One-Parameter At-A-Time Approach*

Optimization of enzymatic synthesis of kojic ester will be carried out following the conventional method of varying one-parameter-at-a-time approach. The reaction is done in screw capped-vials using a horizontal water bath shaker. The shaker is also set up with a temperature and a shaking controller to control the reaction condition. Three reaction parameters were evaluated, reaction time, reaction temperature, and amount of enzymes. Table I shows the selected variable parameters for this study.

TABLE I
SELECTED VARIABLE PARAMETERS

| **Parameters** | **Variables** |
|---|---|
| Effect of Reaction Time | 4; 8; 12; 16; 20; 24; 32 and 48 hours. |
| Effect of Reaction Temperature | 40, 50, 60, 70, and 80°C |
| Effect of Amount of Enzyme | 0.1, 0.2, 0.3, 0.4, and 0.5g |

### *B. Reusability of Enzyme in Screw-Capped Vial*

The reaction system consists of 4.0 mmol of kojic acid, 1 mmol of fatty acids, 2 mL of acetonitrile, and 0.2 g of immobilized lipases (Novozym 435/Lipozyme RMIM). They are placed in a screw-capped vial and then incubate in a water bath shaker at 50°C. The agitation speed is then set at 200 rpm and for 8 hours reaction time. The reaction mixture was extracted from the enzyme after each cycle, and the ester yield was determined according to the method described earlier. Then, the used enzyme was rinsed with an excess of hexane and filtered over Whatman paper. The solvent was evaporated under a fume hood until dried before being placed in a screw-capped vial containing fresh substrate. The percentage conversion of kojic acid monooleate (%) was calculated, as mentioned earlier.

## III. RESULTS AND DISCUSSION

### *A. Enzymatic Synthesis of Kojic Esters and Analysis*

Enzymatic synthesis of kojic acid monooleate (KAMO, $C_{24}H_{38}O_5$) was successfully completed by the esterification reaction between unsaturated fat (oleic acid) and alcohol substrate (kojic acid). This reaction was carried out using a dual enzyme system, consisting of Novozym 435 from Candida Antarctica and Lipozyme RMIM from Rhizomucor miehei in acetonitrile, an organic solvent. Khamaruddin et al. [11] expressed that acetonitrile is a significant solvent as ester's high yields may be well produced. The ester's great transformation in acetonitrile may be due to the substrates' excellent solubility, which would have increased the enzyme and substratum contact.

A new approach of a dual enzyme system was utilized in this study to enhance response performance by increasing the reaction rate. The mixture of a specific enzyme (lipozyme RMIM) and a non-specific enzyme (Novozym 435) was believed to promote higher synergist efficiency towards esterification reaction [12, 13]. This may be due to the synergistic effect between the combined enzymes that increases thermostability and delivers ester high rate conversion [9].

The synergist mechanism involves the reaction between oleic acid with kojic acid as an alcohol substrate [14]. The

unsaturated fatty acid used in this reaction acts as an acyl donor and consolidates with an acyl-enzyme intermediate enzyme. As a result of that formation, the structure is thermodynamically unstable and reacted with kojic acid as a nucleophile and an acyl acceptor that attacks this intermediate structure. Hence, the enzyme will be isolated from the product and become attached to acyl donor to form kojic acid monooleate. The esterification between kojic acid and fatty acid is shown in Figure 2.

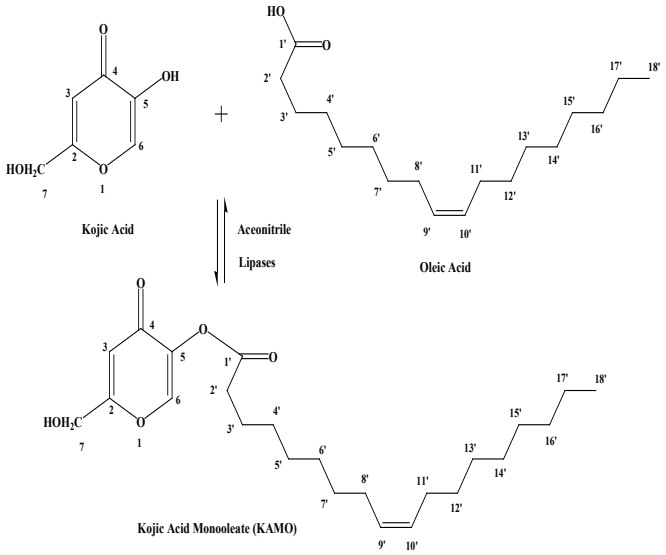


Figure 2. The synthesis of kojic monooleate ester (KAMO).

