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Enzymatic Synthesis of Olive-Based Ferulate Esters: Optimization by Response Surface Methodology

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***Abstract*—**Ferulic acid has widespread industrial potential by virtue of its antioxidant properties. However, it is partially soluble in aqueous media, limiting their usefulness in oil-based processes in food, cosmetic, pharmaceutical, and material industry. Therefore, modification of ferulic acid should be made by producing of more lipophilic derivatives. In this study, a preliminary investigation of lipase-catalyzed trans-esterification reaction of ethyl ferulate and olive oil was investigated. The reaction was catalyzed by immobilized lipase from *Candida antarctica* (Novozym 435), to produce ferulate ester, a sunscreen agent. A statistical approach of Response surface methodology (RSM) was used to evaluate the interactive effects of reaction temperature (40-80°C), reaction time (4-12 hours), and amount of enzyme (0.1-0.5 g). The optimum conditions derived via RSM were reaction temperature 60°C, reaction time 2.34 hours, and amount of enzyme 0.3 g. The actual experimental yield was 59.6% ferulate ester under optimum condition, which compared well to the maximum predicted value of 58.0%.

***Keywords*—**Ferulic acid, Enzymatic Synthesis, Esters, RSM.

I. Introduction

SUN protection or sunscreen has become a top interest since it has been applied by millions daily to protect them from ultraviolet (UV) radiation of the sun [1]. A wide variety of topical sunscreen products flooding our market ranging from moisturizing day creams, hair-care products, lipsticks, aftershaves and hand creams that now carry SPF values [2]. These sunscreen products incorporate with different chemicals that have high UV-light-absorbing properties, which are commonly known as UV filters.

Based on their nature, UV filters can be classified into two groups; inorganic/physical UV filter (reflect and scatter the UV radiation) and organic/chemical UV filter (absorb the light) [3]. Chief innovations nowadays not only focus on UV filters which can filter or block the UV radiation, but also have antioxidants properties to prevent or counteract undesirable effects of the radiation on skin cells [4]. Among many available natural photo-protective agents, ferulate esters have aroused great interest due to its strong antioxidant activity with unique characteristics such as skin lightener, skin anti-wrinkling agent and strong UV absorptive ability [5]-[7]. Ferulate esters are derivative of ferulic acid (4-hydroxy-3-methoxycinnamate) that is widely distributed in plant species but predominantly in the Gramineae family such as wheat, rice, maize, sorghum, barley and oats with 84% frequency of occurrence [8].

Preparation of ferulate esters from a diverse range of raw materials through various synthetic routes has been well researched. Ferulate esters have been produced since decades ago via enzymatic esterification by introducing of ferulic acid to alcohols [9]-[13]. However, direct esterification of ferulic acid can badly affect the catalytic activity of enzymes and possess low stability in various solvent systems [14]. Nowadays, more interests were given to produce ferulate esters by enzymatic trans esterification between ethyl ferulate and triacylglycerols (TAGs) [15]-[18].

Olive oil, in their natural form possesses constituents that function as natural antioxidants. Amongst them are ascorbic acids, α-tocopherol, β-carotene, chlorogenic acids, hydroxytyrosol, flavanols and secoiridoids [19], [20]. Moreover, olive oil has been reported to be an effective option in treating various inflammations including xerosis (dry skin), pruritus (itchiness), seborrhea (dandruff), rosacea (skin sores), eczema or dermatitis (rashes), burns and other cutaneous damage [21].

In enzymatic synthesis, optimization of reaction process is very important. The conventional optimization method of one-factor-at-a-time approach is inefficient as it fails to understand the interplay among the reaction parameters (reaction time, temperature and amount of enzyme) and the response (% yield). Nowadays, a statistical method of Response surface methodology (RSM), receives much attention due to it applications in solving multivariate equations [22]. Instead of requiring limited number of experiments, RSM also offers a mathematical model of overall process which is advantageous over conventional study. This in turn reduces the time and cost of production [23].

Therefore, RSM comprising a five-level-three-factor Central composite rotatable design (CCRD) was applied in this study to evaluate the interactive effects among parameters and to obtain the optimum conditions for the reaction synthesis. The parameters studies were selected based on the previous conventional study. Combination between cosmetic functionality and antioxidant activity of ferulate esters synthesized was believed to develop a multifunctional range of ingredients.

