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# Optimization of Lipase-Catalyzed Synthesis of Sorbitan Acrylate Using Response Surface Methodology

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## Abstract

In this study, we have synthesized sorbitan acrylate through response surface methodology, using sorbitan and vinyl acrylate that catalyze immobilized lipase. In order to optimize the enzymatic synthesis of the sorbitan acrylate, we applied response surface techniques to determine the effects of five-level-four-factors and their reciprocal interactions with the biosynthesis of sorbitan acrylate. Our statistical model predicted that the highest conversion yield of sorbitan acrylate would be approx 100%, under the following optimized reaction conditions: a reaction temperature of 40.1°C, a reaction time of 237.4 min, an enzyme concentration of 8%, and a 4.49 : 1 acyl donor/acceptor molar ratio. Using these optimal conditions in three independent replicates, the conversion yield reached 97.6 ± 1.3%.

**Index Entries:** Bioconversion; central composite rotatable design; esterification; lipase; optimization; response surface methodology; sorbitan ester.

## Introduction

Esterification is the principal process in the synthesis of sugar esters. This process has been extensively studied, and is achievable through both chemical and enzymatic processes. Chemical esterification has been associated with low regioselectivity, which results in poor selectivity, undesirable side reactions, and low yields. However, enzymatic esterification can be applied to the regioselective transformation of several sugars, and does not tend to result in any undue complications *(1–6)*. As compared with conventional chemical processes, enzymatic esterification can be conducted under mild conditions, and exhibits an excellent degree of selectivity, allowing for the generation of pure materials through more efficient and environmentally friendly processes, than are associated with more

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conventional chemical methods. Enzymatic esterification carries the advantages of high material stability, low energy costs, a high degree of selectivity, and low purification costs. Therefore, enzymatic esterification appears to constitute a favorable alternative synthesis technique *(1–6)*.

Recently, a host of reports have noted the myriad possible applications of sugar-harboring polymeric materials synthesized from sugar esters. Sugars constitute an attractive group of multifunctional compounds, as they are both biologically relevant, and harbor multiple hydroxyl groups. Sugar esters have become the focus of increased interest of late, and have already been utilized in several fields, and a variety of applications. They have proven useful in diverse industries, and have been used as detergents, emulsifiers, lubricants, flavorings, and cosmetic additives. Sugar esters are also biodegradable, biocompatible, and nontoxic *(4,7,8)*.

Sorbitan esters are prepared either in one- or two-step reactions. In the one-step reaction, sorbitol and acyl donor are converted simultaneously to the sorbitan ester. In two-step reaction, first, sorbitol is dehydrated to sorbitan with acid catalyst (*p*-toluenesulfonic acid, sulfuric acid, and phosphoric acid). Second, formed sorbitan is esterified to sorbitan ester with the acyl donor and catalyst (alkali catalyst and enzyme) *(2,4–6,9)*.

In this study, we have evaluated two distinct esterification schemes: acylation and alcoholysis (transesterification). Acylation is the esterification of a carboxylic acid (e.g., acrylic acid [AA]) with the hydroxyl group on the acyl acceptor (e.g., 1,4-sorbitan), and generates an ester and a water byproduct. However, transesterification involves the esterification of an ester (e.g., methyl acrylate [MA], ethyl acrylate [EA], and vinyl acrylate [VA]) with the hydroxyl group on the acyl acceptor (e.g., 1,4-sorbitan). Alcoholysis generates another ester, but yields alcohol, rather than water, as a byproduct *(4–6)*. In the enzymatic process utilized in the production of sorbitan acrylate from 1,4-sorbitan, several factors can affect both glycosylation yields and rates. These factors include the reaction solvent, reaction temperature, the type and concentration of the acyl donor, the quantity of enzyme in the reaction, the water content, and the initial concentration of substrate *(3–5)*. In particular, the difficulty inherent to the dissolution of both hydrophobic and hydrophilic substrates in a common reaction solvent of low toxicity has been the principal limitation of biological synthesis *(1,4,6)*.

In all of our experiments, *t*-butanol was used as the reaction solvent owing primarily to its high substrate solubility, remarkable affinity for glycoside, its regioselective effect with glycoside, and the ease inherent to the separation/purification of the products, which is attributable to its low boiling point *(3–5,10)*. In our study, we have conducted the enzymatic synthesis of sorbitan acrylate using Novozym 435, a well-known nonspecific lipase. Novozym 435 facilitates reactions between a wide range of alcohols and vinyl esters, and is a remarkably heat-tolerant enzyme *(11)*.

Response surface methodology (RSM) is an effective statistical technique used in the research of complex processes. RSM uses multiple regression and correlation analyses as tools to test the effects of two or more independent factors on the dependent factors. The primary advantage of RSM is the reduced number of experimental runs required to generate sufficient information for a statistically acceptable result. It is both faster and less expensive than the classical research method. RSM has been successfully applied to the study and optimization of the enzyme synthesis of sugar esters. In addition, central composite rotatable design (CCRD) is a RSM that has previously been successfully used in the optimization of several biotechnological processes *(4,8,12–16)*.

