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Evaluation of Direct Esterification of Fatty Acid Derivative of Kojic Acid in Co-solvent System: A Statistical Approach

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Abstract

The present work deals with direct esterification method to synthesize Kojic acid using immobilized lipase as a biocatalyst in acetonitrile with the addition of dimethylsulfoxide (DMSO) as a solubilizing agent Co-solvent respectively. To increase the esterification yield of KMO, modifications of the process were evaluated, including the use of a co-solvent and the use of Novozyme 435 (*Candida antarctica*) as a catalyst. The KMO synthesis has been developed and optimized by using Response Surface Methodology (RSM) with Central Composite Rotatable Design (CCRD). The optimized condition of enzyme was 3.35 wt% and 1:3.64 molar ratio of kojic acid and oleic acid at 82.39°C for 255.24 min of reaction. With these condition, the maximum percentage yield was 42.73% with R2 value of 0.866914 and indicated that 86.69% of the variability in the response could be explained by the model. The model was significant and fitted well with the experimental data and the lack of fit was not significant. The efficacy for cosmetic application was successfully tested and showed as non-irritating with a Human Irritancy Equivalent score between 0.55-0.83 which safe to be applied in cosmetic ingredient.

Keywords: Kojic acid; Kojic ester; Esterification; Co-solvent; Response surface methodology

Introduction

Kojic acid (5-hydroxy-2-(hydroxymethyl)-1, 4-pyrone), is a production of fungal metabolite from many species of *Aspergillus*, *Acetobacter,* and *Penicilium* [1]. Latterly, the whitening agent in this acid benefits as an essential ingredient in cosmetic cream to block the formation of pigments by the deep cells on the skin [2]. It was studied intensively by several researches due to its potential as a tyrosinase inhibitor to prevent browning in food production as well as a skin lightening agent for cosmetic preparations [3-5]. In Asia, hyroquinone, a well-known whitening agent, was banned for cosmetic usage for its toxicity. Therefore, the cosmetic industry likely opts for kojic acid as an alternative for skin whitening and for its antioxidant properties [6].

However, exposure of sun and air to this compound makes it unstable and reduces its effectiveness. Therefore, to overcome this situation, kojic acid must be modified to improve its properties in terms of storage stability, compatibility, and oil-solubility. Previous studies reported that modifying the esterification method with fatty acid is the best technique to synthesize kojic acid ester [7]. The derivatives have more excellent characteristics than the original starting material, making it more profitable, especially for cosmetic applications. Besides that, solid-phase synthesis of kojic acid-tripeptides and their tyrosinase inhibitory activity, storage stability, and toxicity, concluded that these derivatives were almost 15 times more stable under ambient stored conditions with a lower toxicity than kojic acid itself. Kojic acid derivatives were relatively higher with tyrosinase inhibitory activities than that of kojic acid [4]. Further evidence claimed kojic acid derivatives are major class of many natural and synthetic compounds that own a high activity profile because of their wide range of biological activities [8].

A skin irritancy test and corrosivity potential of an ingredient is necessary for safely assessing cosmetic ingredients. Skin Irritant Assay is one of the methods for assessing chemical irritancy in a product. This assay is a standardized and quantitative *in vitro* test, which works to detect, rank, and predict the dermal irritation potential of product [9]. For the evaluation of skin irritation, physicochemical properties pertinent information that needs to be considered in the assessment. Therefore, the properties of the physicochemical of kojic ester are an important aspect to evaluate their efficacy in the cosmetics field since it is a relatively new compound and research on it is limited.

