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ORIGINAL PAPER

Optimization of Enzymatic Synthesis of Cetyl 2-Ethylhexanoate by Novozym® 435

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Abstract Waxes are esters obtained from long-chain fatty acids and long-chain alcohols which are biodegradable, biocompatible and nontoxic. Seafowl feather oil is a natural wax ester that exists on seafowl feathers. Cetyl 2-ethylhexanoate is the major ingredient of seafowl feather oil. Cetyl 2-ethylhexanoate is widely used in cosmetics as a base oil because of its lubricity, moisture retention and non-toxic properties. An optimal production of cetyl 2-ethylhexanoate by direct esterification of cetyl alcohol with 2-ethylhexanoic acid was developed using an immobilized lipase (Novozym® 435) as a catalyst in *n*-hexane. Response surface methodology (RSM) and 5-level-4-factor central composite rotatable design (CCRD) were employed to evaluate the effects of reaction time, reaction temperature, substrate molar ratio, and enzyme amount on the yield of cetyl 2-ethylhexanoate. The results show that reaction time, reaction temperature, substrate molar ratio, and enzyme amount have significant effects on the yield of the esterification reaction. On the basis of ridge-max analysis, the optimum conditions were as follows: a reaction time of 2.65 days, a reaction temperature of 56.18 °C, a substrate molar ratio of 2.55:1, and an enzyme amount of 251.39%. The predicted and experimental values of molar conversion were 91.95 and 89.75 ± 1.06%, respectively.

Keywords Biocatalysis · Cetyl 2-ethylhexanoate · Lipase · Optimization · Response surface methodology · Wax esters

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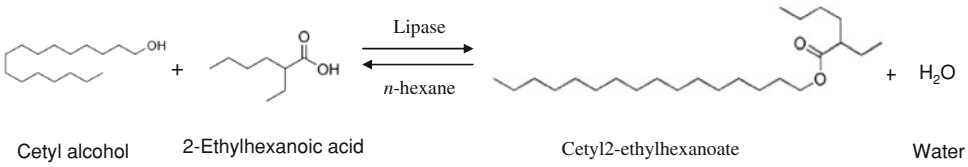
Introduction

Wax esters are esters that are derived from long-chain fatty acids and long-chain fatty alcohols. They are natural chemicals and can be extracted from jojoba oil, sperm whale oil, beeswax, and seafowl feathers [1, 2]. The presence of wax esters on feathers provides the waterproofing properties of the plumage so that seafowl can swim, dive and rest on the water. Cetyl 2-ethylhexanoate is one of the main wax esters found on seafowl feathers, and its chemical structure is shown in Scheme 1.

The compounds are formulated in numerous personal care products due to their excellent wetting behavior at interfaces and non-greasy feeling when applied on the skin surface [3]. They are also important ingredients in cosmetics, pharmaceuticals, lubricants, plasticizers, and polishes [1, 4].

Since the cost and availability of wax esters from natural resources such as jojoba oil and sperm whale oil are limited, the attempts to synthesize wax esters with cheaper starting materials and simple methods have become very important [1]. Wax esters can be synthesized by chemical or enzymatic reactions. Compared to chemical catalysis, lipase-catalyzed synthesis is more substrate specific and therefore suitable for producing high-quality natural products. The enzymatic reaction is conducted under moderate reaction conditions (pH, temperature, atmospheric

Scheme 1 Lipase-catalyzed synthesis of cetyl 2-ethylhexanoate from cetyl alcohol and 2-ethylhexanoic acid



pressure) and requires only simple steps to purify the product from the reaction mixture. The wax esters obtained from the enzymatic reaction can be labeled as 'natural identical' and used in cosmetics or food products.

