Production of Sugar Fatty Acid Esters by Enzymatic Esterification in a Stirred-Tank Membrane Reactor: Optimization of Parameters by Response Surface Methodology

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ABSTRACT: 6-*O*-β-D(+)-Glucose stearate serving as a model product of glucose fatty acid monoesters was synthesized using lipase B from *Candida antarctica* (Chirazyme® L-2) in a mainly solid-phase system in a stirred-tank membrane reactor. Esterification was performed in the presence of a small amount of ethyl methylketone (EMK), maintaining a catalytic liquid phase as well as forming an azeotrope with the reaction water. The azeotrope was evaporated and broken by membrane vapor permeation, then the dried EMK was returned to the reaction medium. The process was optimized by response surface methodology based on five major reaction parameters [time (T_r), substrate ratio (acyl donor to glucose, S_r), temperature (R_t), amount of solvent [solvent to substrates, [w/w], S_a), and enzyme load (E_l)] varied at three levels, resulting in higher yields. Thus, under optimized conditions [T_r = 58 h; S_r = 2.7; E_l = 8.9% (w/w); R_t = 78°C; S_a = 1.9], up to 93% yields of glucose stearate were achieved.

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KEY WORDS: Azeotropic distillation, biosurfactant, food additive, lipase, membrane vapor permeation, response surface methodology, solid-phase synthesis.

Sugar fatty acid esters (SFAE) represent a large group of compounds and consist of two abundant agricultural raw materials—sugars and fatty acids derived from fats or oils. Large-scale synthesis of SFAE still relies on chemical methods performed at high temperatures in the presence of alkaline catalysts. This is accompanied by high energy consumption, coloring of products, and low selectivity toward the various hydroxyl groups in sugars. As a consequence, not all chemically produced SFAE are allowed for applications in, e.g., cosmetics and pharmaceuticals (1,2).

In contrast, enzyme-catalyzed synthesis of SFAE overcomes many of these drawbacks, In the last decade, several approaches for biocatalytic production have been reported (for reviews see Refs. 3,4). This includes the use of polar organic solvents such as pyridine or dimethylformamide (5–8), activated acyl donors (9,10), substrate immobilization (11), or the use of sugar derivatives to increase the miscibility of the substrates (12–16). All of these methods have some disadvantages, e.g., use of toxic solvents, laborious protecting and deprotecting steps, etc., which increase process costs as well as reduce the areas of applications for these SFAE. To overcome these problems, different processes for preparing glucose fatty acid monoesters directly from nonactivated fatty acids and unmodified sugars using a large amount of solvent have been reported (17,18).

In the past few years, our group has developed a process in which the acylation of a solid sugar with a fatty acid is performed *via* lipase catalysis in the presence of a very small amount of organic solvents such as acetone or *t*-butanol (19–21). Synthesis in a solid-phase system is based on an enzymatic reaction in a heterogeneous low-solvent reaction system in which most of the substrate is present as suspended particles. An important feature of the lipase-catalyzed solid-phase synthesis of SFAE is that the SFAE formed during the reaction will crystallize from the liquid phase if its saturation concentration is reached. By this, the equilibrium of the solid-phase synthesis is favored and product inhibition can also be suppressed. This strategy retains the already known advantages of enzymatic synthesis like regio- and stereoselectivity, minimal side-chain protection, and mild reaction conditions. Furthermore, it offers new benefits like high space–time yield and little need for organic solvents. Although very high yields of various sugar esters could be obtained, this method is difficult to scale up owing to the drawback of the method needed for eliminating reaction water (i.e., direct addition of large amounts of activated molecular sieves). In order to establish a system for large-scale synthesis, we designed a process in which ethyl methylketone (EMK) is used as solvent, but at the same time also allows removal of water generated during the reaction by forming a suitable azeotropic mixture (22). EMK has the advantage that it is both easily eliminated and recognized as safe for use in the food industry by, e.g., German authorities. However, molecular sieves are still needed for the recovery of reaction solvent. Unfortunately, this is not practical on a large scale because of the complicated and costly regeneration process for the molecular sieves.

