Developing a High-Temperature Solvent-Free System for Efficient Biocatalysis of Octyl Ferulate

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Abstract: Ferulic acid esters have been suggested as a group of natural chemicals that have the function of sunscreen. The study aimed to utilize an environmentally-friendly enzymatic method through the esterification of ferulic acid with octanol, producing octyl ferulate. The Box-Behnken experimental design for response surface methodology (RSM) was performed to determine the synthesis effects of variables, including enzyme amount (1000–2000 propyl laurate units (PLU)), reaction temperature (70–90 °C), and stir speed (50–150 rpm) on the molar conversion of octyl ferulate. According to the joint test, both the enzyme amount and reaction temperature had great impacts on the molar conversion. An RSM-developed second-order polynomial equation further showed a data-fitting ability. Using ridge max analysis, the optimal parameters of the biocatalyzed reaction were: 72 h reaction time, 92.2 °C reaction temperature, 1831 PLU enzyme amount, and 92.4 rpm stir speed, respectively. Finally, the molar conversion of octyl ferulate under optimum conditions was verified to be 93.2 ± 1.5%. In conclusion, it has been suggested that a high yield of octyl ferulate should be synthesized under elevated temperature conditions with a commercial immobilized lipase. Our findings could broaden the utilization of the lipase and provide a biocatalytic approach, instead of the chemical method, for ferulic acid ester synthesis.

Keywords: ferulic acid esters; octyl ferulate; esterification; Box-Behnken design; response surface methodology; molar conversion; optimum condition

1. Introduction

Ferulic acid (FA) is one of the phenolic acids, which exist in natural fruits and vegetables. FA has been demonstrated as having many bioactivities, such as antioxidation [1], excellent ultraviolet-absorbing activity [2,3], and health benefits for numerous diseases, including cardiovascular-related diseases, inflammatory-related diseases, and cancers [4]. However, FA is a polar and small compound, with poor solubility in oils. Due to the limited solubility of FA in a lipophilic medium, the application of FA in cosmetics, food, and other nutraceutical industries are commonly limited due to their low dissolution rate [5,6]. Thus, it is essential to increase the solubility of FA to increase its practicality. A related study has indicated that the ester form of FA has better antioxidant activity than the original form, especially when compared to butylated hydroxytoluene (BHT) [7]. To overcome the problem of poor solubility, pharmaceutical particle technology of chemical modifications has attracted attention [8–13]. However, it is difficult to chemically synthesize such

derivatives because FA is oxidation-sensitive under certain pH conditions [7,14,15]. Thus, enzymatic biocatalysis of FA as esters has been considered an alternative to chemical processes [16]. However, the environmentally-friendly synthesis process of hydrophobic derivatives, such as FA, is still a significant challenge for researchers.

The hydrophilic feruloylated derivatives have been produced by the esterification reaction of FA with monosaccharides [17]. The water-soluble derivative of glyceryl ferulate with glycerol by pectinase [18] was also synthesized through the esterification of FA. Under solvent-free conditions, the enzymatic transesterification of ethyl ferulate (EF) with triolein had a higher EF conversion of 77% [19]. In addition, hydrophobic feruloylated derivatives can be synthesized by the esterification of FA with alcohols. The strategy for esterifying hydrophilic FA with lipophilic substrates, such as fatty alcohols, can be used to modify its solubility in a lipophilic medium. In previous literature, Katsoura et al. (2009) indicated that ferulic acid esterified with different chain lengths of alkyl—such as methyl, ethyl, and octyl—and possessed a significant antioxidant capacity against lipoprotein oxidation in serum. Moreover, the study also indicated that the protective effect increased the chain length of alkyl from methyl to octyl. [20]. However, because the reaction rate of FA condensation with long chain alcoholic substrates is slow, FA might have conjugated with carboxyls and a sizable noncarboxylic region [21].