There are many reports investigating the synthetic methods for wax esters. Hunter et al. [5] synthesized cetyl myristoleate by chemical reactions between myristoleic acid and cetyl alcohol. Decagny et al. [6] and Hadzir et al. [7] reported the lipase-catalyzed synthesis of wax esters through triolein alcoholysis with stearyl/oleyl alcohol. Wax esters can also be produced by lipase-catalyzed alcoholysis of vegetable oils with different alcohols [8]. Petersson et al. [9] successfully prepared cetyl palmitate in a solvent-free process using an immobilized lipase as catalyst. For the optimal synthesis of wax esters via lipase catalysis, it is important to design a simple and efficient enzymatic biosynthesis system from large experimental variables and to obtain the optimum production of valuable products using an appropriate solvent formulation over a short time period and with minimal trials. Several functional statistical models, such as response surface methodology (RSM), have been successfully applied to investigate the possible interactions and to optimize various valuable wax ester production by lipase [1, 3, 8]. So far, there have been no reports regarding lipase-catalyzed synthesis of cetyl 2-ethylhexanoate. The literature is limited on lipase-catalyzed esterification from ethyl branched acids and long chain alcohol substrates.

In this study, an RSM and a 5-level-4-factor central composite rotatable design (CCRD) were employed to investigate the affinities between the reaction variables (reaction time, reaction temperature, substrate molar ratio, and enzyme amount) and response (yield %), and to obtain the optimal conditions for lipase-catalyzed synthesis of wax esters with branched fatty acids.

Materials and Methods

Materials

Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3; Novozym® 435) from *Candida antarctica*, supported on acrylic resin, was purchased from Novo Nordisk Bioindustrials, Inc. (Bagsvaerd, Denmark). The catalytic activity of Novozym® 435 was 10,000 PLU (Propyl Laurate Units)/g containing 1–2% (w/w) water. Cetyl alcohol (99%) and 2-ethylhexanoic acid (99%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cetyl 2-ethylhexanoate was purchased from Kao Chemicals Co. (Tokyo, Japan). A molecular sieve (4 Å) was purchased from Davison Chemical (Baltimore, MD, USA), and *n*-hexane was obtained from the Merck Chemical Co. (Darmstadt, Germany). All of the other chemicals were of analytical reagent grade.

Experimental Design

A 5-level-4-factor CCRD was employed in this study; 27 experiments were run. The fractional factorial design consisted of 16 factorial points, 8 axial points, and 3 center points. The variables and their levels were: reaction time (1–3 days), reaction temperature (45–65 °C), substrate molar ratio (2-ethylhexanoic acid:cetyl alcohol = 1–3:1, w/w), and enzyme amount (Novozym® 435/cetyl alcohol = 100–300%, w/w). Table 1 shows the independent factors (x_i), levels and experimental design, both coded and uncoded. The 27 runs were performed in a fully random order to avoid bias.

Enzymatic Esterification

All of the reagents were dehydrated by molecular sieves (4 Å) for 24 h. Lipase-catalyzed synthesis of cetyl 2-ethylhexanoate from cetyl alcohol and 2-ethylhexanoic acid is presented in Scheme 1. Cetyl alcohol (0.1 M), different molar ratios of 2-ethylhexanoic acid and different amounts of Novozym® 435 were well mixed in *n*-hexane. The esterification reaction was carried out in an orbital shaking water bath (200 rpm) under various reaction temperatures and reaction times as shown in Table 1.

Determination of Cetyl 2-Ethylhexanoate

The cetyl 2-ethylhexanoate formation was determined by injecting 1 μL of the reaction mixture in splitless mode into a gas chromatograph (Hewlett Packard 7890, Avondale, PA, USA) equipped with a flame-ionization detector (FID) and an MXT-65TG fused silica capillary column (30 m × 0.25 mm i.d.; film thickness 1 μm; Restek, Bellefonte, PA, USA). Injector and FID temperatures were set at 240 and 250 °C, respectively. The oven temperature was maintained at 125 °C for 5 min, increased to 230 °C at

Table 1 Central composite rotatable design and experimental data for 5-level-4-factor and response surface analysis

