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Optimization of Lipase-Catalyzed Synthesis of Octyl Hydroxyphenylpropionate by Response Surface Methodology

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The ability of immobilized lipase *Candida antarctica* (Novozyme 435) to catalyze the direct esterification of hydroxyphenylpropionic acid and octanol in a solvent-free system was investigated in this study. Response surface methodology (RSM) and five-level−four-factor central composite rotatable design (CCRD) were employed to evaluate the effects of synthesis parameters, such as reaction time, temperature, enzyme amount, and pH memory, on percentage molar conversion of phenolic acid esters. Reaction time, temperature, and enzyme amount were the most important variables. On the basis of canonical analysis and ridge max analysis, the optimum synthesis conditions with 95.9% molar conversion were reaction time of 58.2 h, temperature of 52.9 °C, enzyme amount of 37.8% (w/w), and pH memory of pH 7.

KEYWORDS: Phenolic acid ester; lipase; esterification; response surface methodology; optimization

INTRODUCTION

For the past decades, several researchers have focused on the extraction of antioxidants from natural compounds such as vegetable oils, herbs, spices, rice hulls, and tea (*1*). The main reason is the growing concern about the widely used synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), because these compounds may be carcinogenic. Natural phenolic acid compounds are numerous and widely distributed in the plant kingdom. A major portion of the antioxidant activity of oilseeds and oilseed flours and concentrates is attributed to phenolic acids (*2*).

Phenolic acids, including hydroxylated (*p*-coumaric acid, caffeic acid) or methoxylated (ferulic acid) derivatives of cinnamic acids, are present in soybeans, cottonseeds, and peanuts in free forms or as carbohydrate esters (*3*). Due to their phenolic nucleus and an extended side-chain conjugation, they can readily form resonance-stabilized phenoxy radicals, which account for their antioxidant performance (*4*). It is well recognized that phenolic acids or phenolic acid esters exhibit antioxidative activity (*5*), and they are inhibitors of carcinogenesis (*6*).

Many of the natural phenolic acids exhibit low solubility and stability in various solvent systems. For example, the hydrophilicity of phenolic acids reduces their antioxidant effectiveness in stabilizing fats and oils and has been reported as a serious disadvantage if an aqueous phase is also present (*7*). It is thus important to improve the solubility of these compounds to enhance their usefulness as food antioxidants. Therefore, the modification of these compounds via esterification with aliphatic alcohols can be used as a tool to alter solubility in oil-based formulas and emulsions.

The chemical synthesis of benzoic and phenolic acid esters is usually carried out with basic or acidic catalysts under reflux, but these routes do not meet the requirements necessary for food applications. To overcome the disadvantages of the conventional processes, the use of enzymes in nonaqueous media has opened new avenues for producing many valuable products under mild conditions (*8*). Recently the esterification of organic acids via enzymatic routes has been successfully reported in many studies. Guyot et al. (*9*) first reported on the enzymatic esterification of phenolic acids and fatty alcohols with lipase CAL-B from *Candida antarctica*. Buisman et al. (*10*) investigated the esterification of cinnamic acid and some benzoic acid derivatives with fatty alcohols with chain lengths varying between 4 and 12 carbon atoms (C_4−C_{12}). Stamatis et al. (*11*) reported the use of two commercial lipases from *C. antarctica* in various solvent systems for the biotransformation of various cinnamic acid derivatives via esterification with fatty alcohols. Their objective was to investigate the role of various physicochemical parameters (such as the structural characteristics of substrates, the concentration of reactants, and the nature of the organic medium). Stamatis et al. (*12*) described the possibilities of esterification of various cinnamic acid and benzoic acid derivatives with long-chain alcohols and monosaccharides as well as alkyl glucosides catalyzed by various lipases and esterases in organic media. Various reaction parameters affecting the enzyme catalytic behavior such as the structural characteristics of substrates as well as the nature of the organic medium used have been examined.