In this study, we conducted acylation and alcoholysis using immobilized lipase, in order to synthesize sorbitan acrylate. First, several acyl donors were tested in order to select the optimal acyl donor for esterification. Second, the effects of different initial sorbitan concentrations, water contents, and molecular sieves were tested. Finally, RSM, consisting of a five-level-four-factor CCRD, was applied in order to evaluate the interactive effects, and to optimize the conditions for the enzymatic synthesis of sorbitan acrylate, with VA being used as the acyl donor.

## Materials and Methods

### Chemicals

The Novozym 435 (lipase B from *Candida antarctica*, EC 3.1.1.3, a nonspecific lipase immobilized on a macroporous acrylic resin, 1–2% water content, 10,000 propyl laurate units/g) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). The D-sorbitol, VA, and *t*-butanol were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO). The AA, MA, and EA were obtained from Daejung Chemicals & Metals Co., Ltd. (Korea), Junsei Chemical Co., Ltd. (Japan), and Yakuri Pure Chem. Co., Ltd. (Japan), respectively. The acetonitrile was obtained from Fisher Scientific. All other chemicals were of analytical grade, and the solvents were dried using molecular sieves (4 Å, Yakuri Pure Chem. Co., Ltd.) 1 d before use.

### 1,4-Sorbitan Preparation

All dehydration reactions (sorbitol cyclization) for the synthesis of 1,4-sorbitan, using *p*-toluenesulfonic acid in a solvent-free process, were conducted according to the previously reported methods *(3)*.

### Enzymatic Esterification and Experimental Design

In this study, two distinct esterification schemes were conducted: acylation (AA) and alcoholysis (MA, EA, and VA). All esterification reactions for the synthesis of 1,4-sorbitan esters were performed using immobilized lipase (Novozym 435). The reaction temperature was controlled using a water bath equipped with a PID temperature controller. Mixing was performed with a magnetic stirrer, which spun at approx 200 rpm. The condenser prevented the evaporation of the reactants. During all of the reactions, 0.2 mL of the samples were withdrawn at set intervals, and then monitored through high-performance liquid chromatography. In all experiments with no water content, there was no set initial amount of water, with the exception of the water contained in the enzymes themselves. The results of the experiments are expressed as the mean values from at least two independent measurements.

In order to select the optimal acyl donor for enzymatic esterification, we assessed the effects of four acyl donors, as is described below. 30 g/L of prepared 1,4-sorbitan was added to the bottle, and either AA, VA, EA, or MA were subsequently added at a 1 : 3 molar ratio, in order to determine the most favorable acyl donor at 45°C and 5% (w/v) Novozym 435. In order to determine the optimal initial 1,4-sorbitan concentration for enzymatic esterification, 25–100 g/L of prepared 1,4-sorbitan was added to the bottle, at a molar ratio of 1 : 3 with VA, and 5% (w/v) Novozym 435 at 45°C. In order to determine the optimal initial water content and molecular sieve amounts for enzymatic esterification, 0–5% (v/v) water or 0–2% (w/v) molecular sieves were added to the reactant, coupled with 50 g/L of prepared 1,4-sorbitan, at a molar ratio of 1 : 3 with VA, and 5% (w/v) Novozym 435 at 45°C.

In order to apply RSM in the enzymatic synthesis of sorbitan acrylate, a five-level-four-factor CCRD scheme was adopted in this study, requiring 30 experiments, which included 16 factorial points, eight axial points, and six central points in order to provide information regarding the interior of the experiment region, allowing for the evaluation of curvature *(9)*. When applying RSM to our sorbitan acrylate synthesis scheme, we set an initial concentration of 1,4-sorbitan of 50 g/L. The variables, and their levels, selected for the study of sorbitan acrylate synthesis, were as follows: reaction temperature (25–65°C), reaction time (30–240 min), enzyme amount (1–7% [w/v]), and acyl donor/acyl acceptor molar ratio (1 : 1–5 : 1; VA : sorbitan molar ratio). Table 1 provides the coded and uncoded independent factors ($X_i$), the levels, and the experimental design.

### *Statistical Analysis*

Our experimental data (Table 2) were analyzed through RSM, to fit the following second-order polynomial equation generated by Design-Expert 6 software (Stat-Ease, Inc.). Second-order coefficients were generated through regression with stepwise elimination. The response was first fitted to the factors through multiple regression. The quality of the fit of the model was evaluated using the coefficients of determination ($R^2$) and the analysis of variances (ANOVA). The insignificant coefficients were eliminated after examining the coefficients, and the model was finally

Table 1
Factors and Their Levels for Central Composite Design

| Variable | Symbol | Coded factor levels | | | | |
|---|---|---|---|---|---|---|
| | | –2 | –1 | 0 | 1 | 2 |
| Reaction temperature (°C) | $X_1$ | 25 | 35 | 45 | 55 | 65 |
| Reaction time (min) | $X_2$ | 30 | 82.5 | 135 | 187.5 | 240 |
| Enzyme amount (wt%) | $X_3$ | 1 | 3 | 5 | 7 | 9 |
| Substrate molar ratio (acyl donor/acyl acceptor) | $X_4$ | 1 | 2 | 3 | 4 | 5 |

refined. The quadratic response surface model was fitted to the following equation:

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

where $Y$ is the response factor (conversion yield), $x_i$ is the $i$th independent factor, $\beta_0$ is the intercept, $\beta_i$ is the first-order model coefficient, $\beta_{ii}$ is the quadratic coefficient for the factor $i$, and $\beta_{ij}$ is the linear model coefficient for the interaction between factors $i$ and $j$.