Treatment[a]	Factors				Experimental yield (%)	Predicted yield[d] (%)
	Reaction time (day) X_1	Reaction temperature (°C) X_2	Substrate molar ratio (Ac:Al)[b] X_3	Enzyme amount (%)[c] X_4		
1	−1(1.5)	−1(50)	−1(1.5)	1(250)	45.42 ± 0.01	42.86
2	−1(1.5)	−1(50)	1(2.5)	−1(150)	32.50 ± 0.87	33.07
3	−1(1.5)	1(60)	−1(1.5)	−1(150)	63.47 ± 3.34	58.39
4	−1(1.5)	1(60)	1(2.5)	1(250)	72.84 ± 1.11	74.43
5	1(2.5)	−1(50)	−1(1.5)	−1(150)	44.68 ± 2.47	41.83
6	1(2.5)	−1(50)	1(2.5)	1(250)	79.79 ± 0.13	83.61
7	1(2.5)	1(60)	−1(1.5)	1(250)	73.97 ± 2.61	72.15
8	1(2.5)	1(60)	1(2.5)	−1(150)	77.10 ± 0.87	78.40
9	−1(1.5)	−1(50)	−1(1.5)	−1(150)	28.28 ± 0.83	23.43
10	−1(1.5)	−1(50)	1(2.5)	1(250)	65.52 ± 0.22	59.53
11	−1(1.5)	1(60)	−1(1.5)	1(250)	65.43 ± 0.02	62.33
12	−1(1.5)	1(60)	1(2.5)	−1(150)	69.80 ± 3.51	63.47
13	1(2.5)	−1(50)	−1(1.5)	1(250)	59.54 ± 0.41	61.55
14	1(2.5)	−1(50)	1(2.5)	−1(150)	58.08 ± 1.23	56.86
15	1(2.5)	1(60)	−1(1.5)	−1(150)	66.25 ± 2.71	67.92
16	1(2.5)	1(60)	1(2.5)	1(250)	89.11 ± 1.35	89.65
17	−2(1)	0(55)	0(2)	0(200)	36.12 ± 0.43	46.21
18	2(3)	0(55)	0(2)	0(200)	84.33 ± 0.09	79.82
19	0(2)	−2(45)	0(2)	0(200)	36.18 ± 1.36	38.93
20	0(2)	2(65)	0(2)	0(200)	77.10 ± 4.36	79.93
21	0(2)	0(55)	−2(1)	0(200)	40.32 ± 2.77	45.83
22	0(2)	0(55)	2(3)	0(200)	72.89 ± 2.20	72.97
23	0(2)	0(55)	0(2)	−2(100)	39.57 ± 1.01	45.18
24	0(2)	0(55)	0(2)	2(300)	75.90 ± 1.88	75.87
25	0(2)	0(55)	0(2)	0(200)	63.67 ± 2.81	63.54
26	0(2)	0(55)	0(2)	0(200)	63.59 ± 2.99	63.54
27	0(2)	0(55)	0(2)	0(200)	63.35 ± 1.46	63.54

[a] All treatments were run in random order

[b] (Ac:Al) was the molar ratio of 2-ethylhexanoic acid:cetyl alcohol

[c] The enzyme amount is the weight percentage of cetyl alcohol

[d] The predicted yields were calculated by RSREG equation

a rate of 50 °C/min, and then kept at 230 °C for 11.9 min. Nitrogen was used as the carrier gas.

The yield % was defined as (mmol of cetyl 2-ethylhexanoate/mmol of initial cetyl alcohol) × 100% and was estimated using the peak area integrated by on-line software Hewlett Packard 6890 Series II Chem Station (Hewlett Packard 6890, Avondale, PA, USA). A gas chromatogram of the reaction mixture is shown in Fig. 1. There are 4 peaks in the GC analysis: cetyl alcohol (substrate), 2-ethylhexanoic acid (substrate), *n*-hexane (solvent) and cetyl 2-ethylhexanoate (product). The retention time was increased in tandem with the carbon number.

Statistical Analysis

The experimental data (Table 1) were analyzed by the response surface regression (RSREG) procedure of SAS software to fit the following second-order polynomial equation:

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

where Y is the response (yield %), B_{k0}, B_{ki}, B_{kii} and B_{kij} are constant coefficients and x_i and x_j are the uncoded independent variables. The ridge-max option was used to compute the estimated ridge of maximum response for increasing the radii from the center of the original design.

