Feed Batch Addition of Saccharide During Saccharide–Fatty Acid Esterification Catalyzed by Immobilized Lipase: Time Course, Water Activity, and Kinetic Model

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ABSTRACT: A conversion of 80–93% was achieved for esterification of oleic acid and fructose (or sucrose) catalyzed by immobilized *Rhizomucor miehei* lipase (Lipozyme IM; Novozymes, Franklinton, NC) at 65°C using near-stoichiometric amounts of substrates. The product consisted of mono- and diester at a ratio of 9:1 g g^{-1}. The main obstacle for achieving a high rate of reaction, the poor miscibility of the substrates, was overcome by taking advantage of the greatly increased solubility of fructose as the proportion of ester increased. A phase diagram demonstrated that the solubility of fructose increased linearly from 0.002 to 0.07 to 0.13 g g^{-1} as the ester mass fraction increased from 0.00 to 0.47 to 0.80, respectively. Solvent (*tert*-butanol) was present only during the first phase of the time course of the reaction to enhance fructose solubility and was allowed to evaporate away completely on reaching 25% conversion. A conversion higher than 80–93% could not be achieved by reducing the bioreactor's water content through use of vacuum pressure or water activity control. Water adsorption isotherms demonstrate the significant increase of equilibrium liquid phase water content as the reaction progressed, which was due to higher water adsorption by the monoester relative to oleic acid. Increased removal of liquid phase water may result in the loss of water from the lipase, resulting in a reduction of its biocatalytic activity. Initial rate experiments were used to derive a Ping-Pong Bi Bi kinetic model that strongly agreed with measured data for the time course of the reaction. Lipozyme IM did not lose activity when employed for three successive fructose–oleate esterification batch reactions or, equivalently, for a 24-d reaction period.

Paper no. J10971 in *JAOCS 82*, 487–493 (July 2005).

KEY WORDS: Fructose monoester, lipase, Lipozyme RM IM, Ping-Pong Bi Bi kinetic model, saccharide esters, water activity.

Saccharide–FA partial esters have numerous applications in the food and cosmetics industries as biodegradable and biocompatible surfactants and as antibiotics and insecticides (1,2). The general approach for their synthesis, *via* base-catalyzed ester bond formation between saccharide and acyl donor feedstocks, requires a high operating temperature (above 100°C) and results in a broad product distribution (3,4). A more recent chemical method, using disodium hydrogen phosphate catalyst at 40°C, resulted in a high yield and selectivity but required the use of activated acyl donors, vinyl esters, resulting in an increase of time, labor, and materials costs (5). An alternative approach, the use of immobilized lipases in nearly anhydrous media, involves low operating temperatures and other mild reaction conditions and is thus environmentally friendly. As reviewed elsewhere (6,7), early results for this approach demonstrated high regioselectivity toward saccharide mono- and diester formation and high yields for the stoichiometrically limiting substrate but a slow reaction rate due to the poor miscibility of acyl donor and acceptor. Polar solvent, frequently used to increase miscibility, leads to relatively slow rates of reaction for nonaqueous enzymology (8). Improvement of the rate was achieved through a modified form of one or both of the substrates: activated esters of the acyl donor, isopropylidine esters of saccharides, or saccharides complexed to phenylboronic acid (6,7); however, these approaches are impractical for commercial processes. Recent approaches to improve miscibility include the use of nonpolar/polar solvent mixtures (9) and room temperature ionic liquids (10), and acyl acceptors in an amorphous crystalline form to increase the rate of solubilization (11). Alternatively, the use of polar solvents at subambient temperatures leads to high conversion and selectivity owing to the selective precipitation of monoester (ME), which enhances downstream separation and drives ME synthesis in a forward direction (12).

In our laboratory, it was observed that the presence of solid-phase, nonsolubilized saccharide led to a reduced rate of reaction, perhaps owing to its adsorption to immobilized lipase, producing a barrier to mass transfer, and/or increased water content in the bioreactor (Dong, H., and D.G. Hayes, unpublished data). We have increased the reaction rate for fructose–oleic acid ME synthesis by employing a liquid-phase reaction medium that is at or near the saturation limit for the acyl acceptor, achieved by frequently resaturating the liquid phase with saccharide or adding an acyl acceptor in the fed-batch mode, taking advantage of the greatly increased solubility of the acyl acceptor due to the presence of ME (13). A polar solvent (*tert*-butanol, *t*-BuOH) was employed only in the initial period; moreover, it was allowed to evaporate away steadily as a result of operation at 65°C, and was completely removed when the conversion of limiting reactant (fructose) reached

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~25%. The evaporation of *t*-BuOH also enhanced the evaporation of the reaction product, water, driving the thermodynamic equilibrium in a forward direction. Nearly 100% conversion of the rate-limiting substrate, saccharide, was achieved for a reaction mixture consisting of a net mole ratio of oleic acid to fructose of 2:1 (13). In this report, the same approach was employed, but with a nearly stoichiometric substrate ratio of 1:1 to eliminate the need for downstream separation, assuming a high conversion occurs. The solubility of saccharide as a function of the extent of the reaction (in the form of a ternary phase diagram), the partitioning behavior of water, and the modeling of enzyme kinetics were also determined to provide mathematical tools useful for scale-up.