Lipases are commonly used among biocatalysts in synthetic organic chemistry [22,23]. They have been widely used to catalyze carboxylic acids and chiral alcohols, because of their great chiral recognition [24]. Among them, the lipase isoform from *Candida antarctica* (CAL-B) is used most, possessing higher enantioselectivity for a comprehensive range of substrates [25,26]. Novozym® 435 is a commercial enzyme prepared by CAL-B and immobilized on the macroporous acrylic polymer resin. Novozym® 435 is a versatile biocatalyst, mainly conducted to catalyze the hydrolysis of oils and fats, althought it can also be used to catalyze various esterification-related reactions [27]. 3D-structures of the Novozym® 435 that show a short oligopeptide helix acting as a lid and adopting different conformations as a role of detergent, provide higher mobility in its environment [28]. The performance of catalytic activity requires the flexible active site of the enzyme. Although Novozym® 435 is active in many organic solvents, in the esterification process it is needed to react under the condition of lower water activity [29]. This suggests that Novozym® 435 is stabilized mainly by hydrophobic interactions as a biocatalyst for esterification reaction [30,31]. Evidence shows that an increased rate of enzyme hydration is associated with increasing mobility of enzyme molecules [32,33]. Although Novozym® 435 was used in the synthesis of ferulic esters, the final yield remained very low (17%) after a reaction lasting several days [19]. Moreover, the study also reported that, in the ferulic esters by esterification reaction with ferulate synthesis process, the enzyme activity might be affected partly by the hydrophilicity of ferulate [34,35]. In fact, a solvent-free reaction system, which is an eco-friendly method minimizing environmental pollution, could be conducted by lipase in the synthesis system of ferulate esters. This process could reduce the reaction time but increase the synthesized yield of esters [9,23]. Additionally, the solvent-free reaction system commonly contains simple substrates and offers reaction benefits, including the maximization of substrate concentration, a higher volumetric productiveness, less environmental hazard, and a cost savings in both the reactor design of large-scale production and the chemical separation/purification of products [36]. However, to increase the yield of FA esters, it is required to develop a solvent-free system that operates at a high temperature.

As mentioned above, ferulic acid has been suggested as a natural nutraceuticals with a biological function, but because in many solvent systems FA exhibits low stability and poor solubility, its application might be limited. The present work is aimed to develop an environmentally friendly biocatalytic method, instead of chemical synthesis, for the preparation of FA esters. In this study, under solvent-free conditions, the lipase-catalyzed esterification of FA with octanol formed octyl ferulate, followed by optimizing processes that featured an experimental design using response surface methodology (RSM) to evaluate the best experimental conditions. Finally, the thermodynamic and optimum effects on a solvent-free reaction system of lipase-catalyzed biocatalysis of FA was evaluated.

2. Results and Discussion

2.1. Primary Experiment

The synthesis of octyl ferulate, catalyzed by Novozym® 435 (1500 PLU) from ferulic acid (20 mM) and octanol, was performed in a water bath at a reaction time of 72 h. The octyl ferulate catalyzed by Novozym® 435, as well as the liquid samples analyzed by high-performance liquid chromatography (HPLC), are shown in Figure 1.
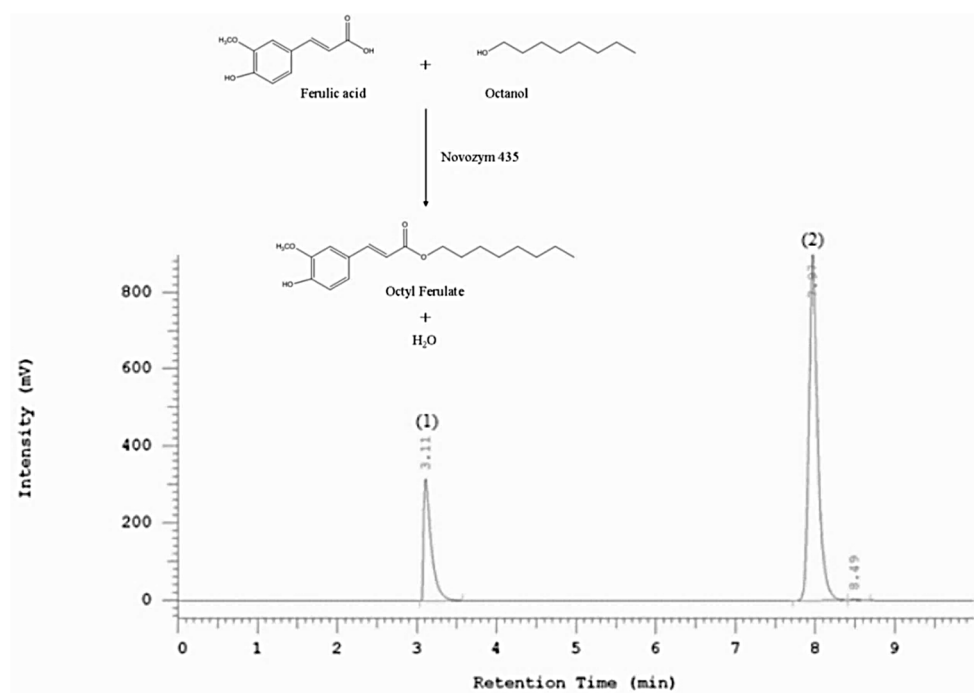
Figure 1. Scheme of octyl ferulate synthesis and high-performance liquid chromatography (HPLC) chromatogram. Peak 1 of HPLC chromatogram is ferulic acid, and peak 2 is octyl ferulate.