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More recently, there has been interest in using pervaporation or vapor permeation membrane separation techniques for the selective separation of organic solvent mixtures because of their high separation efficiency and flux rates coupled with potential savings in energy costs (34). This technique depends on the fact that certain membranes permit easier passage of one fluid than the other, thus changing the composition of the mixture through the permeate-selective, nonporous membrane. In this paper, we describe an integrated process that also allows separation of water from the azeotropic mixture by a vapor permeation membrane module, thus eliminating the requirement for molecular sieves. We chose a batch process for the synthesis of sugar esters because the market demands only small amounts of various kinds of SFAE, resulting in a higher production flexibility. Owing to the various parameters to be considered, we used response surface methodology (RSM) to identify the best process conditions. RSM has been successfully applied in many areas such as food processing (23), enzymatic synthesis of *n*-butyl glycoside (24), and enzymatic modification of fats and oils (25–27). A procedure with good prospects for the food industry is herein proposed and optimized for the production of SFAE.

MATERIALS AND METHODS

Enzyme, membrane, and chemicals. Chirazyme® L-2 (EC 3.1.1.3, immobilized lipase B from *Candida antarctica*) was a gift from Roche Diagnostics GmbH (Penzberg, Germany). The Pervap® 2200 membrane was a donation from Sulzer Chemtech GmbH (Neunkirchen, Germany). Stearic acid was provided by Henkel KGaA (Düsseldorf, Germany). All other chemicals were purchased from Fluka (Buchs, Switzerland).

Apparatus. A diagram of the apparatus is shown in Figure 1. A membrane reactor (top part suitable for a membrane with an area of 23 cm^2: i.d. 58 mm, height 2 mm, bottom part suitable for the enzymatic reaction: i.d. 20 mm × height 7 mm) was provided by the Jülich Forschung GmbH (Jülich, Germany). The Pervap® 2200 membrane was soaked sequentially with water, acetone, and EMK before use. The temperature of the membrane reactor was maintained by circulating heated water from a water bath. Before starting the reaction, the permeate container was cooled to −8°C by a thermostat and connected to a pump maintaining a vacuum of <10 mbar. Both the permeate container and condenser were insulated to reduce heat transmission.

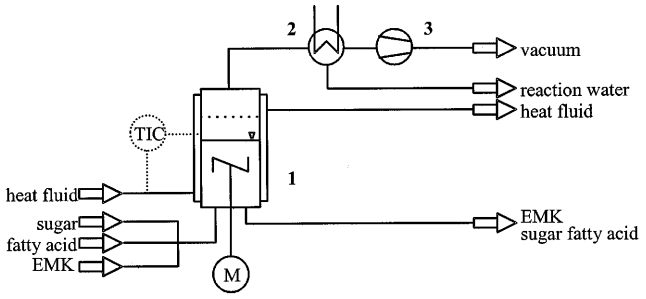

FIG. 1. Schematic diagram of lipase-catalyzed esterification for the production of sugar fatty acid esters in a stirred-tank membrane reactor. (1) Membrane reactor, (2) permeate condenser, (3) vacuum pump. TIC, temperature measurement; M, mixer; EMK, ethyl methylketone.

Determination of membrane efficiency/capacity for water removal. The solvent containing predefined amounts of water was incubated in the membrane reactor at different temperatures. The water was continuously removed through the membrane. Samples from the organic solvent were periodically collected and analyzed by Karl-Fischer titration (Metrohm, Herisau, Switzerland).