Results and Discussion

Effects of Reaction Parameters

The influence of reaction time on the yield of cetyl 2-ethylhexanoate is shown in Fig. 2; when the reaction time increased, the initial yield of cetyl 2-ethylhexanoate also increased. The highest yield (~95%) was obtained from a

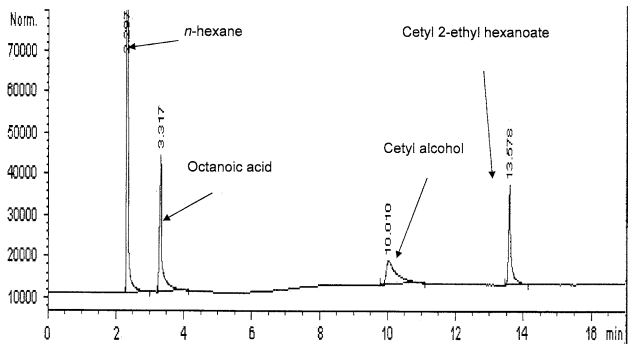


Fig. 1 Gas chromatogram of the reaction mixture. Each peak was identified by comparison with corresponding standards

300% enzyme amount after 3 days. After that, the curve leveled off. In contrast, the yield was only 40% after 5 days at 30% enzyme amount. Many researchers have shown that the addition of a molecular sieve facilitated the reaction rate of esterification because of the removal of excess water [10–12]. However, the yield was not significantly increased in our study when the molecular sieve was added into the reaction mixture. From preliminary experimental data in Fig. 2, the variables and their levels selected in this study were: reaction time (1–3 days); temperature (45–65 °C); substrate molar ratio (1–3:1; Ac:Al); and enzyme amount (100–300%), as shown in Table 1. Table 1 also shows the actual yields obtained from experiments as well as the predicted yields derived from the model. Both values were reasonably close, indicating the effectiveness of the statistical analysis used in this study.

Model Fitting

The RSREG procedure was employed to fit the second-order polynomial Eq. 1 to the experimental data (Table 1). Among the various treatments (Table 1), the highest molar conversion (89.11 ± 1.35%) was treatment no. 16 (a reaction time of 2.5 days, a reaction temperature of 60 °C, a substrate molar ratio of 2.5:1, and an enzyme amount of 250%). The lowest molar conversion (28.28 ± 0.83%) was treatment no. 9 (a reaction time of 1.5 days, a reaction temperature of 50 °C, a substrate molar ratio of 1.5:1, and an enzyme amount of 150%). From the SAS output of RSREG, the second-order polynomial Eq. 2 obtained is given below (X_1: reaction time, X_2: temperature, X_3: substrate molar ratio and X_4: enzyme amount):

$$\begin{aligned} Y(\%) = & -562.99 + 56.318X_1 + 12.353X_2 + 30.377X_3 \\ & + 0.980X_4 - 0.522X_1^2 - 0.887X_1X_2 - 0.041X_2^2 \\ & + 5.395X_1X_3 - 0.456X_2X_3 - 4.142X_3^2 + 0.003X_1X_4 \\ & - 0.016X_2X_4 + 0.070X_3X_4 - 0.0003X_4^2 \end{aligned} \quad (2)$$

The analysis of variance (ANOVA) data are shown in Table 2. The results indicate that the second-order polynomial model is an adequate representation of the actual relationship between the response and the significant variables, with a very small p value (0.0001) and a satisfactory coefficient of determination ($R^2 = 0.9498$). Furthermore, the overall effect of the four synthesis variables on the yield of cetyl 2-ethylhexanoate was further analyzed by a joint test (Table 3). These results revealed that reaction time, reaction temperature, substrate molar ratio, and enzyme amount are important parameters which have a statistically significant overall effect ($p < 0.01$) on the yield of cetyl 2-ethylhexanoate.