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II. Materials and Methodology

A. Materials

Substrates (ethyl ferulate and olive oil) were obtained from Sigma-Aldrich (St. Louis, USA); chemicals (toluene, ethanol, acetone and potassium hydroxide) were purchased from Merck, Germany. Commercial lipases of Novozym 435 (immobilized lipase B from *Candida antarctica*) was procured from Sigma-Aldrich (St. Louis, USA). All chemicals were commercially available and of analytical grade unless otherwise specified.

B. Experimental Design

A five-level-three-factor CCRD was employed in this study, requiring 15 experiments. The fractional factorial design consisted of 4 factorial points, 6 axial points and 5 center points. The variables and their levels selected for ferulate esters synthesis were: temperature (40-80 °C); time (4-12 h) and amount of enzyme (01-0.5 g). The data obtained were fitted to a second-order polynomial:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32} + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y is percentage of yield; 0 is intercept, 1, 2, and 3 are linear coefficients; 11, 22, and 33 are squared coefficients; 12, 13, and 23 are interaction coefficients.

C. Synthesis and Analysis

The transesterification method was modified from Compton et al. [24]. The reaction system consists of 4 g olive oil, 1 g ethyl ferulate and different amount of Novozym 435. They were placed in a screw-capped vial and then incubated in a controlled water-bath shaker at different reaction temperature and reaction time at 200 rpm. The reaction was terminated with 7 mL of ethanol: acetone (7:3 v/v). The percent conversion (%) of ferulate esters were measured by determining the remaining unreacted fatty acids in the reaction mixture by titration with 0.3 M KOH in an automatic tittrator (Methrom, Switzerland). All the samples were assayed in triplicate and the experiment was repeated twice.

$$\text{Conversion of FE (\%)} = \frac{\text{Vol of KOH (control)} - \text{Vol of KOH (sample)}}{\text{Vol of KOH (control)}} \times 100\% \quad (2)$$

D. Data Analysis

The data from the experiments performed were analyzed using Design expert version 7.1 and then interpreted. Three main analytical steps: analysis of variance (ANOVA), a regression analysis and the plotting of response surface were performed to establish an optimum condition for the trans esterification.

III. Results and Discussion

A. Model Fitting and ANOVA

Design matrix of the ferulate esters synthesis with the predicted value is shown in Table I. The predicted values were obtained from model fitting techniques using the software (design expert version 7.1) and were seen to be almost correlated to the observed values. Of the total conditions, standard 7 (temperature 60°C, reaction time 2.34 h, and enzyme amount 0.30 g) resulted in the greatest ferulate esters conversion (59.58%). Fitting of the data to the various models (linear, two factorial, quadratic, and cubic) and their subsequent ANOVA showed that, synthesis of ferulate esters was suitably described with quadratic polynomial model. The quadratic polynomial is given below:

$$\text{Yield (\%)} = 34.41 - 12.82A - 18.12B + 5.60C - 2.68A^2 - 1.02B^2 - 6.12C^2 + 6.96AB - 25.51AC - 6.88BC \quad (3)$$

where A is the temperature; B is time; and C is amount of enzyme.

The P-values were used as a tool to check the significance of each coefficient, which also indicated the interaction strength between each parameter. The smaller the P-values are, the bigger the significance of the corresponding coefficient should be [24]. Here, the P-value of the model was 0.0002, which indicated that the model was statically significant and suitable for use in this experiment. Table II shows the regression coefficients and the corresponding P-values. In this case A, B, C, AB, AC, BC, A^2 and C^2 were the most important factors that represented a statically significant model terms on the ferulate esters conversion. The coefficient of determination (R^2) was 0.989, which indicated that the accuracy of the polynomial model was adequate and sufficient to represent the actual relationship between the response (% yield) and the reaction parameters. The pure error obtained was very low, indicating good reproducibility of the data.