Procedure for the lipase-catalyzed esterification. The reaction mixture consisted of sugar (usually 0.5 mmol) and fatty acid (0.5–3 mmol) in EMK [0.5–2.5-fold of substrate, (w/w)]. The reaction mixture was incubated in the membrane reactor, placed in a magnetic stirrer, and agitated by a magnetic bar (650 rpm). The reaction temperature was maintained (in the range of 74.5–78.5°C) by the thermostat. At these temperatures the azeotropic mixture of EMK and water (b.p. 73.5°C) produced during esterification was vaporized. By passing the Pervap® 2200 membrane, water is removed from the azeotrope, and the remaining condensed EMK is returned to the reaction compartment. The reaction was started by addition of immobilized lipase [6–12% (w/w) of substrate]. Samples from the reaction mixture were periodically collected and analyzed by thin-layer chromatography (TLC) as described previously (19) and high-performance liquid chromatography (HPLC) (see below). At the end of the reaction, the mixture was extracted three times with 20 mL acetone by stirring at room temperature for 30 min. The immobilized enzyme was separated from the reaction mixture by flotation, allowing easy recovery of the biocatalyst. The SFAE ester solution in acetone was cooled to −10°C, and the white crystals that formed were collected by filtration. The purity of the products was determined by HPLC, ^1H and ^{13}C nuclear magnetic resonance spectroscopy to be >99% (data not shown).

HPLC analysis. All reaction mixtures and products were analyzed by an HPLC system equipped with a light-scattering mass detector and a Nucleosil 120-5C_{18} column. The elution was carried out isocratically at 1 mL/min using methanol (70%), acetonitrile (25%), and water (5%) as a mobile phase for glucose palmitate and glucose stearate. For analysis of glucose caprylate, a mobile phase of methanol/acetonitrile/water (50:30:20) was used.

Experimental design. The experiments consisted of 29 runs with five partial factors, each in three levels according to the central composite face design (28,29). According to our earlier works (19,20,22), the following parameters were investigated: time (T_r, h), substrate ratio (S_r, acyl donor to glucose, mol/mol), temperature (R_t, °C), amount of EMK [S_a, solvent to substrates, (w/w)], and enzyme load [E_l, % (w/w) of substrates]. Set ranges are listed in Table 1.

Statistical analysis. Modde 4.0 (Umetri, Umeå, Sweden) was used to analyze the experimental data. Second-order coefficients were generated by regression analysis with backward elimination. Responses were first fitted for the five fac-

TABLE 1
Set Factor Levels, Measured Responses, and the Comparison Between the Calculated Net Incorporation and the Predicted Results by the RSM-Fitted Model[a]

Exp. no.	Run no.	Factors					Conversion* (%)	
		T_r	S_r	E_l	R_t	S_a	Exp.	Pr.
1	5	24	1	6	74.5	2.5	52.3	52.3
2	26	60	1	6	74.5	0.5	64	63.7
3	3	24	3	6	74.5	0.5	52.8	52.9
4	27	60	3	6	74.5	2.5	82.3	82.2
5	21	24	1	12	74.5	0.5	57.8	58
6	23	60	1	12	74.5	2.5	73.4	73.4
7	4	24	3	12	74.5	2.5	69.2	69.6
8	25	60	3	12	74.5	0.5	83.2	83.2
9	24	24	1	6	78.5	0.5	46.3	46.1
10	14	60	1	6	78.5	2.5	83.3	83
11	16	24	3	6	78.5	2.5	67.6	67.7
12	2	60	3	6	78.5	0.5	75.8	75.5
13	17	24	1	12	78.5	2.5	63.5	63.7
14	18	60	1	12	78.5	0.5	76	75.8
15	29	24	3	12	78.5	0.5	63.9	64.1
16	19	60	3	12	78.5	2.5	85	85
17	22	24	2	9	76.5	1.5	72.9	72
18	20	60	2	9	76.5	1.5	89.5	90.5
19	13	42	1	9	76.5	1.5	80.3	80.8
20	10	42	3	9	76.5	1.5	89.2	88.8
21	6	42	2	6	76.5	1.5	78.6	79.4
22	9	42	2	12	76.5	1.5	86.4	85.6
23	1	42	2	9	74.5	1.5	84.6	84.2
24	8	42	2	9	78.5	1.5	87	87.4
25	15	42	2	9	76.5	0.5	76.8	77.2
26	7	42	2	9	76.5	2.5	84.7	84.4
27	28	42	2	9	76.5	1.5	87	86.7
28	11	42	2	9	76.5	1.5	86.5	86.7
29	12	42	2	9	76.5	1.5	86.8	86.7