These esters can be synthesized through chemical or enzyme catalysis. Chemical catalysis might be more economical compared to enzyme catalysis, but this will lead into a complex mixture, thus making it more difficult to purify [10]. Studies from Kobayashi et al. focused on the amino acid derivatives of kojic acid, which showed that the derivatives have stronger activities than kojic acid [11]. In their current study, they attempted to produce a new compound with much stronger inhibitory potency against tyrosinase than that of N-carbobenzoxy-amino acid-kojiate. The synthesis of kojic acid derivatives was performed using DSC (N,N'-disuccinimidyl carbonate) and DMP (4-dimethylaminopyridine). Therefore, ten compounds were obtained by joining the OH group at position C7 of kojic acid with the amino end of an amino acid to form a urethane type bond [11]. This shows that it can be difficult to esterify when a chemical catalyst is used rather than using enzymatic-catalyzed synthesis. Additionally, the high temperature can cause degradation of esters and undesired products, resulting in a high energy cost and low percentage yield [12].

Conversely, enzyme catalysis since is more profitable it requires less amounts of energy, minimum thermal degradation, higher biodegradability, needs a simple step process and therefore, emerges promising substitutes over conventional approaches. Moreover, certain

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aspects such as higher yield, milder reaction conditions, and substrate selectivity can be strong points in favor of biocatalysis production [13]. Raku and Tokiwa, 2003, proposed the regioselective esterification of kojic acid esters using *Bacillus subtilis* protease. They reported that the primary hydroxyl group at the C7 position of kojic acid was easily esterified using a simple one step procedure therefore, giving a higher conversion yield of kojic acid esters. Furthermore, the esters obtained in their study were effective as radical scavenging and improved lipophilicity. It was also indicated that there was no significant change in the biodegradability of kojic acid esters and it can be classified as an environmentally friendly product [14]. In addition, Liu and Shaw improved the lipophilicity of kojic acid by synthesizing lauric and oleic acid esters of kojic acid using nine commercial lipases that were tested [15]. Among all nine lipases, *P. cepacia* and *P. camembertii* showed the best catalytic efficiency and specificity for enzymatic synthesis of kojic acid monolaurate (KAML) and kojic acid monooleate (KAMO). The results show that KAML and KAMO were easily esterified at the hydroxyl group in the C5 position of kojic acid [15].

In enzymatic synthesis, optimization of a reaction is a crucial step in developing an efficient reaction system. The conventional (parameter-at-one-time) method is highly cost and time consuming [16]. For those reasons, optimizing the enzymatic reaction will give the best stage to overcome the lacking of the conventional method. Optimization reaction conditions can be done using Response Surface Methodology (RSM). RSM is defined to be a set of mathematical and statistical methods using a minimal number of experiments for designing experiments, building models, evaluating the effect of variables, and obtaining the optimum condition. The number of experimental trials needed to analyze the interaction between multiple variables can be reduced and thus, save costs and time [7]. This method is more desirable for industrial applications.

The novelty of this research is the usage of Novozym 435 as an enzyme in solvent mixture (acetonitrile and dimethylsulfoxide) for synthesis of kojic ester. The aim of this study is to synthesize, characterize and optimize the reaction condition of kojic ester catalyzed by Novozym 435 in a solvent system. The relationship of reaction variables: enzyme amount, reaction temperature, reaction time, substrate molar ratio and percentage yield, being studied by using Response Surface Methodology (RSM).

Materials and Methods

Materials

Kojic acid (purity 98%) was donated by Tokyo Kasei Kogya Co, Ltd. (Japan). While oleic acid (purity 99%) was purchased from Southern Edible Oil Sdn. Bhd., Selangor, Malaysia. Glycerol tributyrate was used as an internal standard in gas c analysis and was obtained from Sigma-Aldrich (USA). Novozym 435, *Candida antarctica* lipase B immobilized on a macroporous acrylic resin (10,000 propyl laurate units per gram) was purchased from Novo Nordisk A/S (Denmark).

Lipase catalyzed esterification

The esterification was performed by dissolving kojic acid and oleic acid into a solvent mixture of acetonitrile and dimethyl sulfoxide (DMSO) in a 150 ml beaker. The enzyme was added to the mixture and was sealed using aluminium foil. Using a magnetic stirrer, the mixture was stirred at 700 rpm at 80°C for about 360 min. At the end of the reaction, the enzyme was filtered using filter paper (Munktell, 1F) and a rotary evaporator was used to remove undesired solvent. Figure 1 shows the enzymatic synthesis of kojic ester in a solvent system.