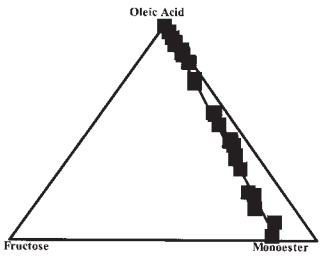

FIG. 1. Ternary phase diagram for fructose/oleic acid/technical-grade monoester (5% diester and 95% monoester) at 60°C. A one-phase liquid mixture exists to the right of the phase boundary, two-phase media to the left of the boundary.

MATERIALS AND METHODS

Materials. Oleic acid (98% pure, determined by HPLC) was purchased from Sigma-Aldrich (St. Louis, MO). Hydranal Coulomat AG and CG Karl Fischer titration solutions were purchased from Fluka-Riedel-de Haan (St. Louis, MO). Solvents and D-fructose were purchased from Fisher Scientific (Pittsburgh, PA). All chemicals were highly pure (>98%) and were used without further purification. Lipozyme IM, lipase (EC 3.1.1.3) from *Rhizomucor miehei* immobilized onto macroporous anionic resin beads (Duolite 568), or RML, was kindly donated by Novozymes, Inc. (Franklinton, NC). Fructose monooleate, used for phase diagram and water adsorption isotherm experiments, was purified from reaction mixture *via* column chromatography on a silica gel column using a positive gradient of ethyl acetate for an ethyl acetate/hexane solvent system. Deionized water was used throughout the experiments.

Methods. Reactions were conducted in vessels placed on a hot plate-stirrer and open to the atmosphere. For a typical reaction, immobilized lipase was suspended in a solution of oleic acid, saccharide, and *t*-butanol (2-methyl-2-propanol) at 65°C using a stirring bar rotating at 400 rpm (41.9 s^{-1}). *t*-BuOH and water freely evaporated during the course of the reaction. Small (100 μL) aliquots of the reaction mixture were removed periodically for analysis. Saturation of the liquid phase media with fructose was achieved by suspending fructose in the media for a few minutes, followed by the removal of excess fructose *via* microfiltration.

Water adsorption isotherms were determined by storage of RML and mixtures of oleic acid and ME at several different proportions in desiccators containing saturated water/salt solutions at 60°C for 2 wk. The salts controlled water equilibration between the phases by maintaining a constant water activity value, a_w: LiCl (a_w = 0.11 at 60°C), $MgCl_2$ (a_w = 0.29 at 60°C), NaBr (a_w = 0.50 at 60°C), $CuCl_2$ (a_w = 0.67 at 40°C), NaCl (a_w = 0.75 at 60°C), and KCl (a_w = 0.80 at 60°C) (14,15). The monophasic–biphasic boundary for the oleic acid/fructose monooleate/fructose ternary system at 60°C depicted in the phase diagram (Fig. 1) was determined *via* visual observation.

HPLC analysis was conducted at 25°C using an analytical (4.6 mm × 25 cm) reversed-phase C_{18} column from Varian (Walnut Creek, CA) and an ELSD (Model MK-III; Alltech Associates, Deerfield, IL) that employed a drift tube temperature of 85°C. The mobile phase used was acetonitrile/acetone/acetic acid 78:20:2 by vol at 1.0 mL min^{-1}. Response factor calibrations for the substrates and products were used to calculate concentrations from the ELSD data. Products were identified using standards.

Karl Fischer titrations were conducted using an Accumet Model 100 coulometric titrator (Denver Instruments, Denver, CO). Solid- and liquid-phase samples were contacted by or dissolved in methanol *via* vortexing or magnetic stirring for several minutes. A specified amount of the resultant methanolic solution was injected into the titrator. The water content of methanol was subtracted in the calculations. Water activity was measured using a Portable MS1model sensor from Novasina (Talstrasse, Switzerland).