## B. *Effect of Reaction Time*

The reaction rate is a fundamental boundary for getting a decent yield inside a brief time frame and minimizing the cost, particularly in massive production. The reaction time range's determination must be exact; otherwise, the ideal state of synthesis may be located outside the experimental region [15]. Fig. 3 shows the effect of reaction time on the percentage yield of kojic ester using consortium enzymes. At various times, reactions were carried out to analyze the effects of reaction time on the esterification reaction. The product of kojic ester increased rapidly with the reaction time from 4 to 8 hours. The highest yield for KAMO was obtained at 8 hours, with 26.6%. After that, the percentage yield was reduced as the selectivity toward kojic ester was decreased. This is probably due to the equilibrium state being approached and the volume of water (by-product) produced by the reaction increased as the time reaction increase [16].

This can also decrease the substrate's solubility and reduce the yield percentage by creating a water-organic solvent immiscibility [17]. It is essential to remove the water during the esterification process to achieve a high product yield. In fact, the rise within the quantity of water in the reaction mixture at first moves the chemical equilibrium towards hydrolysis, causing the decrease of the ester yield as expected [18]. Water has many alternative and presumably contradictory roles in this system. To favour KAMO synthesis, water's thermodynamic activity has to be kept as minimal as possible. However, water plays an essential role in the three-dimensional protein structure, both in the solution and solid-state, as well as in the catalytic function [19]. As a nucleophile in the hydrolysis reaction, water may also be a competitive inhibitor, as stated in hexane dodecyl decanoate for lipase-catalyzed synthesis [19]. The optimum reaction time for this study was 8 hours, an improvement over 24 hours by Khamarudin et al. [11] and 12 hours by Mat Radzi et al. [17] for the production of KAMO. Thus, it is shown that conversion ester improved by using a consortium enzyme system compared to a single lipase system.

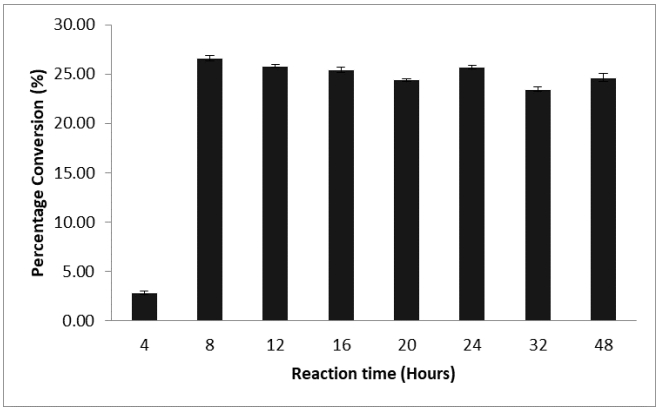


Fig. 3. Effect of reaction time on the enzymatic synthesis of kojic acid ester

## C. *Effect of Reaction Temperature*

Temperature is the most pertinent variable in any biological system. This is often significant for enzymatic processes. It is expected that temperature will have a substantial effect on enzyme activity and stability [20]. The temperature on the esterification reaction was examined at varying intervals: 40, 50, 60, 70, and 80. The effect of temperature on enzymatic activity comprised the decrease of the activation energy by increasing the protein structure's temperature and denaturation by heat [21]. Fig. 4 shows the effect of reaction temperature on the percentage yield of ester. Based on this figure, the product increased with increasing temperature until a maximum of 24.5% of KAMO at 50°C. This result shows an improvement compared to the previous study carried out by Khamarudin et al. [11].