Moreover, the effects of reaction time and reaction temperature on molar conversion of octyl ferulate are shown in Figure 2, which illustrates an increased molar conversion observed after a reaction time of 24 h and an increased reaction temperature. Additinoally, the higher reaction temperature and increased reaction time could be observed without the weakening of enantioselectivities [37]. Although reaction temperature is a critical factor in biocatalysis, an elevated temperature may result in inactive enzymes. In previous literature, producing feruloylated lipids through the transesterification of monostearin and ethyl ferulate by catalysis has been investigated, and the optimal reaction temperature was performed at 74 °C [9]. Under a solvent-free system, a different enzymatic synthesis of feruloylated structured lipids occurred through the transesterification of ethyl ferulate with castor oil, which indicated that a high conversion of ethyl ferulate could be obtained at a reaction temperature of 90 °C [23]. The reaction temperature has a considerable influence on the equilibrium of the reversible thermodynamic reaction. Therefore, experiments performed at 70 °C, 80 °C, and 90 °C investigated the effect of temperature on biocatalysis reaction. As illustrated in Figure 2, the molar conversion increased with an elevating reaction temperature. At a reaction temperature of 90 °C, molar conversion reached a high value of 88.4%; the fastest reaction rate (observed by the slope of the linear curves) was also determined. When the reaction time was over 72 h, the time course was leveled-off. The reason for this may be that the viscosity of the reaction mixture gradually increased over reaction time, leading to a decrease in mutual solubility and inhibiting the diffusion of substrates [38]. Thus, reducing mass transfers and unfavorable interactions between substrates and enzyme particles might occur in the

catalysis process. However, a longer reaction time could lead to a higher molar conversion. Thus, the following experimental design was conducted at a reaction time of 72 h.
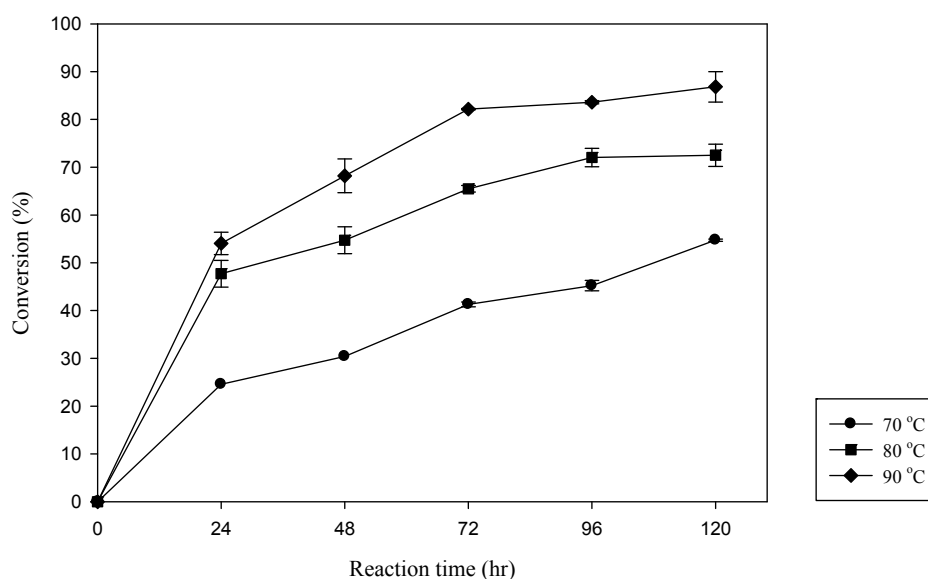
Figure 2. Effects of reaction temperature and time on Novozym® 435-catalyzed synthesis of octyl ferulate. The reaction was employed in a water bath at a reaction temperature of 70–90 °C and enzyme amount of 1500 propyl laurate units (PLU).