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Table 1. Central Composite Rotatable Second-Order Design and Experimental Data for Five-Level–Four-Factor Response Surface Analysis

no.[a]	time (h), x_1	temp (°C), x_2	enzyme (%, by wt of HPPA), x_3	pH memory, x_4	yield (%), Y	SD (%)
1	–1 (36)[b]	–1 (35)	–1 (20)	1 (8)	44.50	0.34
2	–1 (36)	–1 (35)	1 (40)	–1 (6)	64.03	1.44
3	–1 (36)	1 (55)	–1 (20)	–1 (6)	72.04	0.29
4	–1 (36)	1 (55)	1 (40)	1 (8)	83.15	2.18
5	1 (60)	–1 (35)	–1 (20)	–1 (6)	58.37	0.99
6	1 (60)	–1 (35)	1 (40)	1 (8)	79.66	1.34
7	1 (60)	1 (55)	–1 (20)	1 (8)	83.61	5.61
8	1 (60)	1 (55)	1 (40)	–1 (6)	77.07	2.35
9	0 (48)	0 (45)	0 (30)	0 (7)	87.34	1.01
10	–1 (36)	–1 (35)	–1 (20)	–1 (6)	40.13	3.29
11	–1 (36)	–1 (35)	1 (40)	1 (8)	72.71	4.68
12	–1 (36)	1 (55)	–1 (20)	1 (8)	68.89	1.54
13	–1 (36)	1 (55)	1 (40)	–1 (6)	84.82	1.11
14	1 (60)	–1 (35)	–1 (20)	1 (8)	59.94	0.97
15	1 (60)	–1 (35)	1 (40)	–1 (6)	78.42	0.88
16	1 (60)	1 (55)	–1 (20)	–1 (6)	84.23	0.02
17	1 (60)	1 (55)	1 (40)	1 (8)	96.37	1.56
18	0 (48)	0 (45)	0 (30)	0 (7)	86.10	2.71
19	–2 (24)	0 (45)	0 (30)	0 (7)	60.36	2.63
20	2 (72)	0 (45)	0 (30)	0 (7)	94.24	0.18
21	0 (48)	–2 (25)	0 (30)	0 (7)	47.12	1.93
22	0 (48)	2 (65)	0 (30)	0 (7)	94.34	2.79
23	0 (48)	0 (45)	–2 (10)	0 (7)	58.38	0.19
24	0 (48)	0 (45)	2 (50)	0 (7)	95.25	1.16
25	0 (48)	0 (45)	0 (30)	–2 (5)	86.67	0.36
26	0 (48)	0 (45)	0 (30)	2 (9)	58.16	1.87
27	0 (48)	0 (45)	0 (30)	0 (7)	84.50	4.61

[a] Numbers were run in random order. [b] Numbers in parentheses represent actual experimental amounts.

Our purposes in this study were to better understand relationships between the factors (reaction time, temperature, enzyme amount, and pH memory) and the response (percent molar conversion) and to determine the optimal conditions for phenolic acid esters synthesis using central composite rotatable design (CCRD) and response surface methodology (RSM) analyses.

MATERIALS AND METHODS

Materials. Immobilized lipase Novozyme 435 from *C. antarctica* (10000 propyl laurate units/g) supported on macroporous acrylic resin was purchased from Novo Nordisk Bioindustrials, Inc. (Bagsvaerd, Denmark). Hydroxyphenylpropionic acid (HPPA) and octanol (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents were dried extensively with 4 Å molecular sieves (Baltimore, MD). The other chemicals were of analytical reagent grade.

Enzyme Pretreatment. Novozyme 435 was soaked in the different pH buffer solution (pH 5−9) stirred in an orbital shaking water bath (180 rpm) for 24 h and then dehydrated by freeze-dryer (Eyela FD-1000) for 24 h. Enzyme preparations were equilibrated for at least 72 h in saturated CH_3COOK solutions at 6 °C to obtain a uniform initial water activity ($a_w = 0.23$). The water content of equilibrated samples was determined by Karl Fischer titrator (Mettler-Toledo DL31).

Experimental Design. A five-level−four-factor CCRD was employed in this study, requiring 27 experiments. The fractional factorial design consisted of 15 factorial points, 9 axial points (2 axial points on the axis of each design variable at a distance of 2 from the design center), and 3 center points. The variables and their levels selected for the study of phenolic acid ester synthesis were time (24−72 h), temperature (25−65 °C), enzyme amount (10−50%, by weight of HPPA), and pH memory of enzyme (pH 5−9). **Table 1** shows the independent factors (x_i), levels, and experimental design in terms of coded and uncoded.

Esterification Method. All materials were dehydrated by 4 Å molecular sieves for 24 h. HPPA (100 mM) was dispersed in the octanol, followed by different amounts and different pH memories of enzyme. The mixtures of HPPA, octanol, and Novozyme 435 were stirred in an orbital shaking water bath (180 rpm) at different reaction temperatures and reaction times (**Table 1**).