RESULTS AND DISCUSSION

Fructose solubility. A ternary phase diagram for the ternary system saccharide (fructose, *S*)/(oleic acid, FA)/fructose–oleic acid monoester (ME) at 60°C is depicted in Figure 1. Results were within 1% of those reported previously by our group for fructose solubility for systems possessing ME mass fraction, ω_{ME}, between 0 and 0.5 (13). The solubility of saccharide (fructose) at saturation, i.e., the mass fraction, ω_S, increased linearly from 0.002 to 0.07 to 0.13 as ω_{ME} increased from 0.00 to 0.47 to 0.80, in agreement with previously published results for fructose and glucose (13,16). Moreover, the phase boundary of Figure 1 can be described by the following linear equation:

$$\omega_S = A - B\omega_{FA} \quad [1]$$

where *A* and *B* equal 0.125 and 0.122, respectively. The increase of solubility due to the conversion of FA into ME (Figs. 1 and 2C) is a result of either ME acting as a surfactant or the polarity of the reaction medium increasing. Equation 1 can be expressed in terms of concentration of saccharide and FA, [S] and [FA], respectively:

$$[S] = \frac{A \cdot \rho_{mix}}{MW_S} - \frac{B \cdot MW_{FA}}{MW_S} \cdot [FA] \quad [2]$$

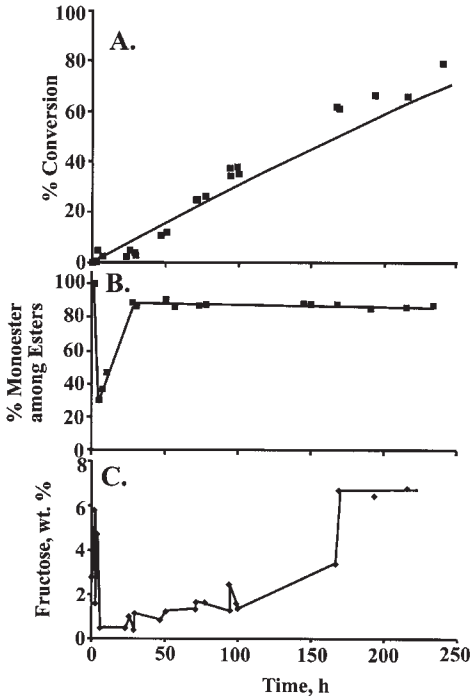

FIG. 2. Time course of lipase-catalyzed fructose ester synthesis (A: conversion of oleic acid, B: product distribution, and C: fructose concentration on a *t*-BuOH-free basis) using a reaction medium saturated with fructose and nearly stoichiometrically equal amounts of the two substrates. Initial conditions: 100 mmol (28.247 g) oleic acid, 25.00 g (32.05 mL) *t*-BuOH, 1.00 g RML, fructose at saturation (*y*-intercept of panel C), 65°C, and a 400-rpm stir rate. The reaction medium was resaturated with fructose periodically (indicated in panel C by increases of fructose concentration). *t*-BuOH and water were allowed to freely evaporate during the time course of esterification. The curve in panel A represents the prediction by the Ping-Pong Bi Bi kinetic model (Eq. 8). RML, *Rhizomucor miehei* immobilized on macroporous anionic resin beads.

where MW_i refers to the M.W. of species *i* and ρ_{mix} to the density of the mixture, calculated according to the following equation:

$$\rho_{mix} = \frac{\rho_{ME} + [FA] \cdot MW_{FA} \cdot \left(1 - B - \frac{\rho_{ME}}{\rho_{ME}}\right)}{1 - A} \quad [3]$$

where ρ_{FA} and ρ_{ME} are the density of oleic acid [895 kg/m^{-3} (17)] and fructose monoleate (936.6 kg/m^{-3}), respectively. The latter value was measured in the authors' laboratory. Equation 3 neglects the contribution of saccharide and *t*-BuOH, the latter present during the initial period only, to the formation of volume and assumes the volumes of FA and ME are additive. Substitution of Equation 3 into Equation 2 yields the following:

$$[S] = \frac{A}{1-A} \cdot \frac{\rho_{ME}}{MW_S} + \left[\frac{A}{1-A} \cdot \left(1 + B - \frac{\rho_{ME}}{\rho_{FA}}\right) + B\right] \cdot \frac{MW_{FA}}{MW_S} \cdot [FA] \quad [4]$$

This equation neglects the effect of *t*-BuOH on fructose solubility and involves concentrations expressed on a *t*-BuOH-free basis.

Time course of reaction for stoichiometric substrate mixture. From our previous investigation, the highest rate of conversion occurred when the liquid phase was saturated with fructose; and a means of maintaining a condition of saturation was implemented, for example, by periodically isolating the liquid phase, introducing further amounts of fructose, removing excess solid-phase fructose by filtration or sedimentation, and returning the biocatalyst to the reaction mixture (13). Solvent [*t*-BuOH, chosen from a series of screening experiments (13)] is present initially to enhance the solubility of fructose; operation at 65°C allows for the evaporative removal of the product, water, and *t*-BuOH, the latter at a constant rate of 0.47 g h^{-1}, independent of the proportion of oleic acid and ME (13). Complete evaporative removal of *t*-BuOH corresponds to ~25% conversion of fructose. The formation of ME negates the requirement of a polar solvent to achieve a significant solubility of fructose, as already discussed herein. In agreement with our previously published results (13), which used the same operating conditions as used herein except for a oleic acyl–fructose mole ratio of 2.0, a linear increase of conversion occurred with respect to time up to about 80% conversion of the limiting reactant (Fig. 2A). Higher conversions could not be achieved, even when the reaction medium was thoroughly dehydrated, as discussed below. The use of a "solid-phase" synthetic approach, in which glucose–ME readily precipitated from the reaction medium by the use of acetone as adjuvant, resulted in a similar degree of conversion as reported herein (78–92%) when using a stoichiometric substrate feed but was effective only for long-chain saturated FA (18).