However, when the reaction temperature was decreased beyond 50°C, the yield decreased as only 12.38% were obtained. As Mat Radzi et al. [17] reported, this decrease was probably because the reaction had proceeded beyond a critical temperature. An increase in temperature expands the pace of the chemical reaction being catalyzed while building the rate at which the enzyme is inactivated [21]. Increasing temperature causes an increase in the acid solubility and dissociation and decreases the binding equilibrium, driving unfavorable esterification reactions [22, 23]. This result may be attributed to the increase in the enzyme substrate's equilibrium dissociation constant (the strength of binding or affinity between the enzyme and substrate) [24] at higher temperatures, resulting in the lower conversion of the ester. Therefore, the elevation of temperature is unfavorable for the desired reaction's selectivity, where the optimum temperature was set at 40°C in this experiment. The lipase

was no longer active or deactivated and reduced the lipase's operational activity because of the movement and vibration of the enzyme molecule, which further influenced the lipase structure's bonding.

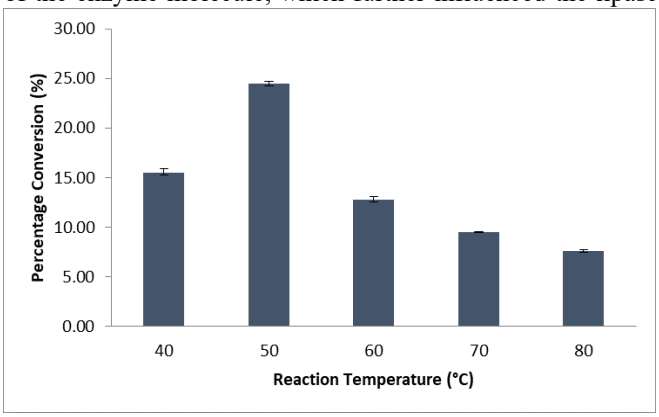


Fig. 3 Effect of reaction temperature on the enzymatic synthesis of kojic acid ester

### *D. Effect of the Amount of Enzyme*

The amount of enzyme is an important parameter that needs to be optimized to increase the KAMO yield. The application of lipases as biocatalyst for esterification is due to the high effectiveness and selectivity, low energy consumption (reaction requires mild conditions), and low waste amounts (more environmentally friendly) [25]. The experiments were conducted at various amounts of enzyme (0.1g, 0.2g, 0.3g, 0.4g, and 0.5g), as shown in Fig. 4. One can observe that kojic acid monooleate production was higher at a higher amount of enzyme used. This effect was substantial, and the percentage of kojic acid ester increased from 0.1g to 0.2g. When 0.3g amount of consortium enzyme was used, the yield of kojic acid ester decreased smoothly. The yield percentage keeps dropping at a range of 0.4g – 0.5g (7.70% - 7.43%). The ratio of kojic acid ester yielded was decreased by increasing the amount of enzyme, probably due to the steric hindrance produced by the excessive enzyme. Besides that, in enzymatic reaction, ester's production will decrease after achieving a 'critical point' whereby all substrates were combined with the enzyme. So, further addition of enzymes will not have a significant effect on the yield.

In fact, the total number of available active sites increased with an increased amount of enzyme as biocatalyst, which resulted in a higher conversion of kojic acid ester. The presence of a higher amount of enzymes gives more dynamic destinations to the acyl-enzyme complex formation and builds the likelihood of an enzyme-substrate collision and subsequent reaction [23, 26]. Besides, a few analysts found that increasing lipase dosage can improve the initial production rate and yield in limited quantities. If too much enzyme is used, particularly the immobilized enzyme could reduce the product yield. In 2009, Ashari et al. [7] stated that further addition of an enzyme might cause substrate limitation and the enzyme itself could also cause mass transfer limitation as all substrates are bound to the enzyme.

The enzyme molecules' active site would also not be presented to the substrate and remain inside the mass enzyme particles, contributing to the reaction rate's decrease [11]. This was apparently due to the rise in viscosity, which decreased the reaction rate to the degree that it was not aided by an additional amount of enzyme [27]. Since lipase costs contribute substantially to the ester's overall production cost, the enzyme's dosage should be reduced as much as possible. Taking the cost and the efficiency into consideration, the optimal condition for this reaction was chosen to be 0.2g of the enzyme at the reaction time of 8 hours.