2.2. Model Fitting

In our preliminary study, we explored the relationship between the influence factors and synthesis yield of octyl ferulate catalyzed by Novozym® 435, testing various independent variables, such as reaction time, reaction temperature, the molar ratio of octanol and ferulic acid, enzyme amount, stirring speed, and so on. Moreover, our preliminary data indicated that the reaction temperature, enzyme amount, and stirring speed were synthesis-dependent variables in response to Novozym® 435-catalysed octyl ferulate. Thus, we chose these factors to investigate the optimal process of octyl synthesis by Box-Behnken design (BBD). In order to systematically realize the interactions between enzyme amount, reaction temperature, and stir speed of the octyl ferulate synthesis, a three-level, three-factor BBD was used to test 15 experiments (treatments). Using statistical response surface methodology (RSM) modelling, we determined the experimental data for process optimization of octyl ferulate production. This study aimed to develop and evaluate a statistical approach in order to thoroughly realize the relationship between experimental variables and lipase-catalyzed responses. Thus, the process could be fully optimized before the scaling-up production, which decreased the cost and time to obtain a high-quality product. Compared with the single-factor-at-a-time design in most studies, our study's combination of RSM and BBD was more effective in reducing experimental runs investigating the optimized process of octyl ferulate biocatalysis.

The experimental conditions and results of a three-level, three-factor BBD are shown in Table 1. The response surface regression (RSREG) process for SAS was employed to fit the second-order polynomial Equation of the experimental molar conversions. Among these treatments, the highest molar conversion (88.4 ± 3.1%) was treatment 8 (reaction temperature 90 °C, enzyme amount 1500 PLU and stirring speed 150 rpm); the lowest molar conversion (42.4 ± 5.7%) was treatment 1 (reaction

temperature of 70 °C, enzyme amount of 1000 PLU and stirring speed of 100 rpm). From the SAS output of RSREG, the second-order polynomial Equation (1) is given below:

$$\begin{aligned} Y = -299.1 + 5.6825X_1 + 0.06625X_2 + 0.22X_3 - 0.00031X_1X_2 \\ -0.0035X_1X_3 - 0.00005X_2X_3 - 0.02025X_1{}^2 \\ -0.0000059X_2{}^2 + 0.00091X_3{}^2 \end{aligned} \tag{1}$$

Table 1. Box-Behnken design experiments and observed data of molar conversion.