The selectivity toward ME over fructose–oleic acid diester (DE) occurred at a ratio of approximately 9:1 g^{-1} throughout the majority of the time course of the reaction (Fig. 2B), in agreement with our previous results that used a 2:1 mole ratio of oleic acid to fructose (13). An exception was noted during the initial period of the reaction (4–29 h, 0–21% conversion), when DE was produced more abundantly than ME; this also occurred previously (13). The underlying cause of this result was the small ratio of fructose to ME (Figs. 2A and 2C), with both competing as acceptors for the acyl group bound to the lipases' active site serine residues.

Esters of sucrose and oleic acid were successfully synthesized from an equimolar ratio of acyl donor and acceptor using a similar approach as conducted for fructose monooleate synthesis. Approximately 88% conversion occurred, with the product distribution consisting of approximately 90% ME and 10% DE (Fig. 3). The time course of sucrose–oleic acid esterification was also nearly linear (Fig. 3).

Effect of enzyme concentration. To optimize productivity, it is best to operate enzymatic reactions under conditions in which the rate of reaction is proportional to the concentration of the biocatalyst, RML. As demonstrated in the inset of Figure 4, under operating conditions comparable to those employed for Figure 2, the threshold of the linear region is 0.45 $g_{RML}\ g_{acyl\ donor}^{-1}$. However, even for the lower enzyme concentration of 0.30 g g^{-1}, a reduction in the reaction rate occurred at 2.0 d, in contrast to the case for 0.15 g g^{-1} (Fig. 4). Two days is approximately the

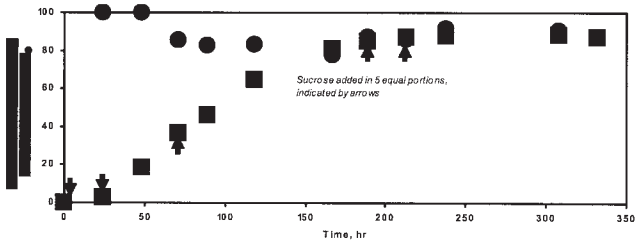

FIG. 3. Time course of lipase-catalyzed sucrose ester synthesis: percent conversion and product distribution, using nearly stoichiometrically equal amounts of the two substrates, with sucrose added in fed-batch mode. The initial reaction medium consisted of 50 mmol (14.13 g) oleic acid, 10 mmol sucrose (17.1 g), 25 g (32.05 mL) *t*-BuOH, and 1.0 g RML. The reaction was conducted at 65°C, and 400 rpm. Four additional 10-mmol increments of sucrose were added at the times indicated in the figure by the arrows. *t*-BuOH and water were allowed to evaporate freely during the time course of esterification. ME, monoester; for other abbreviation see Figure 2.

time at which *t*-BuOH was completely evaporated away (53 h), suggesting an increase of viscosity. Hence, operation at 0.15 $g_{RML}\ g_{acyl\ donor}^{-1}$ is recommended.

Kinetic modeling. The Ping-Pong Bi Bi model, described elsewhere (19), is very applicable to lipase-catalyzed reactions (20), including saccharide esterification in a polar solvent (11). In simple terms, the Ping-Pong Bi Bi model can be conceptualized as consisting of the following reversible steps: the binding of nucleophiles (water, fructose, or ME) to the acyl-enzyme intermediate, the attachment of the nucleophile to the acyl group (resulting in free acid, ME, and DE, respectively), and the simultaneous release of the enzyme. The formation of acyl-enzyme intermediate is described by the reverse reaction steps of the above-described nucleophilic attack of water on the acyl-enzyme intermediate. If one assumes irreversibility of reaction steps and that the concentrations of the primary substrates, fructose [S] and oleic acid [FA], are much greater than those of ME and DE during the initial reaction period, the following equation may be derived (20):

$$\nu = -\left(\frac{d[\mathrm{FA}]}{dt}\right)_{t=0} = \frac{V_{\mathrm{MAX}} \cdot [\mathrm{FA}] \cdot [S]}{K_S \cdot [\mathrm{FA}] + K_{\mathrm{FA}} \cdot [S] + [\mathrm{FA}] \cdot [S]} \qquad [5]$$

where V_{MAX} represents the maximum velocity, K_S and K_{FA} Michaelis constants, and [*S*] and [FA] substrate concentrations.