Analysis of sample

Isolation and purification of Kojic ester: The reaction was terminated by separating the enzyme from mixture by filtration using filter paper. Then, the solvent was removed by rotary evaporator. Preliminary detection were subjected to column chromatography contain silica gel (Silicycle, 60-200 μm particle size). The sample was eluted using a mixture of hexane and ethyl acetate (75:30, vol/vol) and collected in every 2 ml of flow volume. The purification of product was confirmed by Thin Layer Chromatography (TLC) on pre-coated silica gel plate (Merck, 5 × 10 cm) and developed in hexane:ethyl acetate (75:30, vol/vol).

Characterization: The sample of product was put on the TLC plate using the tip and diluted in a mobile phase which is the mixture of hexane and ethyl acetate (75:30 vol/vol). Then, the TLC plate sheet was dip into the $KMNO_4$ solution and dry to make it clearer to see the spotted obtained.

Experimental design

A central composite rotatable design (CCRD) was employed. The study requires 30 experiments of reactions undergone at various reaction temperatures (70-90°C), reaction time (120-360 min), enzyme amount (1.0-5.0 wt%), and substrate molar ratio (1-5 mmole) for kojic acid and oleic acid. The enzymatic synthesis of kojic ester was performed by dissolving kojic acid with oleic acid in a solvent mixture of acetonitrile and dimethyl sulfoxide (DMSO) in a 150 ml beaker. The enzyme was added to the mixture and was sealed using aluminium foil. The mixture was stirred using a magnetic stirrer at 700 rpm at 80°C for about 360 min. From previous work which showed that an immobilized enzyme like Novozyme 435 has high activities and is stable at a high temperature [17]. Table 1 shows the variables in terms of coded and actual values. The R^2 value obtained indicates that the experiment was significant with the model.

Skin irritancy test

An irritancy test of kojic ester was evaluated to determine its potential to cause dermal irritation. Dermal irritancy was conducted at Malaysian Palm Oil Berhad (MPOB) using the *in vitro* International's Irritection Assay System. Four different amounts (50, 75, 100 and 125 mg) of samples were weighed and placed onto a synthetic membrane disc biobarrier. Then, the protein reagent consisting of oligomeric protein, glycoprotein, lipid constituents and globulins plus blanking buffer were added to the 24-well assay plate. The synthetic disc, which

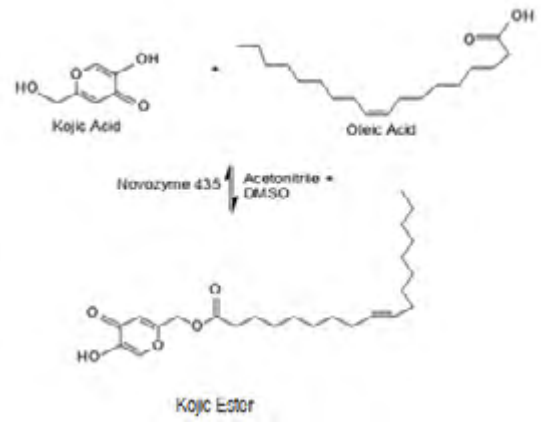

Figure 1: Enzymatic synthesis of Kojic ester in solvent system.

contained various amounts of kojic ester samples, was inserted into the blank and test sample wells of the plate. The assay plate was then incubated at 25°C for 24 h. The optical density (OD) of the protein reagent and blanking buffer were then recorded at 450 nm using an MRX plate reader. The irritancy potential of a test sample was expressed as the Human Irritancy Equivalent (HIE) score. This score is determined by comparing the changes in optical density (OD470) produced by the test material to a standard curve, which is constructed by measuring the increase in OD470 produced by a set of calibration substances. Table 2 shows the relationship of HIE score to the irritancy classification for the Dermal Irritation Test method.

Results and Discussion

Model fitting and Analysis of Variance (ANOVA)

The CCRD design was used in order to achieve a preferable model for the optimization of kojic ester synthesis in solvent system. Table 3 shows the experimental data and the percentage yield obtained.