| Treatment No. [a]	Experimental Factors [b]			Molar Conversion [c] (%)
	X_1 (°C)	X_2 (PLU)	X_3 (rpm)	
1	−1(70)	−1(1000)	0(100)	42.4 ± 5.7
2	1(90)	−1(1000)	0(100)	75.2 ± 0.7
3	−1(70)	1(2000)	0(100)	61.7 ± 3.6
4	1(90)	1(2000)	0(100)	88.3 ± 0.5
5	−1(70)	0(1500)	−1(50)	49.4 ± 1.1
6	1(90)	0(1500)	−1(50)	88.3 ± 0.1
7	−1(70)	0(1500)	1(150)	56.5 ± 0.7
8	1(90)	0(1500)	1(150)	88.4 ± 3.1
9	0(80)	−1(1000)	−1(50)	56.4 ± 0.1
10	0(80)	1(2000)	−1(50)	80.2 ± 3.8
11	0(80)	−1(1000)	1(150)	64.7 ± 4.9
12	0(80)	1(2000)	1(150)	83.5 ± 1.1
13	0(80)	0(1500)	0(100)	71.1 ± 1.4
14	0(80)	0(1500)	0(100)	70.2 ± 2.8
15	0(80)	0(1500)	0(100)	70.2 ± 3.5

[a] The treatments were employed in a random order. [b] X_1: reaction temperature; X_2: enzyme amount; X_3: stir speed.
[c] Molar conversion for octyl ferulate shows means ± SD of duplicated experiments.

Furthermore, the analysis of variance (ANOVA) indicated that the second-order polynomial equation had a significant correlation between the experimental response (molar conversion) and dependent variables, which had a very small *p*-value of 0.0001 and a satisfactory coefficient of $R^2 = 0.9887$. Additionally, the total effects of these three experimental variables on the molar conversion were investigated by a joint test. The results indicated that the reaction temperature (X_1) and enzyme amount (X_2) were the most critical factors, statistically showing a significant effect ($p < 0.001$) on the responded molar conversion. The stirring speed (X_3) was statistically insignificant on the responded molar conversion.

2.3. Optimal Synthesis Conditions

The optimal biocatalysis of octyl ferulate was further determined by the ridge max analysis, indicating that the highest molar conversion was 91.74 ± 2.3% at 72 h, temperature 92.2 °C, enzyme amount 1831 PLU, and stir speed 92.4 rpm (Figure 3). Figure 4 indicates the correlation between the predicted and experimental values of the average cutting speed in the RSM model. A certification experiment, performed in the investigated optimal conditions, could obtain a 93.2 ± 1.5% molar conversion, which was similar to the RSM-predicted molar conversion, thus indicating that the predicted model in this study was successfully established. Thus, the lipase-catalyzed biocatalysis of octyl ferulate could be carried out with an easy method.
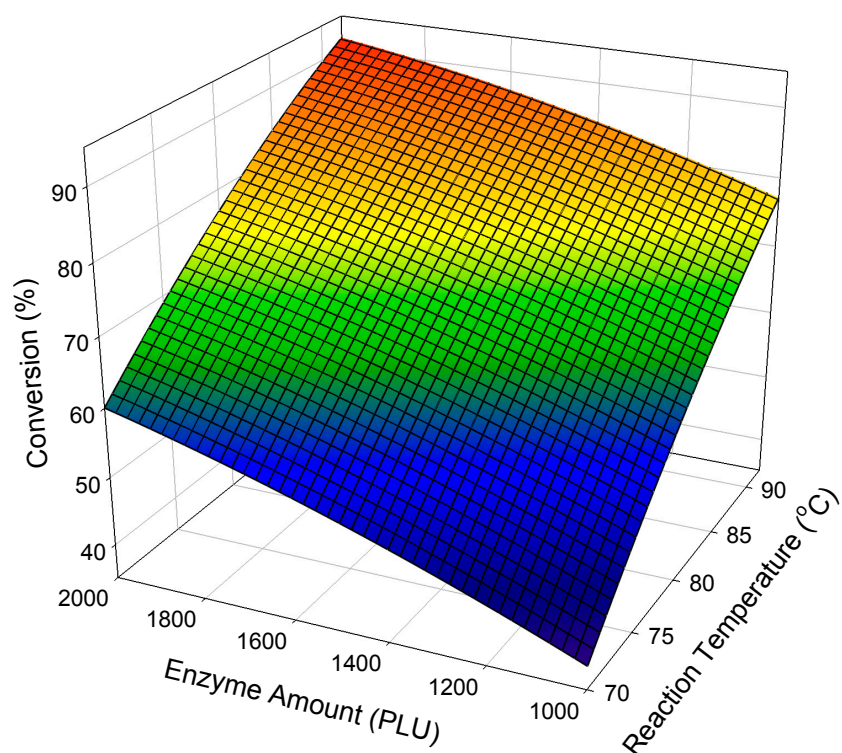
Figure 3. Response surface plots show the correlation between the molar conversion of octyl ferulate synthesis and reaction parameters (enzyme amount and reaction temperature).
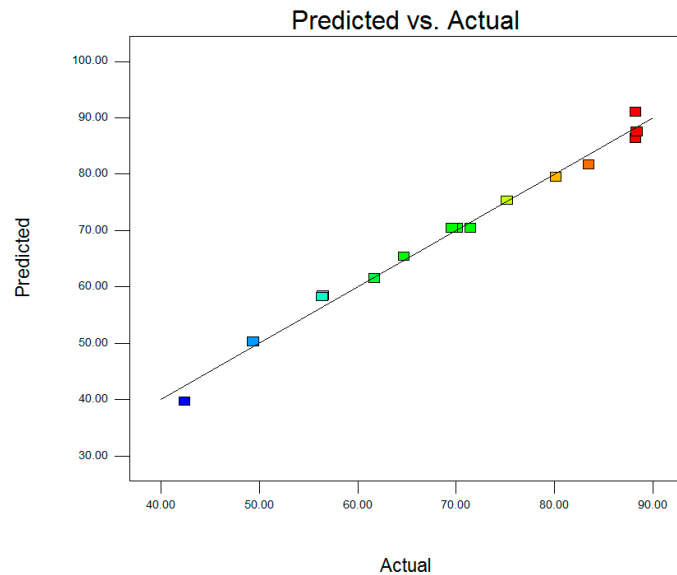
Figure 4. Comparison of the experimental data with those predicted by the response surface methodology (RSM) model.