### *Quantitative Analysis*

The enzymatic reactions were monitored through analysis of the conversion yield of 1,4-sorbitan. The reactant and product measurements were obtained through high-performance liquid chromatography with a ZORBAX carbohydrate column (5 µm, 120 Å, 250 × 4.6 mm$^2$, Agilent), and maintained at a constant 35°C. In order to measure the amount of sorbitan and its ester concentrations, we used a mixture of acetonitrile : water (75 : 25 [v/v]) as a mobile phase, at a flow rate of 1.0 mL/min. A 0.2 mL sample was extracted from each of the reaction mixtures at set time intervals throughout the reaction. The enzymes were removed through the filtration and dilution of the sample with buffer solution (acetonitrile : water, 50 : 50 [v/v]). 20 µL of a prepared sample was then administered. Detection was conducted using an RI detector (Shimazu RID-10A, Japan).

## Results and Discussion

In the enzymatic process used in the synthesis of sorbitan acrylate from 1,4-sorbitan, several factors can affect both the conversion yield and rate, including the reaction solvent, reaction temperature, acyl donor type and concentration, enzyme amount, water content, and initial substrate

Table 2
Central Composite Rotatable Second-Order Design, Experimental, and Estimated Data for 5-Level-4-Factor Response Surface Analysis

| Standard | Run | Temperature (°C), $X_1$ | Time (min), $X_2$ | Enzyme amount (wt%), $X_3$ | Substrate molar ratio (acyl donor/acceptor), $X_4$ | Conversion yield (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Experimental | Estimated |
| 1 | 25 | –1 | –1 | –1 | –1 | 23.4 | 19.5 |
| 2 | 19 | 1 | –1 | –1 | –1 | 14.3 | 10.0 |
| 3 | 17 | –1 | 1 | –1 | –1 | 40.5 | 40.4 |
| 4 | 11 | 1 | 1 | –1 | –1 | 15.2 | 14.1 |
| 5 | 7 | –1 | –1 | 1 | –1 | 49.8 | 48.4 |
| 6 | 16 | 1 | –1 | 1 | –1 | 38.7 | 38.9 |
| 7 | 6 | –1 | 1 | 1 | –1 | 73.8 | 77.2 |
| 8 | 26 | 1 | 1 | 1 | –1 | 43.5 | 50.9 |
| 9 | 3 | –1 | –1 | –1 | 1 | 32.6 | 32.7 |
| 10 | 23 | 1 | –1 | –1 | 1 | 24.1 | 23.2 |
| 11 | 20 | –1 | 1 | –1 | 1 | 50.8 | 53.6 |
| 12 | 12 | 1 | 1 | –1 | 1 | 23.4 | 27.3 |
| 13 | 18 | –1 | –1 | 1 | 1 | 60.7 | 61.6 |
| 14 | 21 | 1 | –1 | 1 | 1 | 52.5 | 52.1 |
| 15 | 2 | –1 | 1 | 1 | 1 | 86.3 | 90.4 |
| 16 | 10 | 1 | 1 | 1 | 1 | 65.2 | 64.1 |
| 17 | 9 | –2 | 0 | 0 | 0 | 51.7 | 49.9 |
| 18 | 27 | 2 | 0 | 0 | 0 | 14.6 | 14.0 |
| 19 | 28 | 0 | –2 | 0 | 0 | 26.0 | 32.0 |
| 20 | 13 | 0 | 2 | 0 | 0 | 73.4 | 65.0 |
| 21 | 15 | 0 | 0 | –2 | 0 | 11.2 | 14.1 |
| 22 | 30 | 0 | 0 | 2 | 0 | 85.2 | 79.8 |
| 23 | 5 | 0 | 0 | 0 | –2 | 34.3 | 35.5 |
| 24 | 1 | 0 | 0 | 0 | 2 | 65.5 | 61.9 |
| 25 | 4 | 0 | 0 | 0 | 0 | 62.6 | 58.6 |
| 26 | 8 | 0 | 0 | 0 | 0 | 59.9 | 58.6 |
| 27 | 22 | 0 | 0 | 0 | 0 | 57.2 | 58.6 |
| 28 | 24 | 0 | 0 | 0 | 0 | 56.5 | 58.6 |
| 29 | 29 | 0 | 0 | 0 | 0 | 57.9 | 58.6 |
| 30 | 14 | 0 | 0 | 0 | 0 | 57.2 | 58.6 |

concentration *(3)*. The first step of this study was the identification of factors likely to affect the conversion yield. Therefore, we assessed several screening factors for the RSM experiments, including several different acyl donors, initial sorbitan concentrations, water contents, and molecular sieves.