Fitting of the data to the various models (linear, two factorial interactions quadratic and cubic) and their subsequent ANOVA shows that the reaction of kojic acid and oleic acid in a solvent system is most suitable described with a quadratic polynomial model. The final equation in terms of coded factors is shown in Equation (1):

$$\text{Actual Yield (\%)} = +42.77 + 1.59A + 0.87B + 0.12C + 3.18D + 0.19AB + 291AC + 6.67AD + 4.31BC + 3.58BD + 5.61CD - 7.26A^2 - 9.36B^2 - 9.00C^2 - 7.16D^2 \quad (1)$$

Where A is the reaction temperature, B is the reaction time, C is the enzyme amount, and D is the substrate molar ratio. The positive sign in front of the terms indicates a synergetic effect whereas the negative sign indicates an antagonistic effect. From the ANOVA shown in Table 4, the model of P-values was significant and lack-of-fit with prob>P less than 0.05 values, of which there was only a 0.01% chance that model P-values this large could occur due to noise. Generally, the lack-of-fit test should not significant. Variables in Table 4, state that A, B and C are referred to as the main linear term effects, while AB, AC, AD, BC, BD and CD are the interaction terms and A^2, B^2, C^2, D^2 are the quadratic terms in the responses. In this study, the main linear term (D) is significant ($p<0.05$) whereas interaction term (AD), (CD) and (BC) is significant ($p<0.05$) while for quadratic term A^2, B^2, C^2, D^2 are all significant ($p<0.05$).

According to Bezerra et al. a not significant lack-of-fit indicates the model is good because the model was fitted well to the experimental data [18]. Based on the coefficient value (R^2), the fit between the development model and experimental data can be determined. According to Keng et al. R^2 value >0.900 indicates that the model describes the situation well and indicates that the model has a very high correlation [19]. In this case, the R^2 was equal to 0.866914, and this coefficient value indicated that 86.69% of the variability in the response could be explained by the model. Therefore, the model was preferable to represent the real relationship among the parameters studied.

Effect of reaction parameters

By studying the three-dimensional (3D) response surface plot, the relationship between reaction parameter and responses as well as the optimum level of each variable for production of kojic ester can be determined. Figure 2A illustrates the response surface plots for the interaction between reaction time (B) and reaction temperature (A) with fixed 3.00 wt% of enzyme amount and 1: 3.00 mole substrate molar ratio (KA : OA). According to the Figure 2, the highest yield (43.6%) was obtained at 240 minutes of reaction time and 80°C of reaction temperature. At the beginning, it was seen that when the reaction time increases, the percentage yield also increases. Then, a lower percentage yield is obtained when the reaction time goes further to 250 minutes. Therefore, this result shows that by prolonging the time of reaction (>250) will not increase the production of kojic ester because the esterification reaction will produce water as a side product. The yield of kojic ester was increased by increasing the temperature up to 80°C. When the temperature was raised up to 90°C, the yield becomes decreased which was probably beyond the critical temperature (>90°C), the lipase may have undergone some inactivation and become denatured. These results were similar with the reviewed paper by using the same lipase, which is from *Candida antarctica* (Novozym 435) as optimally used at a temperature of 83°C [19]. Figure 2B represents the effect of the enzyme amount and reaction temperature in the range of 2.00 - 4.00 wt% and 75-85°C by fixing the reaction time 240 minutes and 1 : 3.00 mmole (KA:OA) substrate molar ratio. From the Figure 2C the enzyme amount of 3.00 wt% and a moderate temperature at 80°C appeared to be a favorable condition for the esterification reaction. This phenomenon is related to the active site of enzyme molecules, where the large excess of enzyme cannot be exposed to the substrates due to possible protein aggregation and hence, reducing the conversion of yield [20]. The esterification activity becomes slow as temperature increases more than 80°C at a high amount of enzymes due to the inactivation of the enzyme at a high temperature (Figure 2D). This was proven by Khamarudin et al. in a conventional study of temperature effect on the synthesis of palm-based kojic acid ester [21].