[a]T_r, reaction time (24–60 h); S_r, acyl donor to glucose molar ratio (1–3); E_l, enzyme load [6–12%, (w/w), of substrates]; R_t, reaction temperature (74.5–78.5°C); S_a, solvent (ethyl methylketone) amount (0.5–2.5-fold of substrates); Exp., experiment; Pr., prediction. Conversion* as referred to glucose. Experiments were run in run number. RSM, response surface methodology.

tors by multiple regression. The fit of the model was evaluated by the coefficients of determination (R^2—the fraction of the variation of the response explained by the model, an overestimated measure of the goodness of fit of the model; and Q^2—the fraction of the variation of the response predicted by the model, an underestimated measure of the goodness of fit of the model) and analysis of variance (ANOVA). The insignificant coefficients were eliminated after examining coefficients, and the model was finally refined. The polynomial equation to be fitted to the measured, dependent variables Y was of the form:

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1}^{5} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i=1}^{5} \beta_{ij} X_i X_j \qquad [1]$$

where Y is the value of the response, X_i and X_j are the independent variables, β_0 is the intercept, β_i is the first-order model coefficients, β_{ii} are the quadratic coefficients for the ith variable, and β_{ij} are the interaction coefficients for the interaction of variables i and j.

The performance of the enzymatic synthesis of SFAE was evaluated by analyzing the response—conversion. Optimization of the reaction parameters was calculated using the predictive models from RSM. The synthesis of SFAE was carried out at the predicted optimal conditions. The observed response obtained at these conditions was analyzed and compared to the predicted value.

RESULTS AND DISCUSSION

In our previous publication (22), we could show that an efficient lipase-catalyzed synthesis of SFAE is possible by using a mainly solid-phase system. Further optimizations identified EMK as a suitable solvent, because it forms an azeotropic mixture with the water generated during the esterification. However, from a process point of view, continuous water removal without large consumption of solvent is desired. This can be achieved by using membranes that are highly selective for the removal of water from an organic solvent. To obtain high permeation rates and sufficient separations while maintaining high product yields, the reaction system was now optimized in detail as outlined in the following sections.

Membrane selection. The choice of membrane material is crucial for a successful substrate conversion in this type of reactor. For dehydration of an organic solvent, hydrophilic polymers appear to be most suitable for the selective removal of the highly polar water (30–33). Preliminary investigations (data not shown) revealed that the water content of the enzymatic reaction mixture must be reduced to about 0.02–0.4% to ensure high concentrations of product. On the other hand, even at high conversions, the water content of EMK was rather low, in a range of 2–4% (vol/vol). Thus, the membrane must be very selective for water and should not allow permeation of EMK. These conditions were fulfilled by the Pervap® 2200 membrane. The dehydration process can be divided into three steps: (i) water sorption into membrane at the upstream side, (ii) diffusion through the membrane, and (iii) water desorption into the vapor phase at the downstream side (34). The efficiency of the membrane is governed by the water concentration, the feed flow rate, pressure difference between up- and downstream, and temperature. The water content is not constant during esterification and rises with increasing conversion. To ensure maximal water removal, we applied a vacuum of <10 mbar (usually, 5 mbar).