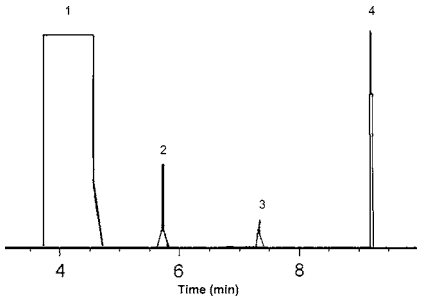

Figure 1. GC of esterification of hydroxyphenylpropionic acid with octanol by Novozyme 435. Peaks: 1, octanol; 2, internal standard; 3, hydroxyphenylpropionic acid; 4, octyl hydroxyphenylpropionate.

Extraction and Analysis. The enzyme and any residual water were removed by passing reaction media through an anhydrous sodium sulfate column. Before sample analysis, the reactant was taken to mix with an equal volume of an internal standard solution (50 mM tributyrin). Analysis was done by injecting a 1 μL aliquot in a splitless mode into a Hewlett-Packard 6890 series gas chromatograph (Avondale, PA) equipped with a flame ionization detector. An MXT-65TG capillary column (30 m × 0.25 mm i.d.; Restek) was used. Injector and detector temperatures were set at 280 and 300 °C, respectively. The oven temperature was maintained at 70 °C for 1 min, elevated to 230 °C at a rate of 40 °C/min, held for 1 min, and then increased to 300 °C at a rate of 70 °C/min and held for 3 min. Nitrogen was used as carrier gas. The relative percentage conversion (yield) was defined as (millimoles of esters/millimoles of initial HPPA) × 100% and was estimated using peak area integrated by on-line software Hewlett-Packard 3365 Series II ChemStation.

Statistical Analysis. The experimental data (**Table 1**) were analyzed by the response surface regression (RSREG) procedure to fit the second-order polynomial equation

$$Y = \beta_{k0} + \sum_{i=1}^{4}\beta_{ki}x_i + \sum_{i=1}^{4}\beta_{kii}x_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{kij}x_i x_j \qquad (1)$$

where Y is response (percent molar conversion), β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant coefficients, and x_i are the uncoded independent variables. Canonical analysis was one part of the RSREG SAS output, and the RIDGE MAX option was used to compute the estimated ridge of maximum response for increasing radii from the center of the original design.

RESULTS AND DISCUSSION

RSM and CCRD. The major objective of this paper is the development and evaluation of the RSM approach to better understanding the relationships between the variables of a lipase-catalyzed direct esterification reaction. Compared with one-factor-at-a-time design, which has been adopted most often in the literature, RSM combined with a five-level−four-factor CCRD is a useful statistical technique for investigating complex synthesis processes, especially for the optimum synthesis of lipase-catalyzed reaction. In this way, the process can be optimized before the scale-up procedure in order to save experimental work, money, and time, allowing economically important phenolic acid esters to be obtained at lower cost.

The GC chromatogram for octyl hydroxyphenylpropionate (OHPP) synthesis catalyzed by Novozyme 435 is presented in **Figure 1**. Besides the reactants (octanol and HPPA), the peak of OHPP was clearly observed after reaction. The time course for the direct esterification of HPPA with octanol by Novozyme

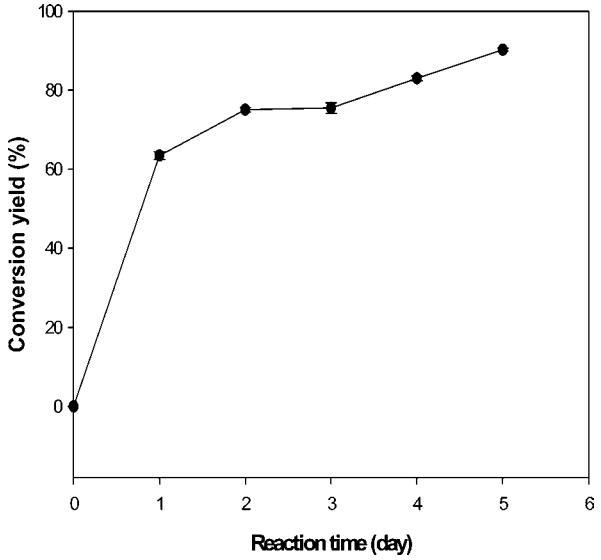

Figure 2. Time course of esterification of hydroxyphenylpropionic acid with octanol by Novozyme 435. The reaction was carried out at 45 °C in octanol containing 100 mM hydroxyphenylpropionic acid and 30% (by weight of hydroxyphenylpropionic acid) Novozyme 435.