Parameters contained in Equation 5 were estimated from a series of kinetic experiments performed in *t*-BuOH-rich media, where the FA concentration was varied for a given fructose concentration in a series and initial rates of reaction were measured. Lineweaver–Burk plots for each of three series, depicted in Figure 5, are linear and parallel, demonstrating the validity of the Ping-Pong Bi Bi model. From the value of the slope for the Figure 5 plots, and the slope and intercept of a reciprocal plot of the *y*-intercepts vs. the fructose concentration (Fig. 5 inset), values for $V_{MAX}\,[E]^{-1}$, K_S, and K_{FA} were determined to be 0.46 ± 0.02 mmol $h^{-1} g_{RML}^{-1}$, 401 ± 11 mM, and 387 ± 11 mM, respectively, where [E] represents the enzyme concentration. Note that the enzyme concentration used for the experiments depicted in Figure 5 falls in the linear region of enzyme concentration vs. initial rate (Fig. 4 inset).

To simulate reaction conditions in which the saccharide concentration is maintained at its saturation limit, i.e., the experimental conditions corresponding to Figure 2, the phase boundary relationship (Eq. 4) is substituted for the saccharide concentration in Equation 5. Substitution for [FA] in terms of fractional conversion, X,

$$[\mathrm{FA}] = \frac{\rho_{\mathrm{FA}}}{\mathrm{MW}_{\mathrm{FA}}} \cdot (1 - X) \qquad [6]$$

followed by separation of the variables and integration leads to

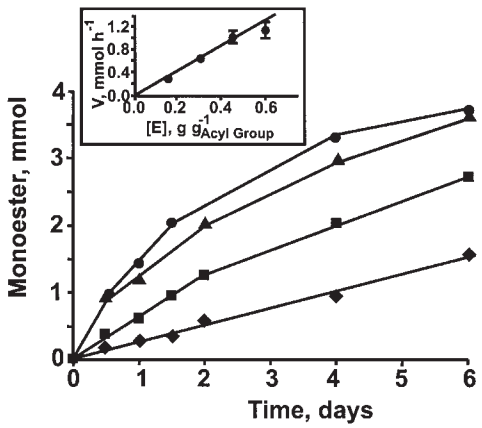

FIG. 4. Effect of ME production as a function of lipase concentration. Inset: Initial reaction rate *V* vs. lipase [*E*] concentration. The reaction mixture consisted of 1.00 g (3.54 mmol) oleic acid, 0.422 g (2.34 mmol) fructose, 23.4 g (30 mL) *t*-BuOH, and RML concentrations of (◆) 0.15, (■) 0.30, (▲) 0.45, and (●) 0.60 g $g_{oleic\ acid}^{-1}$. Reactions were conducted at 65°C with stirring at 350 rpm. *t*-BuOH and water were allowed to freely evaporate during the time course of esterification. For other abbreviations see Figures 2 and 4.

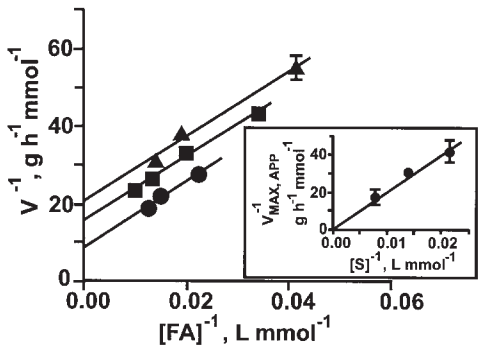

FIG. 5. Lineweaver–Burk plots used to generate the Ping-Pong Bi Bi kinetic model. Inset: reciprocal plot: inverse of *y*-intercepts, or equivalently, $V_{MAX,APP}$ (per mass of lipase), vs. the inverse of saccharide concentration, [*S*]. Each plot represents a series of experiments in which the initial oleic acid concentration was varied and the initial saccharide concentration was held constant at (●) 127.5 mM, (■) 71.5 mM, and (▲) 46.4 mM. Other conditions: 23.4 g (30 mL) *t*-BuOH, 0.3 g RML, 65°C, stirring rate of 350 rpm. *t*-BuOH and water were allowed to freely evaporate during the time course of esterification.

$$t = \frac{1}{V_{MAX}} \cdot \left(\frac{\frac{\rho_{FA}}{MW_{FA}} \cdot X + K_{FA} \cdot \ln\left(\frac{1}{1-X}\right) + \frac{K_S MW_S}{\left(\frac{A}{1-A} \cdot \left(1 - B - \frac{\rho_{ME}}{\rho_{FA}}\right) - B\right) \cdot MW_{FA}} \ln\left\{ \frac{\rho_{ME} + \rho_{FA} \cdot \left[1 - B - \frac{\rho_{ME}}{\rho_{FA}} - \frac{B \cdot (1-A)}{A}\right]}{\rho_{ME} + \rho_{FA} \cdot \left[1 - B - \frac{\rho_{ME}}{\rho_{FA}} - \frac{B \cdot (1-A)}{A}\right] \cdot (1-X)} \right\} \right) \quad [7]$$