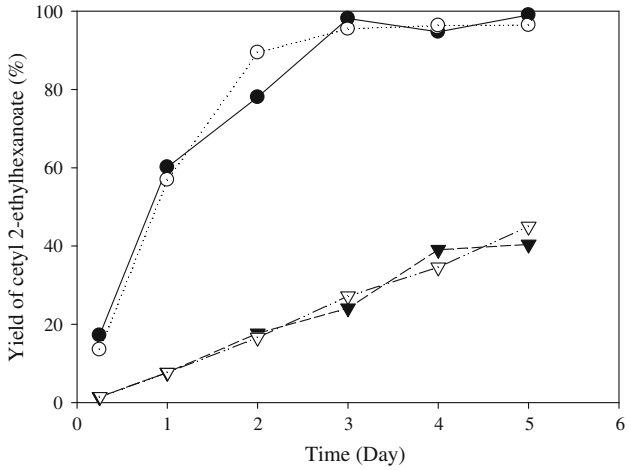


Fig. 2 The effects of reaction time on the yield of cetyl 2-ethylhexanoate. Reaction conditions: reaction temperature of 55 °C, substrate molar ratio of 2:1 (acid:alcohol), *open circles* enzyme amount of 300%, no molecular sieve; *closed circles* enzyme amount of 300%, molecular sieve of 15 mg; *open inverted triangles* enzyme amount of 30%, no molecular sieve; *close inverted triangles* enzyme amount of 30%, molecular sieve of 15 mg

Table 2 Analysis of variance for synthesis variables pertaining to the response of percent yield

Source	Degree of freedom	Sum of squares	F value	Pr > F
Linear	4	6733.6609	53.13	<0.0001[a]
Quadratic	4	40.4515	0.32	0.8598
Cross product	6	418.2584	2.20	0.1155
Total model	14	7192.3709	16.21	<0.0001[a]
Lack of fit	10	380.1856	1370.86	0.0007[a]
Pure error	2	0.0555		
Total error	12	380.2411		
R^2	0.9498			

[a] $p < 0.01$, significant at 1% level

Mutual Effect of Parameters

Reaction times and reaction temperatures were investigated in the range of the reaction time of 1–3 days and

temperatures of 45–65 °C, respectively. Fig. 3a represents the effect of varying reaction time and reaction temperature on esterification efficiency at a substrate molar ratio of 2:1 and an enzyme amount of 200%. With the highest reaction temperature (65 °C) and highest reaction time (3 days), cetyl 2-ethylhexanoate yield of 89% was obtained. Whereas, when the reaction temperature was decreased to 45 °C and the reaction time shortened (1 day), only 13% yield remained.

Figure 3b shows the effects of enzyme amount, reaction temperature and their mutual interaction on cetyl 2-ethylhexanoate synthesis at a reaction time of 2 days and a substrate molar ratio of 2:1. At the lowest reaction temperature (45 °C) and enzyme amount (100%), the yield was 5%. The yield increased remarkably when the reaction temperature and enzyme amount increased. The effect of substrate molar ratio and reaction temperature on esterification efficiency at a constant reaction time (2 days) and a constant enzyme amount (200%) is shown in Fig. 3c. At any given substrate molar ratio (1–3:1; Ac:Al), an increase in reaction temperature tends to give higher yields of cetyl 2-ethylhexanoate. At the highest substrate molar ratio (3:1) and highest reaction temperature (65 °C), the yield of cetyl 2-ethylhexanoate was 84%.

The relationships between reaction factors and response can be better understood by examining the planned series of contour plots (Fig. 4) generated from the predicted model by holding constant enzyme amounts (150, 200, 250%) and substrate molar ratios (1.5:1, 2:1, 2.5:1). Fig. 4a–c represent the same enzyme amount (150%); and a, d, and g represent the same substrate molar ratio (1.5:1). In general, all nine contour plots in Fig. 4 exhibit similar behavior, in that predicted yield increased according to increased reaction time and reaction temperature. The reaction with a 250% enzyme amount and substrate molar ratio 2.5:1 (Fig. 4i) is suggested as the optimal condition

Table 3 Analysis of variance for the joint test

Factor	Degree of freedom	Sum of squares	*F* value	Pr > *F*
Reaction time (X_1)	5	1803.0113	11.38	0.0003[a]
Temperature (X_2)	5	2883.7068	18.20	<0.0001[a]
Substrate molar ratio (X_3)	5	1226.9909	7.74	0.0018[a]
Enzyme amount (X_4)	5	1714.2826	10.82	0.0004[a]