Influence of temperature on water removal. In principle, higher temperatures lead to enhanced water removal in the membrane pervaporation process. On the other hand, the maximum temperature is set by the thermostability of the lipase employed. Indeed, we found that with increasing temperatures more water could be removed through the membrane. At 75°C, more than 4% water can be removed efficiently, which also corresponds to the water content maximum to be generated during the enzymatic reaction. According to our previous results (22), we used 25, 40, 50, 60, and 75°C. The influence of the operating temperature on the water permeate flux through the membrane is shown in Figure 2. The amount of water permeate significantly in-

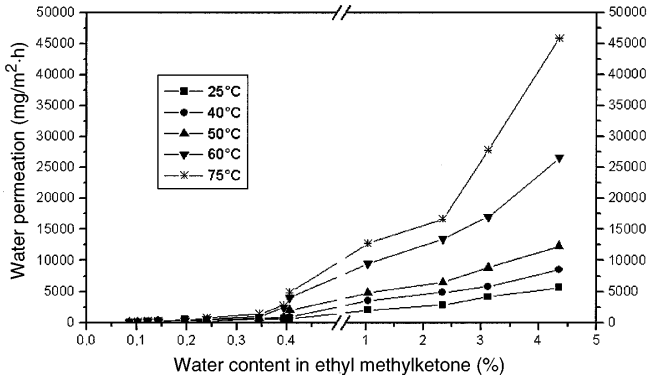

FIG. 2. Relationship between water content in EMK and water permeate at different temperature. Test condition: 20 mL EMK with 4.3–4.6% water, membrane module (Φ 58 × 8 mm, membrane area 23 cm^2), vacuum 8–12 mbar (downstream), collection container –8°C, operating temperature (upstream) at 25, 40, 50, 60°C membrane at bottom of module; at 75°C membrane at top of module. For abbreviation see Figure 1.

creased at temperatures above 60°C especially when the feed water content in the EMK was above 1.0%. For a technical application of the vapor permeation technology, the operating temperature should be taken as high as possible to achieve maximum permeation rates and to minimize the required membrane area and plant costs. On the other hand, the high optimal temperature of 75°C (above the boiling point of the azeotropic mixture) leads to low monoester formation due to the high solubility of the sugar fatty acid ester in the solvent, thus preventing the crystallization necessary for the shift of the reaction equilibrium. As a consequence, synthesis of sugar esters from medium-chain fatty acids should be performed in a normal stirred-tank reactor that is connected to a membrane module for dehydration, which then can be thermostated separately to appropriate temperatures.

A series of experiments were carried out to investigate the esterification of glucose with stearic acid in a stirred-tank membrane reactor, in which the reaction water was removed by azeotropic distillation and the solvent was continuously dehydrated by membrane vapor permeation at 75°C. We found that at a stearic acid/glucose ratio of 1:1 (mol/mol), the conversions of monoesters were generally lower [75% for 6-*O*-β-D(+)-glucose-stearate] compared to our previous experiments (90%), which were performed at 60°C. Since a temperature around 75°C is a pre-condition for a successful azeotropic distillation, higher yields can only be obtained if all other reaction parameters (i.e., reaction time, substrate ratio, amount of EMK, and enzyme load) are further optimized. Because of the complicated interaction of these parameters, we used RSM to identify the best reaction conditions.

Optimization of the synthesis of glucose stearate as a model product by RSM. (i) Model fitting. By using the normalized results as the responses for the fitting of the RSM models, we could set up the quadratic relationships required for modeling. The ANOVA for the five process variables indicated that the SFAE yields can be well-described by a polynomial model. The second-order polynomials fitted to the measured data, which were computed by stepwise regression analysis using the standardized variables X_i. Standardized values were used to attain proper preference between the variables in the calculation of the models. The data shown in Table 1 are, however, computed with those actual variables which also occur in the standardized models. The coefficient of determination (R^2) of the model for the response is 0.996 (Q^2 = 0.899). According to the ANOVA, there was no lack of fit, and there was a satisfactory coefficient of determination. Predicted results were close to the observed, and all absolute errors of the predictions were less than 1.0. This indicates that the model represents the real relationships between reaction parameters well.