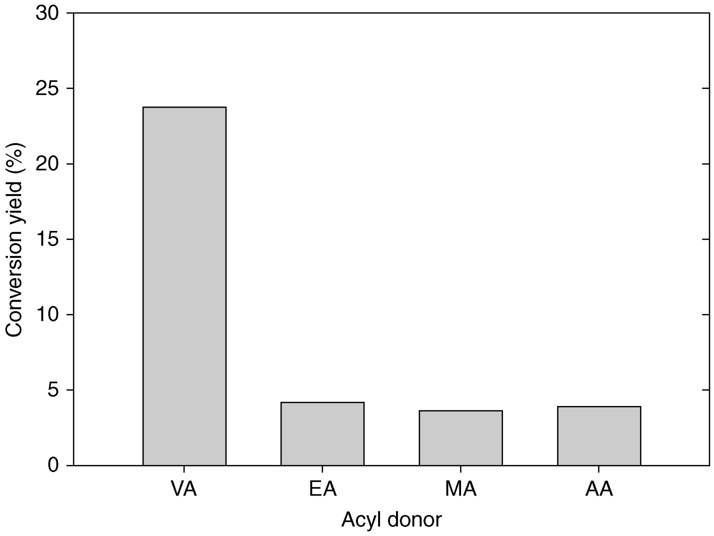

**Fig. 1.** Effect of acyl donor on enzymatic synthesis of sorbitan acrylate. Sorbitan concentration 30 g/L, acyl acceptor : acyl donor molar ratio 1 : 3, enzyme amount 5% (w/v), and reaction temperature 45°C. AA, MA, EA, and VA.

## *Effect of Acyl Donor*

In this study, we carried out two distinct esterification schemes (acylation and alcoholysis). In the acylation, AA was used as an acyl donor. In the alcoholysis, MA, EA, and VA were used as acyl donors. Figure 1 shows the effects of different acyl donors on the enzymatic esterification of sorbitan acrylate. The application of VA to the reaction was shown to result in a higher conversion yield than was observed with AA, MA, or EA. In cases in which VA was used as an acyl donor, vinyl alcohol was produced during periods in which glycosylation occurred. Therefore, we were able to determine that this process facilitates an irreversible glycosylation, which results in higher conversion yields *(3,5,10,17)*.

## *Effects of Initial Sorbitan Concentration*

In a previous experiment, we had identified VA as the optimal acyl donor. The effects of initial sorbitan concentrations on the conversion of 1,4-sorbitan with VA to sorbitan acrylate were investigated using Novozym 435. As has been shown in Fig. 2, the conversion of 1,4-sorbitan to sorbitan acrylate was approx 19.2% at 135 min, with a 25 g/L sorbitan concentration. High initial sorbitan concentrations, in excess of 50 g/L, resulted in low conversion yields. Sorbitan acrylate was synthesized at a conversion yield of approx 31.1% at an initial 1,4-sorbitan concentration of 100 g/L. In contrast to the final conversion yield of 55%, the initial conversion rate was found to be higher at a sorbitan concentration of 50 g/L within 135 min than was seen when 100 g/L of sorbitan was applied.

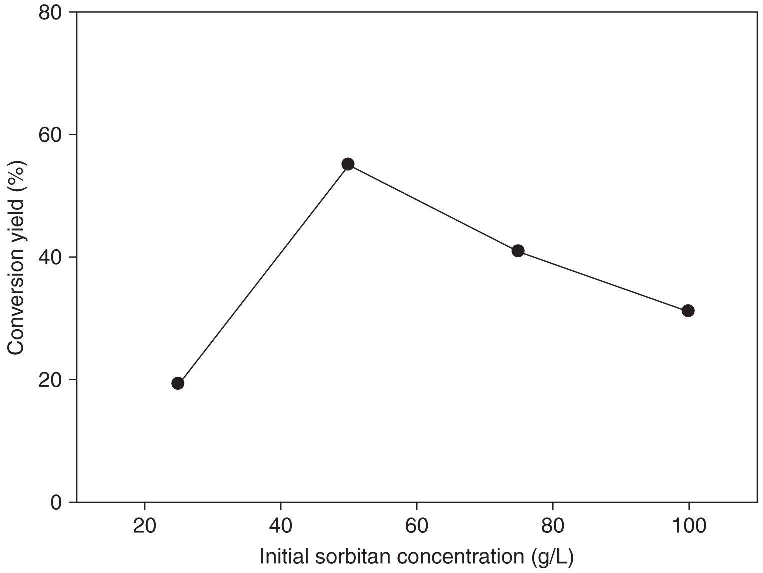

**Fig. 2.** Effect of initial sorbitan concentration on enzymatic synthesis of sorbitan acrylate. Sorbitan : VA molar ratio 1 : 3, enzyme amount 5% (w/v), and reaction temperature 45°C.