[a] $p < 0.01$, significant at 1% level

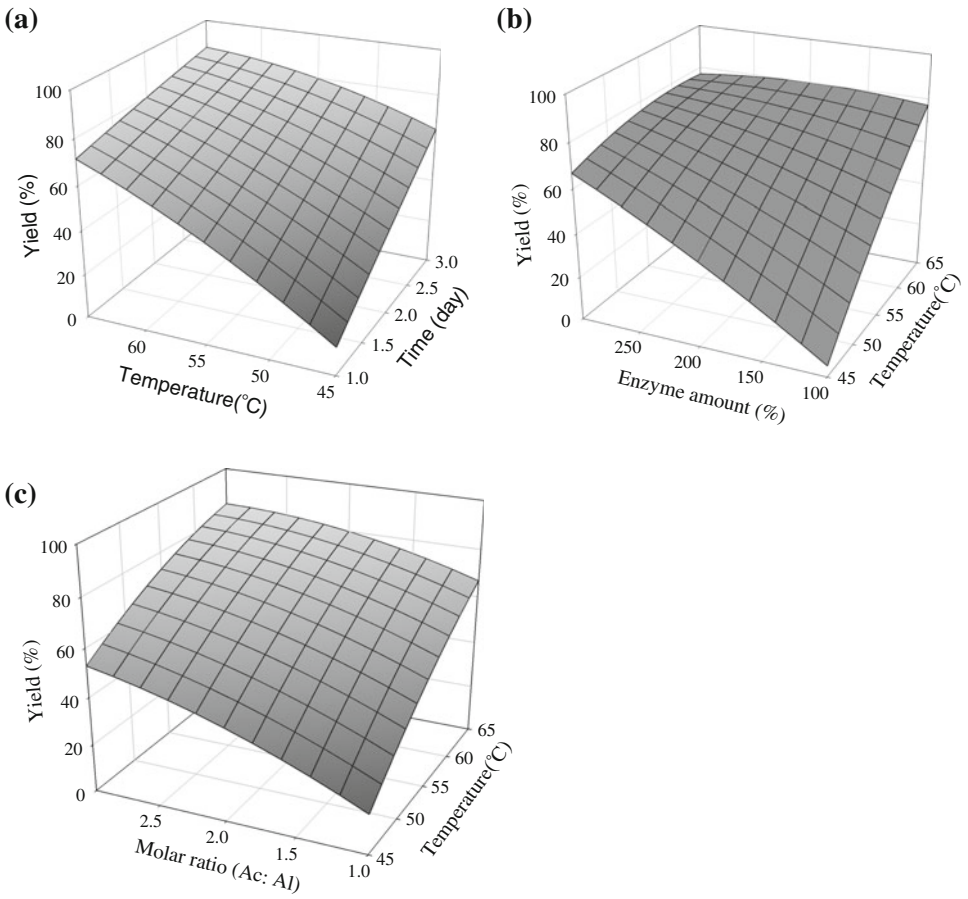


Fig. 3 Response surface plots showing the relationships between cetyl 2-ethylhexanoate yield and reaction parameters: **a** reaction time and temperature; **b** enzyme amount and temperature; **c** substrate molar ratio and temperature

for enzymatic biosynthesis of cetyl 2-ethylhexanoate which represented higher predicted yield than the others' in Fig. 4.

Obtaining Optimal Synthesis Conditions

The optimal point of synthesis was determined by ridge-max analysis, which approximates the estimated ridge of maximum response for increasing radii from the center of the original design. The ridge-max analysis indicates that maximum molar conversion was 91.95% at a reaction time of 2.65 days, a reaction temperature of 56.18 °C, a substrate molar ratio of 2.55:1, and an enzyme amount of 251.39%.

Model Verification

The adequacy of the predicted model was examined by additional independent experiments at the suggested optimal synthesis conditions. The predicted yield was 91.95%, and the actual experimental value was 89.75 ± 1.06%. Thus, the optimization of lipase-catalyzed synthesis for cetyl 2-ethylhexanoate was successfully developed by RSM and CCRD design.