Table 2. Analysis of Variance for Joint Test

factor	degrees of freedom	sum of squares	prob > F^a
time, x_1	5	1428.31	0.0091
temp, x_2	5	3212.48	0.0003
enzyme amount, x_3	5	1655.93	0.0051
pH memory, x_4	5	450.01	0.2238[b]

[a] prob > F = level of significance. [b] Not significant at $p \geq 0.1$.

435 is shown in **Figure 2**. The percentage molar conversion of OHPP increased to 75% after 2 days, so a range of reaction times from 1 to 3 days was chosen in this study. The reaction time range needs to be carefully defined in CCRD; otherwise, the optimal condition of synthesis cannot be found inside the experimental region through the analyses of statistics and contour plots.

Model Fitting. The RSREG procedure from SAS was employed to fit the second-order polynomial eq 1 to the experimental data—percent molar conversions (**Table 1**). Among the various treatments, the greatest molar conversion (96.37%) was from treatment 17 (60 h; 55 °C; enzyme amount, 40%; and pH memory, 8), and the smallest conversion (only 40.13%) was for treatment 10 (36 h; 35 °C; enzyme amount, 20%; and pH memory, 6). In addition, the overall effect of the four synthesis variables on the percent molar conversion of OHPP was further analyzed by a joint test (**Table 2**). The results revealed that time (x_1), temperature (x_2), and enzyme amount (x_3) were the important variables, exerting a statistically significant overall effect ($p < 0.01$) on the response molar conversion of OHPP; pH memory in the experimental scale (pH 5−9) of this study was the least important factor ($p = 0.22$) compared to the other reaction parameters from the statistical analysis. Therefore, the factor of pH memory was removed from the original model and the final second-order polynomial equation is

$$Y = -359.557 + 2.490x_1 + 6.227x_2 + 3.793x_3 - 0.010x_1^2 - 0.0184x_2x_1 - 0.043x_2^2 - 0.010x_3x_1 - 0.029x_3x_2 - 0.028x_3^2 \quad (2)$$

(s_1, time; x_2, temperature; x_3, enzyme; x_4, pH memory). With a very small p value (0.0004) from the analysis of variance (ANOVA) and a suitable coefficient of determination ($R^2 = 0.903$), the second-order polynomial model (eq 2) was highly significant and sufficient to represent the actual relationship between the response (percent molar conversion) and the significant variables.

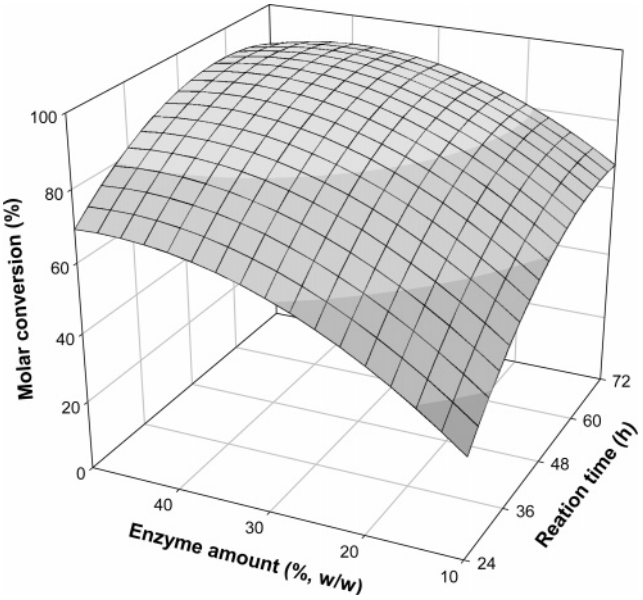

Figure 3. Response surface plot showing the effect of enzyme amount, reaction time, and their mutual interaction on phenolic acid ester synthesis. Reaction temperature is constant at zero level.