Equation 7 provides a good fit to the time course of the reaction data (up to 80% conversion; Fig. 2A), even though the derived model used many assumptions; did not account for the effect of polarity, or log *P*, on the values of kinetic constants; and neglected the conversion of ME into DE. The linearity of the conversion vs. time plot suggests the first term of Equation 7 is the most influential. A substrate inhibition term was not required in the fitting of the data, suggesting its absence or near absence, in agreement with results for lipase-catalyzed glucose–lauric acid esterification (11). In contrast, FA substrate inhibition was present for octyl glucoside–octanoic acid esterification (21), perhaps reflecting the greater frequency of inhibition for short-chain FFA (20,22,23). The value of productivity, or V_{MAX} $[E]^{-1}$, achieved herein, 0.46 mmol h^{-1} $g_{RML}{}^{-1}$, is lower than that obtained for the same reaction operated at a 2:1 mole ratio of oleic acid to fructose, 1.6 mmol h^{-1} g^{-1}, owing to the difference in biocatalyst type [immobilized *Candida antarctica* lipase, Novozym 435 (Novozymes, Inc.), for the previous result] (13).

Effect of water content. Under similar operating conditions as used herein, we demonstrated that the removal of water from reaction medium components before initiation of the reaction did not increase the rate or extent of the reaction; moreover, the rate was lowered in cases when RML was significantly dehydrated (13). Similarly, the water content of enzyme was recently reported to be a minor factor for a large range of this parameter for saccharide ester synthesis in organic solvent mixtures (9). To determine whether water removal from the reaction medium *via* evaporation would lead to a significant increase in the conversion from the apparent ~80% threshold, we combined the reaction medium from the experiment depicted in Figure 2 with fresh RML and subjected it to vacuum pressure. Although the water content of the liquid phase was decreased from 1.6 to 1.0 wt%, the increase in conversion was very small, within 2% (data not shown).

We hypothesize that the limitation for the conversion at 80–90% is due to the thermodynamic equilibrium. Moreover, equilibrium-limited conversion has been observed for lipase-catalyzed saccharide–FA ester synthesis (11,21,24). To examine the effect of water content on equilibrium, experiments were operated in closed reaction systems in which the water activity of the reactor air headspace was controlled by saturated salt solutions. According to the results, a decrease in the water content of the medium led to a slight increase in the percent conversion (Table 1). Values of the concentration-based equilibrium constant, K_C, ranged between 4.2 and 6.1 (Table 1) for fructose–oleic acid esterification at 65°C, calculated according to the following equation:

TABLE 1
Effect of the Control of Water Activity, $a_{w'}$ on the Equilibrium Conversion of Fructose–Oleic Acid Esterification[a]

Agent used for a_w control	$a_{w'}$ 24°C	% Conversion of oleic acid[b]	K_C[c]
$CaSO_4$[d]	<0.022	93.1 ± 6.7	4.2
Uncontrolled	0.311	89.9 ± 1.0	4.5
NaCl (aq)[d]	0.752	84.8 ± 4.8	6.1

[a]Reaction conditions: Initial medium contained about 70% monoester, fructose (at a saturation level in accordance with the phase diagram of Fig. 1), oleic acid, and *Rhizomucor miehei* immobilized on macroporous anionic resin beads (RML). The reaction was conducted at 60°C. Fructose was continually added in small (batch) increments to maintain saturation.
[b]Error limits calculated based on a 95% confidence interval using a *t*-distribution.
[c]Calculated according to Equation 8.
[d]The reaction mixture was contained in a beaker, which was placed in a sealed desiccator that also contained $CaSO_4$ or NaCl (aq), and placed on top of a hot plate/stirrer.

$$K_C = [ME]_{eq} [H_2O]_{eq} [S]_{eq}^{-1} [FA]_{eq}^{-1} \quad [8]$$

In agreement, the K_C value for the experiment depicted in Figure 2 was calculated to be 6.4. The values obtained herein are an order of magnitude higher than values reported for octyl glucoside octanoic acid ester synthesis in acetonitrile, 0.55 (21), and glucose–lauric acid synthesis in 2-methyl-2-butanol, (0.30) (11).