### *Effects of Water Content and Molecular Sieve*

In a previous experiment, the optimal initial sorbitan concentration was determined to be 50 g/L, and so this concentration was used in all subsequent experiments. Generally, at least a trace of water is required for enzymatic bioconversion. Enzymes manifest different reaction and selectivity behavior in organic solvents than in water *(3–6,17)*. In this experiment, the effects of water content (0–5% [v/v]) and molecular sieve amount (0–2% [w/v]) on the synthesis of sorbitan acrylate were investigated under the following conditions: 50 g/L of 1,4-sorbitan, a 1 : 3 molar ratio of VA, 0.15% (w/v) Novozym 435, and a reaction time of 135 min at 45°C.

As shown in Fig. 3, the addition of water resulted in an inhibition of the conversion of sorbitan acrylate. High water content induced low-conversion yields in a range of 1–5% (v/v). At 5% (v/v) water content, sorbitan acrylate was synthesized with a yield of approx 29.8%. Compared with the yields achieved under other conditions, the final conversion yield was 57.2% under conditions in which no water had been added, except for the inherent water content of the immobilized enzymes used.

Figure 4 shows the effects of the application of molecular sieves as water absorbents on the enzymatic synthesis of sorbitan acrylate, when using VA as the acyl donor. The addition of molecular sieves was found to generally inhibit sorbitan conversion. As compared with the 57.2% conversion yield achieved when no molecular sieves were added, the application of 2% (w/v) molecular sieves resulted in an approx 48.8% sorbitan

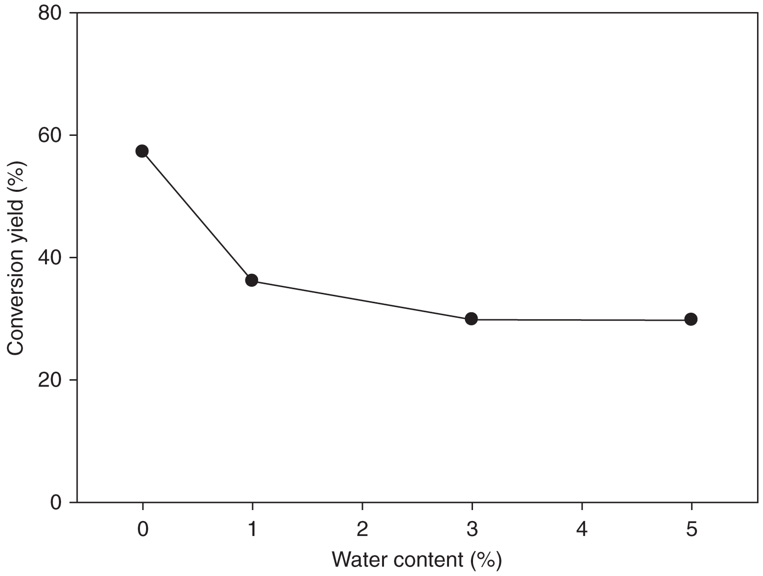

**Fig. 3.** Effect of water content on enzymatic synthesis of sorbitan acrylate. Sorbitan concentration 50 g/L, sorbitan : VA molar ratio 1 : 3, enzyme amount 5% (w/v), and reaction temperature 45°C.

acrylate conversion rate. As is shown in Figs. 3 and 4, neither the addition of water nor the dehydration of water with molecular sieves was required for the enzymatic synthesis of sorbitan acrylate using Novozym 435. In subsequent experiments, then, the reaction mixtures contained no water, except for the inherent water content of the enzymes used.

## *RSM of Esterification*

In order to construct a proper model for the optimization of sorbitan acrylate synthesis, we elected to use a CCRD, generally considered to be the best design for response surface optimization. This design featured the following five-level four-factors: reaction temperature, reaction time, enzyme amount, and acyl donor/acyl acceptor molar ratio. Table 2 shows the experimental parameter settings and results on the basis of the experimental design. A total of 30 designed experiments were then conducted, and the results were analyzed through multiple regression. The coefficients of the full model were then evaluated through regression analysis, and tested for their significance. The insignificant coefficients were eliminated in a stepwise fashion on the basis of p-values after the coefficients had been tested. Finally, the best fitting model was determined through regression and stepwise elimination. Ultimately, four linear coefficients (X1, X2, X3, and X4), three quadratic coefficients ($X_1^2$, $X_2^2$, $X_3^2$, and $X_4^2$, and two cross-product coefficients (X1X2 and X2X3) were determined to be significant (Tables 3 and 4).