Mutual Effect of Parameters. Enzyme amount and reaction time were investigated in the range of 10−50 wt % of HPPA and 24−72 h, respectively. **Figure 3** shows the effect of enzyme amount, reaction time, and their mutual interaction on OHPP synthesis at 45 °C. At the lowest reaction time (24 h) with lowest enzyme amount (10%), molar conversion was only 30%. A reaction with an enzyme amount of 40% and a reaction time of 60 h led to the stationary molar conversion (∼90%). The effect of varying the enzyme amount and reaction temperature on esterification at constant reaction time (48 h) is shown in **Figure 4**. At any given temperature from 25 to 65 °C, an increase of enzyme amount led to higher yields. A reaction with high reaction temperature (65 °C) and highest enzyme amount favored maximal yield, which indicated the same report from Novo Nordisk Bioindustrials that the enzyme is stable at higher temperature (>65 °C).

The relationships between reaction factors and response can be better understood by examining the planned series of contour plots (**Figure 5**) generated from the predicted model (eq 2) by holding constant the enzyme amount (10, 20, and 30%). Reaction time (x_1) and temperature (x_2) were the most important variables for OHPP synthesis with the small p values (see **Table 2**) and therefore considered to be indicators of effectiveness performance. Overall, all three contour plots in **Figure 5** exhibited similar behaviors in which predicted molar conversion increased with the increase of the enzyme amount. The reaction of 30% enzyme amount (**Figure 5C**) resulted in higher predicted molar conversion than the others.

Attaining Optimization. The optimum synthesis of OHPP was determined by the ridge maximum analysis and the canonical analysis (*13*). The method of ridge analysis computes the estimated ridge of maximum response for increasing radii from the center of original design. The ridge maximum analysis indicated that maximum molar conversion was 95.9 ± 3.9% at

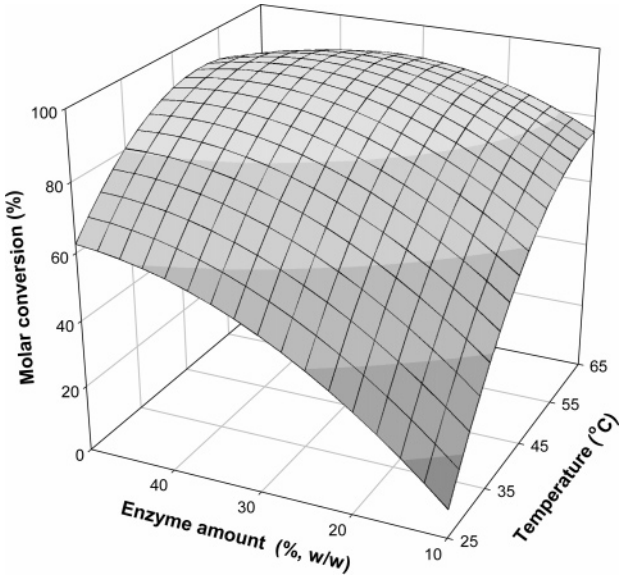

Figure 4. Response surface plot showing the effect of enzyme amount, reaction temperature, and their mutual interaction on phenolic acid ester synthesis. Reaction time is constant at zero level.

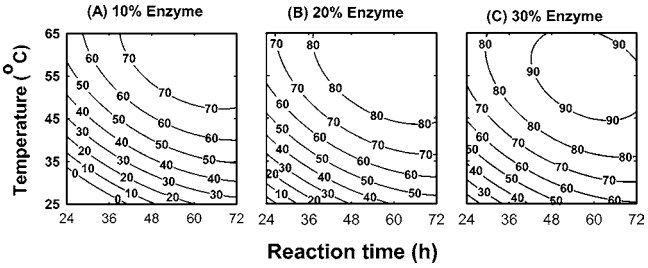

Figure 5. Contour plots of percent molar conversion of octyl hydroxyphenylpropionate. Enzyme amount was by weight of hydroxyphenylpropionic acid. The numbers inside the contour plots indicate molar conversions at given reaction conditions.