To help monitor the water content of RML during the course of the reaction, water adsorption isotherms at 60°C were determined from water concentration measurements of the reaction mixture and RML stored over saturated salt solutions for several days (Fig. 6). The values of the water content for RML vs. a_W, the latter dictated by the salt type, are in strong agreement with the literature (25). As an example of its utility, Figure 6 predicts the water content of the solid phase of the final product for the reaction depicted in Figure 2 (80% ME and 20% FFA, liquid phase water concentration of 1.6%) is 8%, which is near, but slightly lower than, the manufacturer's suggested water content, 10%. This result may indicate that RML is

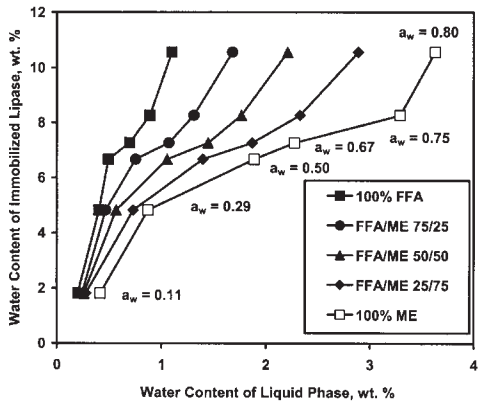

FIG. 6. Water isotherms at 60°C as a function of liquid phase composition. Ordinate values correspond to a specific water activity, or $a_{w'}$ value at 40°C, indicated in the figure. For abbreviation see Figure 3.

TABLE 2
Enzyme Activity Retention of RML During Successive Batch Runs of Lipase-Catalyzed Esterification of Fructose and Oleic Acid[a]

Batch reaction number	$V_{MAX, Batch\ n}/V_{MAX, Batch\ 1}$	Reaction time, h (d)
1	—	173 (7.2)
2	1.11 ± 0.25	239 (10.0)
3	1.21 ± 0.24	173 (7.2)
Total		585 (24.4)

[a]Reaction conditions: Initial medium contained about 70% monoester, fructose (at a saturation level in accordance with the phase diagram of Fig. 1), oleic acid, and RML. The reaction was conducted at 60°C. Fructose was continually added in small (batch) increments to maintain saturation, such that the overall mole ratio of fructose to oleic acyl groups was near 1:1. At the end of a batch reaction, RML was isolated by filtration, washed with acetone, allowed to dry, and reused in the subsequent batch reaction. For abbreviation see Table 1.

slowly dehydrating and that a means of resupplying water to the biocatalyst may be required for long-term operation. Figure 6 suggests that the liquid phase water content will increase during the course of the reaction for a fixed value of a_W, further suggesting the difficulty in removing water from the reaction medium without dehydrating RML in order to drive the reaction toward 100% conversion.

Enzyme activity retention. To examine the activity retention of RML during the synthesis of saccharide esters as conducted using the methodology described herein, 0.5 g of RML was used in three successive reactions operated in batch mode. The initial reaction medium contained fructose-saturated 70 wt% fructose monoleate/30% oleic acid. Fructose was added in a fed batch mode during the course of the reaction until a 1:1 mole ratio of fructose to oleyl acyl groups was achieved; saturation or near-saturation conditions of fructose were maintained. On achievement of at least 90% conversion of the oleyl acyl groups, RML was isolated by filtration, washed with acetone, and reused in a successive batch reaction. The time course of reaction in all cases was linear, in agreement with the trend shown in Figure 2.

According to the results, there was no loss of activity during the three successive batches; in fact, the activity increased slightly in batches 2 and 3 relative to batch 1 (Table 2). The results do not take into account small amounts of RML that were lost between batches during its recovery and reuse, which would result in an even higher relative activity for the second and third successive batch reactions. In sum, RML remained fully active for three successive batch experiments, amounting to 585 h (24.4 d) of operation.

ACKNOWLEDGMENT

Financial support was provided by USDA Grant 2001-01450. April R. Kelly helped collect experimental data. Dr. Hans Christian Holm, Novozymes North America Inc., kindly donated the Lipozyme IM for this work.