TABLE I
Central Composite Rotatable Quadratic Polynomial Model, Experimental Data, Actual and Predicted Values for Five-Level-Three-Factor Response Surface Analysis

No	A (℃)	B (h)	C (g)	Actual (%)	Predicted (%)
1	80.00	12.00	0.10	25.83	27.41
2	80.00	4.00	0.50	8.33	9.91
3	40.00	12.00	0.50	35.00	36.58
4	40.00	4.00	0.10	22.92	24.50
5	31.72	8.00	0.30	48.75	46.42
6	88.28	8.00	0.30	12.50	18.92
7	60.00	2.34	0.30	59.58	58.00
8	60.00	13.66	0.30	8.33	15.28
9	60.00	8.00	0.02	15.83	22.70
10	60.00	8.00	0.58	31.67	33.89
11	60.00	8.00	0.30	33.75	34.41
12	60.00	8.00	0.30	33.50	34.41
13	60.00	8.00	0.30	33.75	34.41
14	60.00	8.00	0.30	32.50	34.41
15	60.00	8.00	0.30	35.42	34.41

TABLE II
RESULTS OF REGRESSION ANALYSIS OF CENTRAL COMPOSITE DESIGN EXPERIMENT

Source	Estimate	Standard Error	F-value	Prob> F
A	-12.82	1.25	105.28	0.0002*
B	-18.12	1.25	210.43	<0.0001*
C	5.60	1.25	20.10	0.0065*
AB	6.96	1.77	15.50	0.0110*
AC	-25.51	1.77	208.62	<0.0001*
BC	-6.88	1.77	15.15	0.0115*
A^2	-2.68	0.90	8.89	0.0307*
B^2	-1.02	0.90	1.28	0.3094
C^2	-6.12	0.90	46.29	0.0010*

*Significant at "Prob>F" less than 0.05

B. Effect of Parameters

Fig. 1 shows response surface plots for interaction between reaction temperature (A) and reaction time (B) with enzyme's amount fixed at their centre point (0.30 g). From the plot, an increased in reaction temperature (40-80°C) led to lower production yields of ferulate esters. One possible explanation is that higher temperature has reduced the operational stability of the enzyme used and resulted in less trans-esterification at any given reaction time [25]. The selectivity towards ferulate esters also decreased with the increased in reaction time (4-12 h) due to the hydrolysis of ferulate esters at extended reaction time.

The effect of varying reaction temperature (A) and amount of enzyme (C) on trans esterification at constant reaction time (8 h) is shown in Fig. 2. An increased in reaction temperature (40-80°C) at lower amount of enzyme from 0.1-0.3 g led to higher production yields of ferulate esters. Conversely, increased amount of enzyme from 0.3-0.5 g with increasing the reaction temperature, the ferulate esters yield was lower. This was due to the presence of larger amounts of enzyme resulting in increasing of the fraction of acyl donor molecules that forms acyl-enzyme complexes, simultaneously increasing the percentage yield of ferulate esters. However, too much of enzyme molecules may cause a decrease in the yield due to the substrate limitations. Enzyme itself could also cause mass transfer limitation [26].

Fig. 3 represents the effect of varying reaction time (B) and amount of enzyme (C) on trans-esterification at 60°C. A decreased in reaction time (12-4 h) and an increased in enzyme's amount (0.1-0.5 g) led to higher production yields of ferulate esters. Gunawan et al. [27] had reported that there was a linear increase in wax esters production with increase in amount of enzyme and incubation time. In contrary for this research, we found that the value of conversion of ferulate esters was increased as the amount of enzyme increased and the reaction time decreased. Enzymes contribute to the increasing of product's yield by increasing the formation of acyl-enzyme complexes but increasing reaction time led to the hydrolysis of the product occurred.

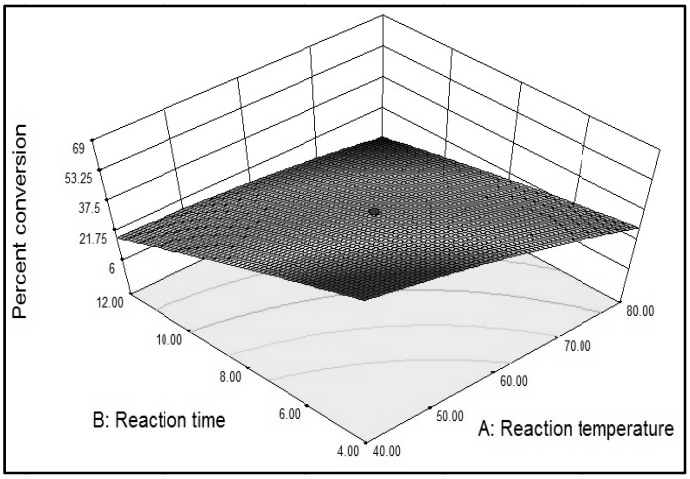

Fig. 1 Response surface plot showing the effect of reaction time, temperature and their mutual effect on the synthesis of ferulate esters