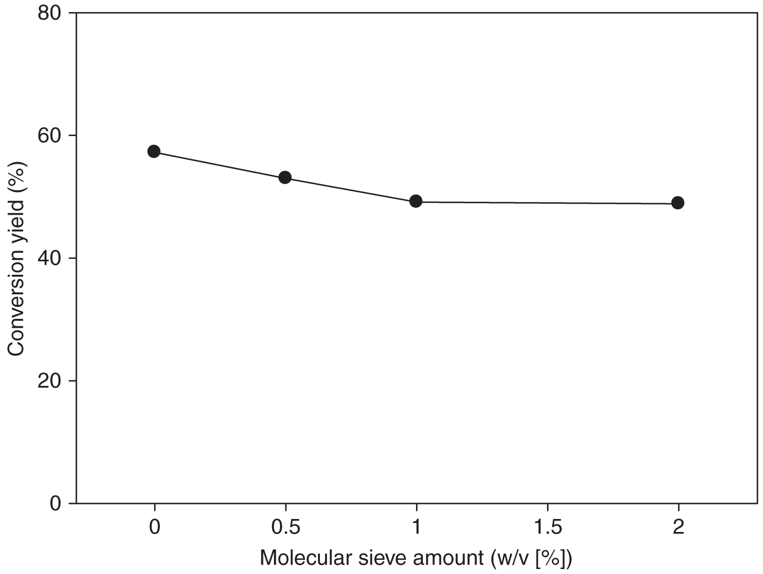

**Fig. 4.** Effect of molecular sieve on enzymatic synthesis of sorbitan acrylate. Sorbitan concentration 50 g/L, sorbitan : VA molar ratio 1 : 3, enzyme amount 5% (w/v), and reaction temperature 45°C.

Table 3
Regression Coefficients and Significance of Response Surface-Reduced Quadratic Model After a Stepwise Elimination

| Factor | Coefficient estimate | DF | Standard error | 95% CI low | 95% CI high |
|---|---|---|---|---|---|
| Intercept | 58.56 | 1 | 1.721 | 54.954 | 62.156 |
| $X_1$ | –8.96 | 1 | 0.860 | –10.760 | –7.159 |
| $X_2$ | 8.23 | 1 | 0.860 | 6.432 | 10.034 |
| $X_3$ | 16.43 | 1 | 0.860 | 14.626 | 18.227 |
| $X_4$ | 6.61 | 1 | 0.860 | 4.810 | 8.411 |
| $X_1^2$ | –6.65 | 1 | 0.805 | –8.337 | –4.968 |
| $X_2^2$ | –2.51 | 1 | 0.805 | –4.199 | –0.831 |
| $X_3^2$ | –2.90 | 1 | 0.805 | –4.582 | –1.213 |
| $X_4^2$ | –2.46 | 1 | 0.805 | –4.147 | –0.778 |
| $X_1X_2$ | –4.21 | 1 | 1.054 | –6.411 | –2.000 |
| $X_2X_3$ | 1.97 | 1 | 1.054 | –0.240 | 4.171 |

CI, confidence interval.

The final estimative response model equation, after the elimination of the insignificant variables in order to estimate the enzymatic synthesis of sorbitan acrylate, was as follows:

$$\begin{aligned} Y = & -210.833 + 6.173\,X_1 + 0.670\,X_2 + 12.929\,X_3 \\ & + 21.3859\,X_4 - 0.067\,X_1^2 - 0.001\,X_2^2 - 0.724\,X_3^2 \\ & - 2.462\,X_4^2 - 0.008\,X_1X_2 + 0.019\,X_2X_3 \end{aligned} \qquad (2)$$

Table 4
ANOVA for Response Surface-Reduced Quadratic Model

| Source | Sum of squares | DF | Mean square | *F*-value | Probability > $F^{a,b}$ |
|---|---|---|---|---|---|
| Model | 12828.48 | 10 | 1282.848 | 72.217 | <0.0001 |
| $X_1$ | 1926.58 | 1 | 1926.579 | 108.455 | <0.0001 |
| $X_2$ | 1626.74 | 1 | 1626.742 | 91.576 | <0.0001 |
| $X_3$ | 6475.72 | 1 | 6475.721 | 364.545 | <0.0001 |
| $X_4$ | 1048.74 | 1 | 1048.743 | 59.038 | <0.0001 |
| $X_1^2$ | 1213.83 | 1 | 1213.834 | 68.332 | <0.0001 |
| $X_2^2$ | 173.48 | 1 | 173.478 | 9.766 | 0.0056 |
| $X_3^2$ | 230.26 | 1 | 230.260 | 12.962 | 0.0019 |
| $X_4^2$ | 166.31 | 1 | 166.310 | 9.362 | 0.0064 |
| $X_1X_2$ | 283.00 | 1 | 282.997 | 15.931 | 0.0008 |
| $X_2X_3$ | 61.82 | 1 | 61.819 | 3.480 | 0.0776 |
| Residual | 337.51 | 19 | 17.764 | – | – |
| Lack of fit | 310.83 | 14 | 22.202 | 4.160 | 0.0621 |
| Pure error | 26.69 | 5 | 5.337 | – | – |
| Correlation total | 13165.99 | 29 | – | – | – |

[a] *Probality* > *F*, level of significance.

[b] Values of probality > *F* < 0.0500 indicate model terms are significant. Values more than 0.1000 indicate the model terms are not significant.

where Y is the response factor, and conversion yield (%). X1, X2, X3, and X4 represent the real values of the independent factors—reaction temperature (°C), reaction time (min), enzyme amount (wt%), and acyl donor/acyl acceptor molar ratio (–), respectively. The model coefficients and probability values are shown in Table 4. All p-values of the coefficients were less than 0.05, and the coefficient of determination (R2) was 0.974 (R2 = 0.90 estimated by model Eq. 2), thereby indicating that the model adequately represented the real relationship among the factors selected. The ANOVA for the response surface-reduced quadratic model is given in Table 4. The coefficients of the response surface model, as are provided in Eq. 1, were evaluated. A Probability > F-value indicated that all of the linear coefficients were more highly significant than were their quadratic and cross-product terms. According to the results of our analysis of factors, there was a low lack of fit. This indicates that the model represents the actual relationships of the reaction parameters, well within the selected ranges.