In conclusion, the reaction was not favorable in the highest amount of enzyme. This was proven by Ashari et al. because as the amount of enzyme increases, the production yield decreases and when time increases, the conversion yield also increases [7]. At a certain time, a small decrease of percentage yield was seen as the reaction time goes

Table 1: Range of variables and their levels for the CCRD.

Variable	Level				
	-2	-1	0	+1	+2
Reaction temperature, A (°C)	70	75	80	85	90
Reaction time, B (min)	120	180	240	300	360
Enzyme amount, C (wt %)	1	2	3	4	5
Substrates molar ratio (kojic acid : oleic acid), D (mmole)	1	2	3	4	5

Table 2: Relationship of Human Irritation Equivalent (HIE) Score to Irritancy Classification for the Dermal Irritection Test Method.

Human Irritation Equivalent (HIE)	Predicted Dermal Irritancy Classification
0.00-0.90	Non-irritant
0.91-1.20	Non-irritant/irritant
1.21-5.00	Irritant

Table 3: Central composite rotatable design of kojic ester.

un	A: Reaction Temperature (°C)	B: Reaction Time (min)	C: Enzyme Amount (wt%)	D: Substrate Molar Ratio (mmole)	Actual Yield (%)
1	80 (0)	240 (0)	3 (0)	3 (0)	30.3
2	80 (0)	240 (0)	3 (0)	3 (0)	39.36
3	75 (+1)	180 (+1)	2 (+1)	2 (+1)	28.55
4	75 (+1)	300 (-1)	2 (+1)	2 (+1)	18.4
5	90 (-2)	240 (0)	3 (0)	3 (0)	20.25
7	80 (0)	240 (0)	3 (0)	3 (0)	34.09
8	75 (+1)	180 (+1)	4 (-1)	2 (+1)	8.88
9	75 (+1)	300 (-1)	4 (-1)	2 (+1)	0.08
10	75 (+1)	300 (-1)	4 (-1)	4 (-1)	26.68
11	80 (0)	240 (0)	3 (0)	3 (0)	48.44
12	75 (+1)	300 (-1)	2 (+1)	4 (-1)	2.97
13	85 (-1)	180 (+1)	4 (-1)	4 (-1)	15.91
14	85 (-1)	300 (-1)	4 (-1)	4 (-1)	42.72
15	80 (0)	240 (0)	1 (+2)	3 (0)	7.25
16	85 (-1)	180 (+1)	2 (+1)	4 (-1)	22.02
17	75 (+1)	180 (+1)	2 (+1)	4 (-1)	2.32
18	85 (-1)	300 (-1)	2 (+1)	4 (-1)	6.43
19	80 (0)	240 (0)	5 (-2)	3 (0)	1.48
20	80 (0)	240 (0)	3 (0)	3 (0)	55.98
21	85 (-1)	180 (+1)	2 (+1)	2 (+1)	0.87
22	70 (+2)	240 (0)	3 (0)	3 (0)	2.34
24	80 (0)	240 (0)	3 (0)	1 (+2)	7.67
25	80 (0)	240 (0)	3 (0)	5 (-2)	15.78
26	85 (-1)	300 (-1)	4 (-1)	2 (+1)	0.82
27	80 (0)	240 (0)	3 (0)	3 (0)	48.44
28	80 (0)	120 (+2)	3 (0)	3 (0)	2.32
29	80 (0)	360 (-1)	3 (0)	3 (0)	3.49
30	85 (-1)	300 (-1)	2 (+1)	2 (+1)	0.81

Table 4: ANOVA for the quadratic model developed for synthesis of kojic ester.