(ii) Main effects of parameters on responses. Optimization of the yield of glucose fatty acid monoester was performed by using the following parameters: reaction time, temperature, glucose/stearic acid molar ratio, solvent amount, and enzyme load. The yield of glucose fatty acid monoester was increased by longer reaction time, higher enzyme load, and higher substrate ratio. Reaction temperature had to be kept in a narrow range (between 74.5 and 78.5°C) in order to allow evaporation of the azeotrope and to change the gaseous EMK to a liquid when the azeotrope was broken. The different effects of the changing parameters are shown in Figure 3. From this, it can be observed that all five parameters had positive effects. The reaction time had significant influence on conversion for the first-order factors, which indicated longer reaction times were favored. For the second-order factors, solvent amount, reaction time, and enzyme load had negative effects on conversion. No significantly effective interactions in the model were observed, meaning that the production of SFAE can be optimized by one-factor design.

The merits of using RMS are to evaluate the relationships between each parameter and to predict the result and behavior under given reaction conditions. Moreover, optimal parameters can be obtained by iterative calculation with more than one response and targets. Optimization of the yield of glucose fatty acid monoester was performed by taking into account the

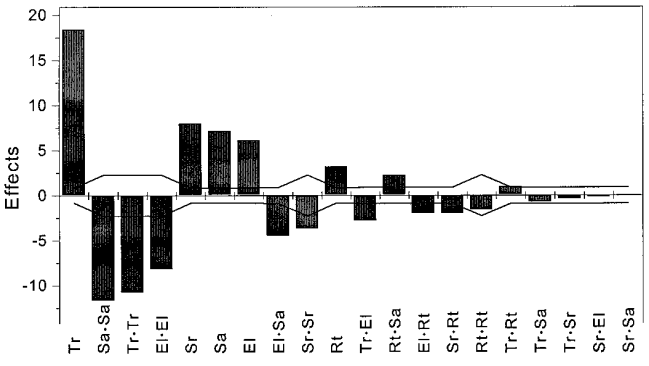

FIG. 3. Main effects of the factors and their significance. T_r, time (h); S_a, amount of EMK solvent/substrate (w/w); E_l, enzyme load, % (w/w) of substrates; S_r, substrate ratio (acyl donor to glucose, mol/mol); R_t, temperature (°C). For other abbreviation see Figure 1.