Figure 5 shows the responses of surface plots representing the effects of reaction temperature, acyl donor/acceptor molar ratio, reaction time, and enzyme amount as well as their reciprocal interaction with the synthesis of sorbitan acrylate, by holding two factors constant at zero level. The relationships between the reaction factors and responses can be better understood

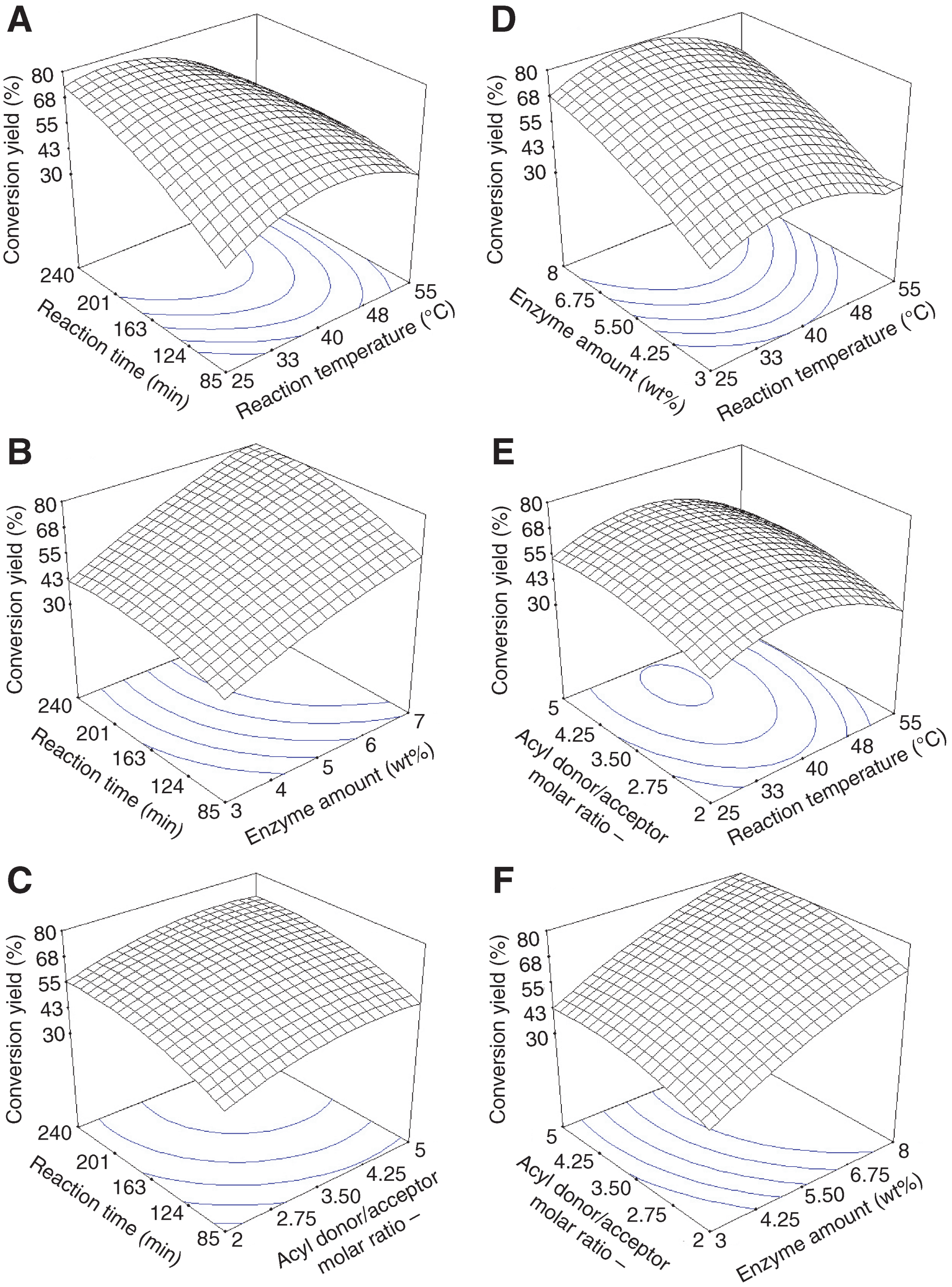

**Fig. 5.** Response surface plots representing the effect of reaction temperature, enzyme amount, substrate molar ratio, reaction time, and their reciprocal interaction with sorbitan acrylate synthesis. Other factors are constant at zero levels.

with an explanation of the planned series of plots generated from the predicted model, in Eq. 2. As shown in Fig. 5A, the low reaction temperature (35–40°C) results in an increased conversion yield, with an increase in reaction time with the other factors at zero level. Conversion was affected slightly by reaction temperature, and the optimal reaction temperature for sorbitan acrylate synthesis was determined to be between 30 and 40°C.