Source	Sum of Squares	df	Mean Square	p-value
Model	7459.508	14	532.822	0.0003
A-Reaction temp	60.5155	1	60.5155	0.3874
B-Reaction time	18.2876	1	18.2876	0.6316
C-Enzyme amount	0.357704	1	0.357704	0.9463
D-Substrate molar ratio	242.3797	1	242.3797	0.0950
AB	0.566256	1	0.566256	0.9325
AC	135.1988	1	135.1988	0.2031
AD	712.4896	1	712.4896	0.0080
BC	296.7868	1	296.7868	0.0674
BD	205.4206	1	205.4206	0.1217
CD	504.3393	1	504.3393	0.0213
A^2	1447.147	1	1447.147	0.0006
B^2	2403.595	1	2403.595	< 0.0001
C^2	2219.812	1	2219.812	< 0.0001
D^2	1404.629	1	1404.629	0.0006
Residual	1145.164	15	76.34429	
Lack of Fit	663.891	10	66.3891	0.7119
Pure Error	481.2733	5	96.25466	
Cor Total	8604.672	29		

further [7]. According to Virto et al. and Ismail et al. by prolonging the reaction time, the synthesis of water and concentration of products increases gradually, this promoted the reverse esterification. Therefore, the percentage yield of the product may reduce [22,23].

Optimum conditions

The highest percentage yield was obtained from the optimal combination of parameters by response surface methodology. The predicted highest yield of 44.30% at reaction conditions of 82.39°C,