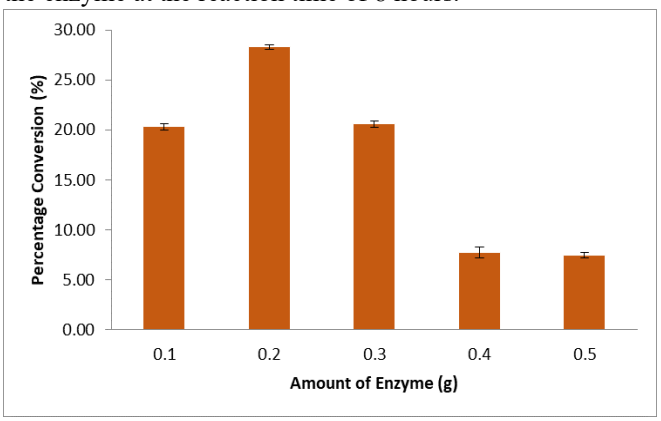


Fig. 4 The effect of enzyme amount on the enzymatic synthesis of kojic acid ester

### *E. Reusability of Enzyme*

In some instances, the enzyme's cost remains too high to justify its disposal when the synthesis is completed. Issues such as enzyme operational stability, recovery, and reuse are critical in assessing the process's commercial viability. Therefore, the enzyme's activity and stability can only be optimized with a proper study of reaction parameters.

Several workers examined the ability of immobilized lipase to retain its synthetic activity during recycling. In this work, the retention of consortium enzyme activity after repeatedly used in small scale reaction was assessed in terms of % conversion of kojic acid ester at the end of the cycle. Fig. 5 demonstrates the reusability of consortium enzyme (Novozym 435 and Lipozyme RMIM) on the enzymatic esterification reaction using screw-capped vials. The enzymes retain a high activity of more than 25% even after five uses. Enzyme immobilization and low water content can be related to stability. Water content plays an essential role in enzyme stability, as water is responsible for internal structural flexibility and inactivation caused by heat. However, the percentage conversion was decreased from 19% to 10% at ten cycles. Even after nine uses, the enzyme activity remained high, which could be contributed by a lower shear effect to the enzyme molecules. The substrate was reacted in the vial in a small-scale reaction, which was incubated using a horizontal water bath shaker. There were no direct mechanical forces like agitator that may affect the rupturing of the enzyme particle.

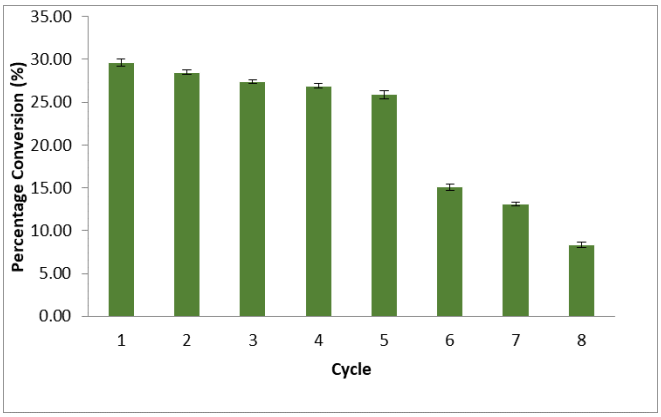


Fig. 4 Reusability of enzyme

## IV. CONCLUSION

Kojic ester, specifically kojic acid monooleate (KAMO), was successfully synthesized via an enzymatic esterification reaction between oleic acid and kojic acid reactant in the presence of dual enzyme systems, which are Novozym 435 and Lipozyme RMIM at the ideal conditions. Compared to the single lipase system, the dual enzyme system used in the research showed a greater degree of enzymatic esterification. This study used a traditional analysis of one parameter in the synthesizing reaction at a time approach. Three parameters have been optimized, including reaction time, temperature, and enzyme quantity. The optimum conditions for synthesizing KAMO were reaction time at 8 hours, temperature 50°C, and amount of enzyme 0.2 g (1:1 ratio of Novozym 435 to Lipozyme RMIM). Novozym 435 and Lipozyme RMIM were also stable in heat and organic solvent, where the activity was maintained at >25% up to 5 cycles.

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