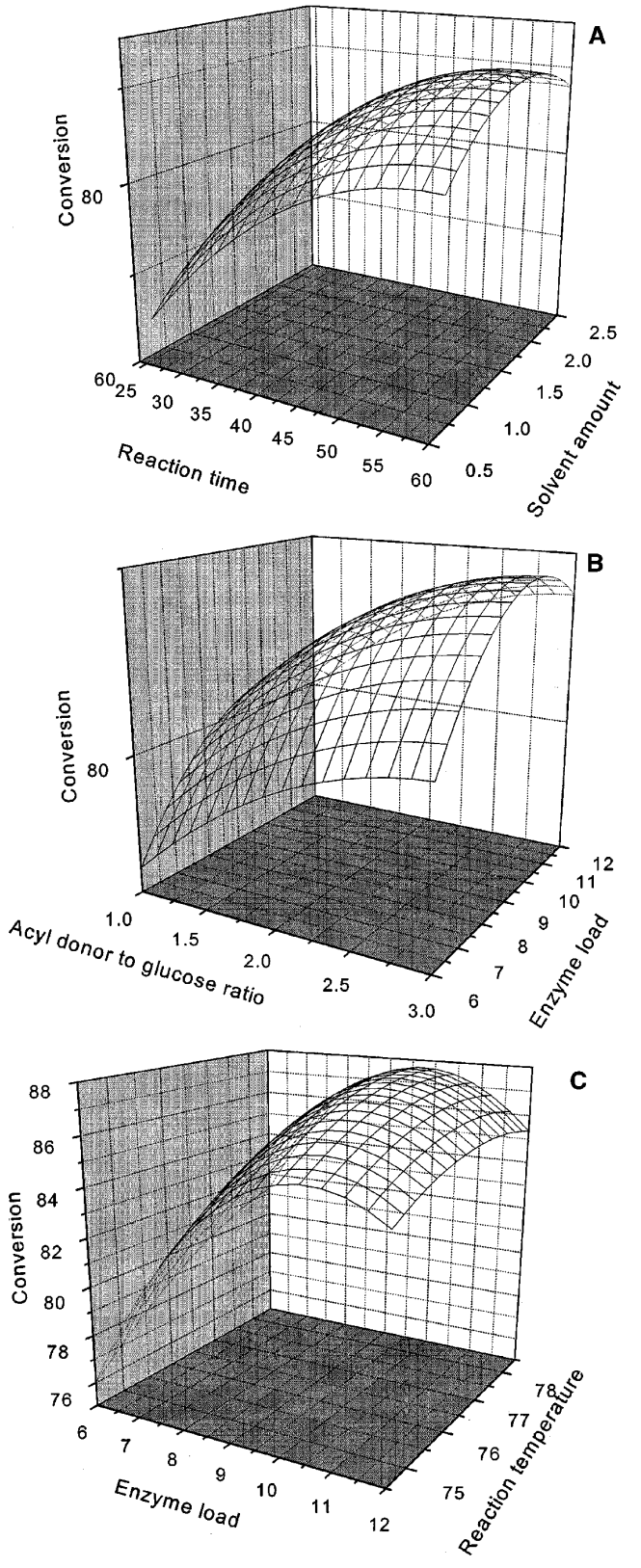

FIG. 4. Response surface plots for the evaluation of effects of parameters. Units: time (h); solvent amount, solvent to substrate ratio (w/w); enzyme load, % (w/w) of substrates; temperature (°C); conversion (%); substrate ratio (acyl donor to glucose, mol/mol).

TABLE 2
Optimum Conditions Generated by *Modde 4.0* in the Set Parameter Ranges and Targets Through Iterative Calculation[a]

						Conversion (%)		
No.	T_r	S_r	E_l	R_t	S_a	Pr.	Exp.	Iter.
1	57.18	2.96	9.38	77.53	1.81	93.4	93.2	226
2	57.35	2.80	9.02	78.18	1.86	93.6	93.5	136
3	60.00	2.12	8.01	78.50	2.19	92.1	92.3	171
4	58.02	2.72	8.89	78.27	1.86	93.5	93.5	129
5	52.80	2.16	9.18	76.62	1.56	91.2	91.4	11
6	60.00	2.40	9.00	76.50	1.50	91.7	91.8	0
7	49.20	3.00	9.00	76.50	1.50	91.5	91.5	0
8	49.20	2.00	9.00	78.50	1.70	91.1	91.0	7

[a]Set parameter ranges: T_r, reaction time, 24–60 h; S_r, acyl donor to glucose molar ratio, 1–3 mol/mol; E_l, enzyme load, 6–12% w/w; R_t, reaction temperature, 74.5–78.5°C; S_a, solvent (ethyl methylketone) amount, 0.5–2.5-fold of substrates. Set targets: conversion 92.7% (minimum 88.2%). Pr., prediction; Exp., experiment; Iter., iteration.

five parameters mentioned above. However, it should be noted that the range of each parameter has to be predecided before optimization, e.g., a shorter reaction time always results in lower conversion. On the other hand, shorter reaction times with a sufficient monoester yield allow for a higher space-time yield which is very important in terms of industrialization. To evaluate the relationships and interactions of parameters, surface plots give good prescriptions. The behavior of the product conversion under the process conditions is selectively presented in Figures 4 A–C. The optimization with one response and five variables cannot just be calculated mathematically through Equation 1 as more than one optimal condition may exist. Several optimal conditions have been proposed by RSM and are summarized in Table 2. It shows that optimal conditions are different and a conversion up to 93% can be achieved under the given reaction condition.

To verify whether the predictive model could be applied to a preparative synthesis, eight experiments were carried out in a stirred-tank membrane reactor using the optimal conditions predicted by RSM. From the data shown in Table 2, it is obvious that predicted and experimental conversion match perfectly.

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