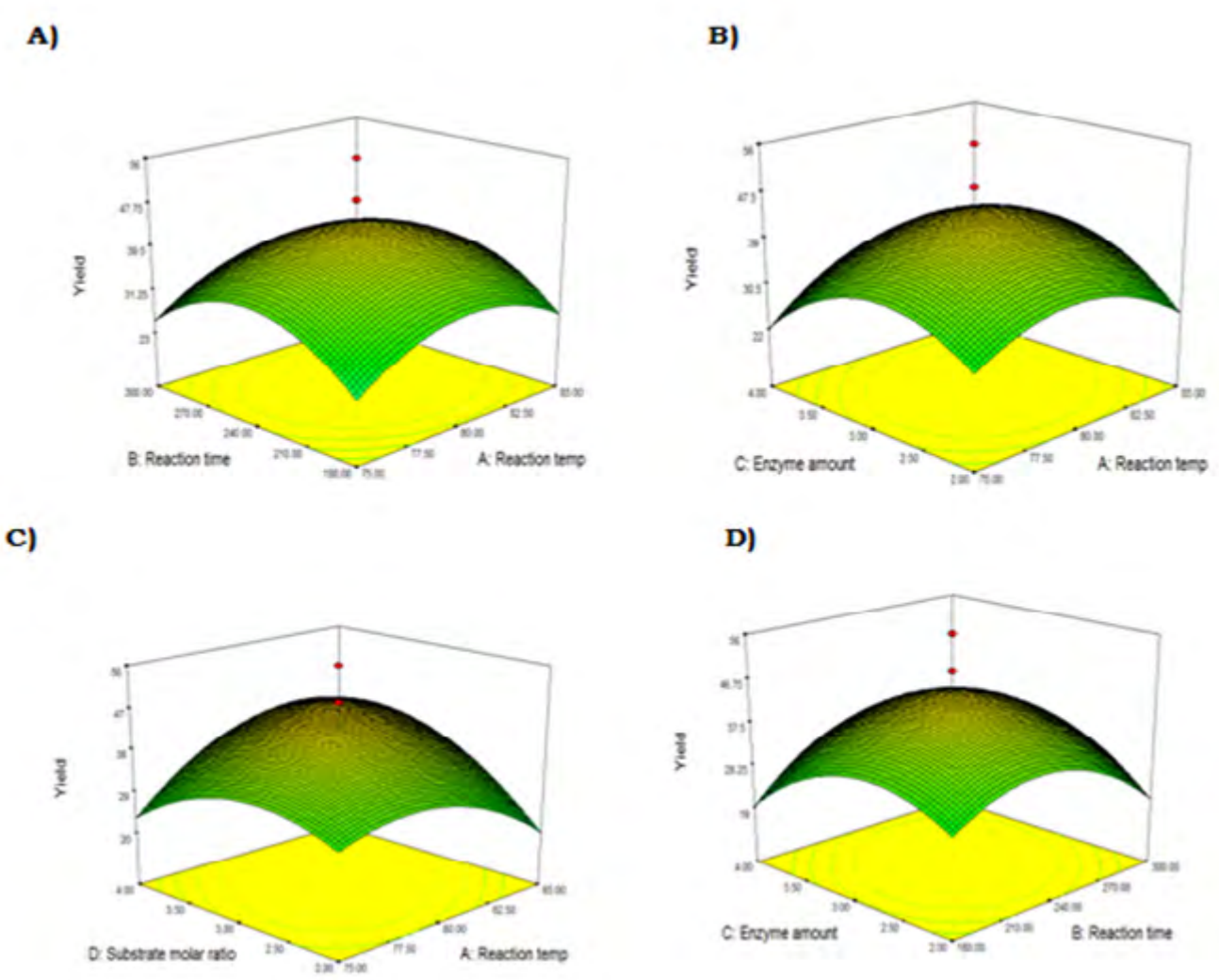

Figure 2: Three-dimentional response surface plot. (A) reaction time (min) versus reaction temperature (°C), (B) enzyme amount (%wt) versus reaction temperature (°C), (C) substrate molar ratio (KA:OA) (mmole) versus reaction temperature (°C) and (D) enzyme amount (%wt) versus reaction time (min) on percentage yield as response.

255.24 min, 3.35 wt% of enzyme and molar ratio of substrate of 1: 3.64 was predicted by using the optimized function of the Design Expert software. As shown in Table 5, the actual experimental value collected was 42.73% with a small difference of 1.57. Hence, these results support the validity of the quadratic model and response surface methodology can be practiced adequately to optimize the lipase-catalyzed synthesis of kojic ester.

Skin irritancy test

A safety evaluation of an ingredient is essential for consumer safety protection. For a novelty ingredient, the toxicity of the ingredients used is of concern. This may give rise to restrictions on their cosmetic applications if the toxicity is highly detrimental and could be eliminated or stopped from further development. In order to assess the safety of cosmetic ingredients, a tiered-approach was suggested [24]. The efficacy of kojic ester in cosmetic formulation was investigated with the use of Irritection Assay System. Table 6 indicates the results of *in vitro* dermal irritancy assay for four samples of kojic ester.

Based on the results, all samples had Human Irritancy Equivalent (HIE) scores of between 0.55-0.83, which is below the non-irritant level. Therefore, the skin compatibility of kojic ester is safe for cosmetic formulation. Previous studies also employed similar dermal irritection Assay methods to test the irritation potential of engkabang wax ester, palm oil esters and dihydroxystearic acid (DHSA) synthesis, respectively [9,18,25]. All new compounds tested were predicted to be non-irritant with the highest HIE scores of 0.31, 0.18, and 0.90, respectively.

Conclusions

Kojic ester was synthesized by enzymatic esterification using Novozym 435 to speed up the reaction and obtained excellent conversion of yield. Optimization was successfully predicted by Response Surface Methodology (RSM) and well-fitting models were established through the quadratic polynomial model. The optimal conditions obtained were 3.35 wt% of enzyme and 1:3.64 molar ratios of kojic acid and oleic acid at 82.39°C for 255.24 min of reaction. The

Table5: Optimum condition for Novozym 435 catalysed synthesis of kojic ester.

Exp	Reaction Temperature (°C)	Reaction Time (min)	Enzyme Amount (wt%)	Substrate Molar Ratio (KA:OA) (mmole)	Yield (%) Actual
1	82.39	255.24	3.35	01:03.6	42.73

Table 6: Irritancy Test of Kojic Ester.

Dose (mg)	Irritancy Score (HIE)	Classifiation
50	0.55	Non-irritant
75	0.63	Non-irritant
100	0.52	Non-irritant
125	0.83	Non-irritant

actual yield obtained was 42.73%. The efficacy for cosmetic application was successfully tested and showed as non-irritating with a Human Irritancy Equivalent score between 0.55-0.83 which safe to be applied in cosmetic ingredient.

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