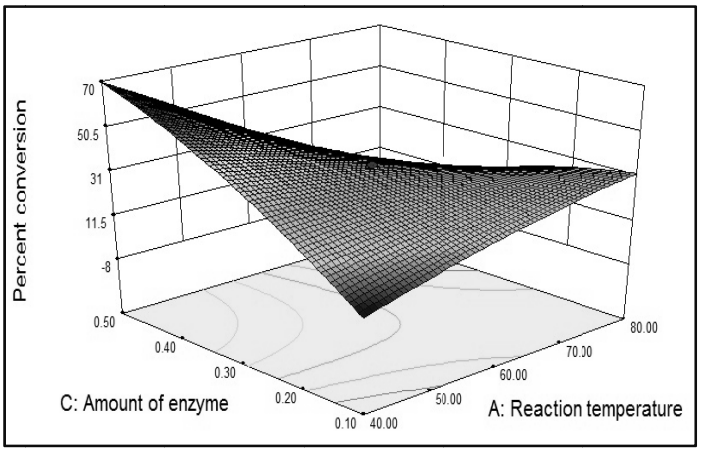

Fig. 2 Response surface plot showing the effect of reaction temperature, amount of enzyme and their mutual effect on the synthesis of ferulate esters

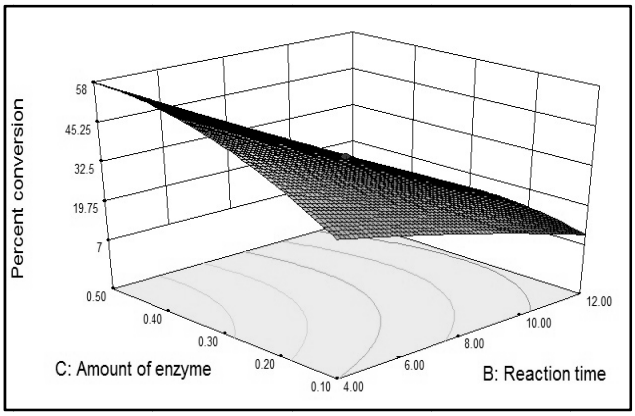

Fig. 3 Response surface plot showing the effect of reaction time, amount of enzyme and their mutual effect on the synthesis of ferulate esters

C. Optimization of Reaction

In order to verify the prediction of the model, the optimal reaction conditions were applied to three independent

replicates for olive-based ferulate esters synthesis (Table III). Comparison of predicted and experimental values revealed good correspondence between them implying models derived from the RSM could be used adequately to describe the relationship between the factors and response in this enzymatic synthesis. Among the various optimum conditions, the highest % yield (59.58%) was from the 3rd experiment. This is an improvement over Xin et al. [16] and Compton et al. [28] in the synthesis of ferulate esters which offer considerably high yield of ester produce in a reasonable amount of time.

TABLE III
OPTIMAL CONDITIONS DERIVED BY RSM

No	A (°C)	B (h)	C (g)	Predicted (%)	Actual (%)
1	60.00	8.00	0.30	34.41	35.42
2	31.72	8.00	0.30	46.42	48.75
3	60.00	2.34	0.30	58.00	59.58

IV. CONCLUSION

The modelling and optimization of Novozym-catalyzed trans-esterification to synthesis ferulate esters was successfully performed using a RSM based on a central composite rotatable design. The effect of three main reaction parameters (reaction temperature, time and amount of enzyme) and the response (% yield) were evaluated over the given ranges. Ferulate esters conversion of 59.58% was obtained when optimal conditions for maximal ferulate esters synthesis were: 60°C, 2.34 h and 0.30 g of enzyme. The established quadratic model generated by RSM can be used for future upscale enzymatic synthesis of ferulate esters.

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