Conclusions

We developed an optimal system for the production of wax esters by direct esterification of cetyl alcohol with 2-ethylhexanoic acid using immobilized lipase (Novozym® 435) in *n*-hexane. RSM and 5-level-4-factor CCRD were employed for the optimization of esterification reactions. The ridge-max analysis indicates that maximum yield was 91.95% at a reaction time of 2.65 days, a reaction temperature of 56.18 °C, a substrate molar ratio of 2.55:1, and an enzyme amount of 251.39%. The enzymatic synthesis production of cetyl 2-ethylhexanoate can be used as a base oil in cosmetics. In this study, we found that with branched acid substrates (2-ethylhexanoic acid) it was more difficult to achieve esterification via lipase- catalysis. Prolonged reaction time and large enzyme amounts were required to achieve a high conversion of cetyl 2-ethylhexanoate.

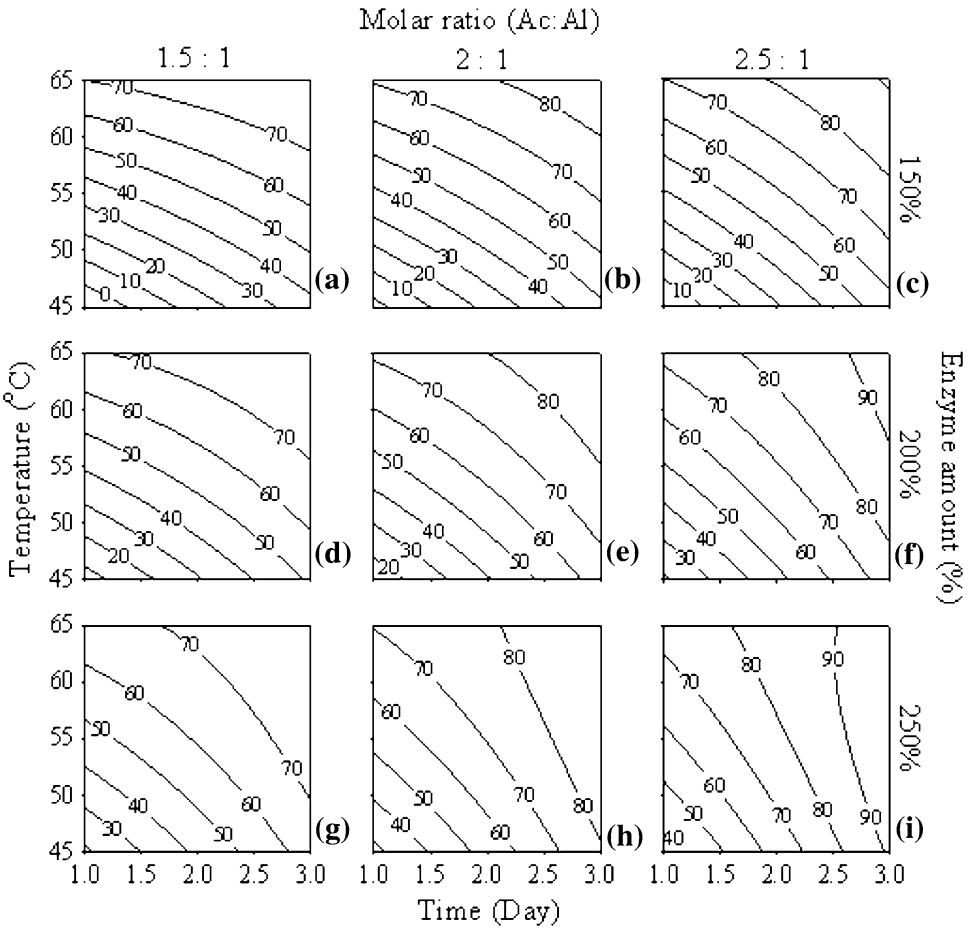


Fig. 4 Contour plots of yield of cetyl 2-ethylhexanoate. The *numbers* inside the contour plots indicate yield under given reaction conditions

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