Additionally, to confirm the enzyme reusability, the immobilized lipase used for octyl ferulate synthesis was determined under optimum conditions. Novozym® 435 was recovered from the reaction medium after synthesis, followed by a direct reuse in the next batch. When the immobilized lipase was reused five times, the molar conversion of octyl ferulate remained higher than 90% with very little loss of enzymatic activity (Figure 5).
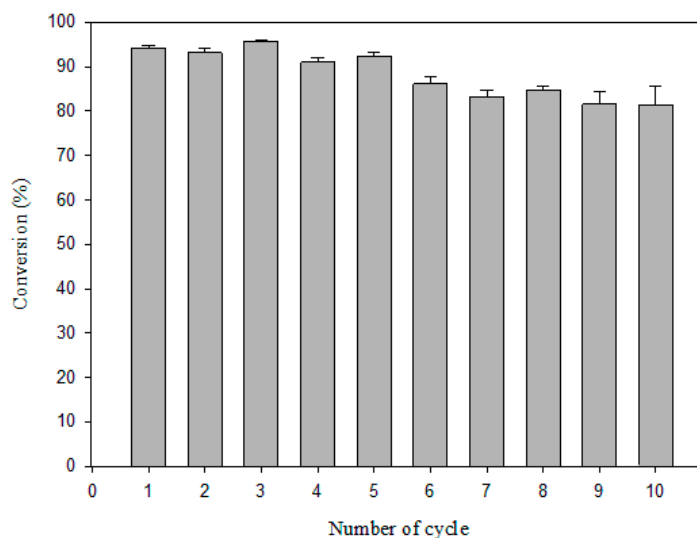
Figure 5. Reusable cycles of Novozym® 435 in the synthetic process of octyl ferulate under indicated optimal condition.

The data indicated that the immobilized lipase could keep enzymatic stability under the conditions of long-term octanol exposure and high reaction temperature (90 °C). Therefore, the data confirmed that the Novozym® 435 could be efficiently used for the synthesis of octyl ferulate and that the enzymatic stability was enough to reuse.