Figure 5B represents the effects of different enzyme amounts and reaction times on sorbitan conversion at constant reaction temperature (45°C) and substrate molar ratio (3 : 1). At any enzyme concentration between 3 and 7%, an enhancement of conversion yield leads to an increase in reaction time. An increase in the amount of introduced enzyme amount results in a linear increase in conversion yield at a constant reaction time. The maximal yield was obtained through the administration of a high enzyme amount and a long reaction time, using a moderate reaction temperature. At any of the reaction temperatures within the limits of the experimental design, an increase in the enzyme concentrations enhances the conversion yield in a linear fashion. Also, with increases in the acyl donor/acceptor molar ratio, conversion yields were enhanced in a linear fashion with reaction time, when other factors were maintained at zero level (Fig. 5C).

The effects of reaction temperature and differing enzyme concentrations on the enzymatic synthesis of sorbitan acrylate, with a acyl donor/acceptor molar ratio of 3 : 1 and a 135 min reaction time, are shown in Fig. 5D. Within the entire range of introduced enzyme amounts, maximal sorbitan conversion was accomplished at a mild reaction temperature, of between 35 and 40°C. At constant reaction temperature, an increase in the amount of introduced enzymes resulted in a linear increase in sorbitan conversion. Reaction temperature also evidenced a high degree of influence on the conversion rate. High reaction temperatures clearly tended to induce enzyme inactivation. Increases in the reaction temperature more than 55°C resulted in low-conversion yields at any enzyme amount because of the inactivation of enzymes at such high temperatures. This showed that the optimal temperature for Novozym 435 was between 40 and 60°C, as previously reported *(4–6)*.

Figure 5E shows the effects of different reaction temperatures and acyl donor/acceptor molar ratios on sorbitan conversion conducted with a constant reaction time (135 min) and enzyme amount (5%). An increase in the introduced acyl donor/acceptor molar ratio resulted in a general increase in conversion yields at constant reaction temperature. The optimal yield was obtained at a 4.25 : 1 acyl donor/acceptor molar ratio and a 40°C reaction temperature.

The effects of different enzyme amounts and acyl donor/acceptor molar ratios on the enzymatic synthesis of sorbitan acrylate at zero level are shown in Fig. 5F. Increases in the amount of introduced enzymes or in the acyl donor/acceptor molar ratio both resulted in a linear increase in conversion yields, when reaction temperature and time were constant. Maximal conversion was achieved using a high enzyme content and a

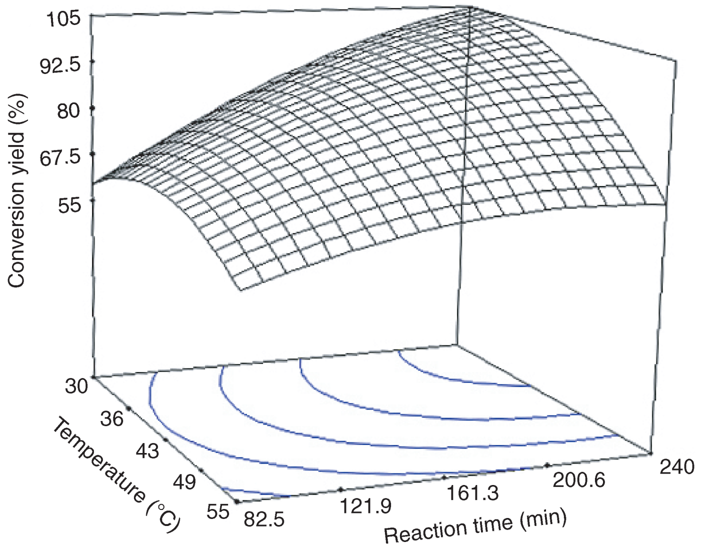

**Fig. 6.** Response surface plot representing the effect of reaction temperature and reaction time of the optimal enzymatic synthesis condition at the stationary point (enzyme amount 8%, and acyl donor/acceptor molar ratio 4.49 : 1).

high acyl donor/acceptor molar ratio. As compared with the results shown in Fig. 5, the optimal conversion yields were achieved using a high enzyme amount and a high acyl donor/acceptor molar ratio, as well as a long reaction time, at a reaction temperature of between 40 and 45°C. This pattern is similar to the previous report *(4)*.

The optimum values of the selected factors were obtained by solving the regression equation (Eq. 2), using Design-Expert 6 software. The optimal conditions for the lipase-catalyzed enzymatic synthesis of sorbitan acrylate, as estimated by the model equation, were as follows: $X_1 = 40.1°C$, $X_2 = 237$ min, $X_3 = 8\%$, and $X_4 = 4.49 : 1$ (Fig. 6). The theoretical conversion yield predicted under the above conditions was $Y = 100.1\%$. In order to conform the prediction value by the model, using these optimal conditions in three independent replicates, the average yield reached 97.6 ± 1.3%. This confirms that RSM coupled with proper experimental design can be effectively used in the optimization of lipase-catalyzed processes.

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