58.2 h, 52.9 °C, 37.8% enzyme amount, and pH memory of 7.1 at the distance of the coded radius 0.7. The optimum point was also determined by canonical analysis. The stationary point (reaction time, 58.2 h; reaction temperature, 52.9 °C; 37.8% enzyme amount; and pH memory, 7), values of variables at which the first derivative of response was zero, was located exactly in the experimental region with the predicted value of 95.5%. The canonical analysis based on the stationary point resulted in the equation

$$Y = 95.93 - 7.37W_1^2 - 8.25W_2^2 - 15.50W_3^2 - 24.01W_4^2 \quad (3)$$

where W_1, W_2, W_3, and W_4 are eigenvalues based on coded data and Y is the molar conversion of OHPP (percent). All eigenvalues were negative, indicating that the predicted response surface of the stationary point is shaped like a maximum. The response behavior of reaction time and synthesis temperature (**Figure 6**) was followed while the other reaction parameters were held constant at the suggested optimum point. The maximum value (95%) was predicted to be near a combination of 58 h and 53 °C. Because both results from maximum ridge analysis and canonical analysis obtained the same conclusions, the reaction condition (reaction time, 58.2 h; synthesis temperature, 53 °C; 37.8% enzyme amount; and pH memory, 7.1) was recommended as the optimization for OHPP synthesis with 95.9% molar conversion in this study. Stamatis et al. (*12*)

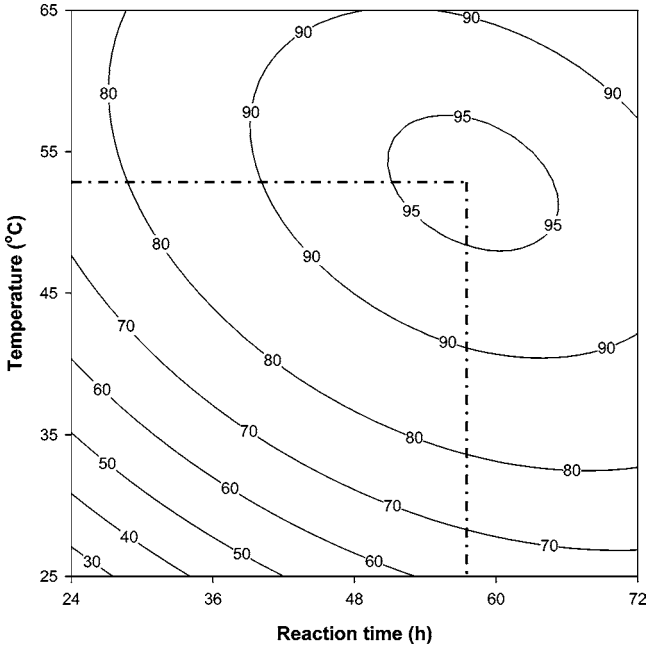

Figure 6. Contour plots showing response behavior of reaction time and temperature of the optimum synthesis condition at the stationary point (enzyme amount, 37.8%; pH memory, 7.1) suggested by canonical analysis.

synthesized OHPP by direct esterification in a solvent-free system and attained an ester yield of 95% in 96 h at 45 °C in the presence of 30 mg/mL (w/v octanol) of the immobilized lipase from *C. antarctica* (Novozyme 435) and 0.5 mol^{-1} of substrate. Our results were in agreement with their investigation. Both studies produced similar results, regardless of the different types of acyl donors (by direct esterification or by transesterification) and lipases employed in the studies. Compared with our work in which the optimized reaction time for OHPP in a solvent-free system was 58.2 h with 95.9% molar conversion, higher reaction rate and molar conversion of OHPP were observed in this synthesis through the response surface analysis. In addition, the optimal pH memory for Novozyme 435 in this study was 7, which indicated that the enzyme as received is already immobilized at optimum pH by Novo Nordisk Bioindustrials. There is no need to alter the pH further before the reaction for industry application.

Model Verification. The adequacy of the predicted model here was examined by additional independent experiments at the suggested optimal synthesis conditions. The predicted value was 95.9% molar conversion, and the actual experimental value was 94.3%. A Chi-square test (p value = 0.96, degrees of freedom = 3) indicated that observed values were statistically the same as the predicted values and that the generated model adequately predicted the percent molar conversion (*14*). Thus, the optimization of lipase-catalyzed synthesis for OHPP by Novozyme 435 was successfully developed by CCRD and RSM.

Conclusion. The biocatalysis of phenolic acid esters by direct esterification was successfully achieved using Novozyme 435 from *C. antarctica* lipase in a solvent-free system. Thus, the optimization of lipase-catalyzed synthesis for OHPP by Novozyme 435 was successfully developed by CCRD and RSM. On the basis of ridge maximum analysis, the optimum synthesis conditions with 95.9% molar conversion were as follows: reaction time, 58.2 h; temperature, 52.9 °C; enzyme amount, 37.8% (w/w); and pH memory, pH 7.

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