3. Materials and Methods

3.1. Materials

A total of 10,000 U/g (propyl laurate units, PLU) of immobilized lipase Novozym® 435 from *Candida antarctica* B (EC3.1.1.3) (on a macroporous acrylic resin) was purchased from Novo Nordisk Bioindustrials Inc. (Copenhagen, Denmark). Ferulic acid (FA), acetic acid, 2-methyl-2-butanol, methanol, hexanol, and octanol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). A 4-Å molecular sieve was purchased from Davison Chemical (Baltimore, MD, USA). All chemicals and reagents were of analytical grade.

3.2. Enzymatic Synthesis of Octyl Ferulate

In this study, the synthesis process was performed under a solvent-free system in order to biocatalyze ferulic esters, using the maximum concentration of ferulic acid, which helped its participation in esterification. However, the best solubility of ferulic acid in octanol was also determined by a maximum concentration of 20 mM. Therefore, 20 mM of ferulic acid was employed in this study. All materials were dehydrated using molecular sieve (4 Å) for 24 h before use. The 20 mM of ferulic acid and Novozym® 435 were thoroughly mixed with octanol (1 mL) in sealed dark vials, and reacted in a water bath for 72 h, under different experimental conditions of temperature, enzyme amount, and stir speed, as shown in Table 1. The reacted samples were further analyzed by high-performance liquid chromatography (HPLC). Samples were centrifuged and diluted with hexanol/2-methyl-2-butanol (1:100). Analyses were done according to the procedure modified by Huang et al. [36]. The flow rate was set at 1.0 mL/min, followed by detection at a wavelength of 325 nm. Molar conversion was calculated based on the peak areas of the sample.

3.3. Experimental Design

In this study, a three-level, three-factor Box-Behnken design requiring 15 experiments was employed. To avoid bias, the 15 runs were done in a random order. The variables and their response levels selected for the synthesis of octyl ferulate were: Reaction temperature (70–90 °C), enzyme amount (1000–2000 PLU), and stir speed (50–150 rpm). All of the experiments were performed at a reaction time of 72 h. Table 1 shows the independent factors (X_1), levels, and experimental design of the coded and un-coded values.

3.4. Statistical Analysis

All data were analyzed by the procedure of response surface regression (RSREG) with SAS software to fill in the second-order polynomial equation, as shown in Equation (2):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j \tag{2}$$

Y is the molar conversion of octyl ferulate; β_0 represents a constant; β_i, β_{ii}, and β_{ij} represent constant coefficients; and *xi* and *xj* are uncoded variables. The suffixes *i* and *j* are shown in Equation (1) with the three variables representing: x_1 for reaction temperature, x_2 for enzyme amount, and x_3 for stir speed. The decision of ridge max in the SAS software was used to calculate the estimation of maximum response ridge, in which the radius increased from the center of the original design.

4. Conclusions

In this study, the esterification of ferulate with octanol catalyzed by lipase in a solvent-free system was well investigated. The immobilized Novozym® 435 could be used to synthesize octyl ferulate. Both three-level, three-factor BBD and RSM were employed successfully for the experimental design. An environmentally friendly experimental model for the octyl ferulate synthesis was built, and the optimal conditions for biocatalysis had a reaction time of 72 h, a reaction temperature of 92.2 °C, an enzyme amount of 1831 PLU, and a stir speed of 92.4 rpm. In order to obtain a molar conversion of 93.2 ± 1.5%, we employed a synthesis of octyl ferulate by lipase biocatalysis under the optimal condition. Notably, our results indicated that a high reaction temperature significantly affected the efficiency of a lipase-catalyzed ester synthesis under a solvent-free system. Overall, as compared to the chemical methods, our present study suggests that the lipase-catalyzed and environmentally-friendly synthesis of esters, when under a solvent-free reaction system at a high reaction temperature and without a marked loss of enzymatic activity, could offer a significant reference for an industrial preparation process.

Author Contributions: C.-J.S., J.-H.C. and C.-H.K. conceived and designed the experiments; P.-Y.W. performed the experiments; S.-M.H. and P.-Y.W. analyzed the data; S.-M.H. and C.-H.K. wrote and revised the paper.

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