REFERENCES

1. Nakamura, S., Using Sucrose Esters as Food Additives, *INFORM 8*:866–874 (1997).
2. Plou, F.J., M.A. Cruces, M. Ferrer, G. Fuentes, E. Pastor, M. Bernabe, M. Christensen, F. Comelles, J.L. Parra, and A. Ballesteros, Enzymatic Acylation of Di- and Trisaccharides with Fatty Acids: Choosing the Appropriate Enzyme, Support, and Solvent, *J. Biotechnol. 96*:55–66 (2002).
3. Kaufman, V.R., and N. Garti, Organic Reactions in Emulsions—Preparation of Glycerol and Polyglycerol Esters of Fatty Acids by Transesterification Reaction, *J. Am. Oil Chem. Soc. 59*:471–474 (1982).
4. Feuge, R.O., H.J. Zeringue, Jr., T.J. Weiss, and M. Brown, Preparation of Sucrose Esters by Interesterification, *Ibid. 47*:56–60 (1970).
5. Cruces, M.A., F.J. Plou, M. Ferrer, M. Bernabe, and A. Ballesteros, Improved Synthesis of Sucrose Fatty Acid Monoesters, *Ibid. 78*:541–546 (2001).
6. Lang, S., C. Syldatk, and U. Rau, Enzymatic Synthesis and Modification of Glycolipids, in *Enzymes in Lipid Modification*, edited by U.T. Bornscheuer, Wiley-VCH, Weinheim, Germany, 2000, pp. 363–393.
7. Sarney, D.B., and E.N. Vulfson, Enzymatic Synthesis of Sugar Fatty Acid Esters in Solvent-Free Media, in *Enzymes in Nonaqueous Solvents: Methods and Protocols (Methods in Biotechnology Vol. 15)*, edited by E.N. Vulfson, P.J. Halling, and H.L. Holland, Humana Press, Totawa, NJ, 2001, pp. 531–543.
8. Laane, C., S. Boeren, K. Vos, and C. Veeger, Rules for Optimization of Biocatalysis in Organic Solvents, *Biotechnol. Bioeng. 30*:81–87 (1987).
9. Degn, P., and W. Zimmermann, Optimization of Carbohydrate Fatty Acid Ester Synthesis in Organic Media by a Lipase from *Candida antarctica*, *Ibid. 74*:483–491 (2001).
10. Park, S., F. Viklund, K. Hult, and R.J. Kazlauskas, Ionic Liquids Create New Opportunities for Nonaqueous Biocatalysis with Polar Substrates: Acylation of Glucose and Ascorbic Acid, in *Ionic Liquids as Green Solvents: Progress and Prospects (ACS Symposium Series No. 856)*, edited by R.D. Rogers, American Chemical Society, Washington, DC, 2003, pp. 225–238.
11. Flores, M.V., and P.J. Halling, Full Model for Reversible Kinetics of Lipase-Catalyzed Sugar-Ester Synthesis in 2-Methyl 2-Butanol, *Biotechnol. Bioeng. 78*:794–800 (2002).
12. Yan, Y., U.T. Bornscheuer, G. Stadler, S. Lutz-Wahl, R.T. Otto, M. Reuss, and R.D. Schmid, Regioselective Lipase-Catalyzed Synthesis of Glucose Ester on a Preparative Scale, *Eur. J. Lipid Sci. Technol. 103*:583–587 (2001).
13. Zhang, X., and D.G. Hayes, Increased Rate of Lipase-Catalyzed Saccharide–Fatty Acid Esterification by Control of Reaction Medium, *J. Am. Oil Chem. Soc. 76*:1495–1500 (1999).
14. Rockland, L.B., Saturated Salt Solutions for Static Control of Relative Humidity Between 5° and 40°C, *Anal. Chem. 32*:1375–1376 (1960).
15. Greenspan, L., Humidity Fixed Points of Binary Saturated Aqueous Solutions, *J. Res. Natl. Bureau Stds. A, Phys. Chem. 81A*:89–96 (1977).
16. Tsavas, P., S. Polydorou, I. Faflia, E.C. Voutsas, D. Tassios, M.V. Flores, K. Naraghi, P.J. Halling, F. Chamouleau, M. Ghoul, J.-M. Engasser, M. Ferrer, and F.J. Plou, Solubility of Glucose in Mixtures Containing 2-Methyl-2-butanol, Dimethyl Sulfoxide, Acids, Esters, and Water, *J. Chem. Eng. Data 47*:807–810 (2002).
17. Noureddini, H., B.C. Teoh, and L.D. Clements, Densities of Vegetable Oils and Fatty Acids, *J. Am. Oil Chem. Soc. 69*:1184–1188 (1992).
18. Cao, L., A. Fischer, U.T. Bornscheuer, and R.D. Schmid, Lipase-Catalyzed Solid Phase Synthesis of Sugar Fatty Acid Esters, *Biocatal. Biotrans. 14*:269–283 (1997).
19. Segel, I.H., *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, John Wiley & Sons, New York, 1975.

20. Paiva, A.L., V.M. Balcao, and F.X. Malcata, Kinetics and Mechanisms of Reactions Catalyzed by Immobilized Lipases, *Enzyme Microb. Technol. 27*:187–204 (2000).
21. Kobayashi, T., S. Adachi, and R. Matsuno, Kinetic Analysis of the Immobilized-Lipase-Catalyzed Synthesis of Octanoyl Octyl Glucoside in Acetonitrile, *Biochem. Eng. J. 16*:323–328 (2003).
22. Paiva, A.L., D. Van Rossum, and F.X. Malcata, Kinetics of Lipase-Mediated Synthesis of Butyl Butyrate in *n*-Hexane, *Biocatal. Biotrans. 20*:43–51 (2002).
23. Mensah, P., J.L. Gainer, and G. Carta, Adsorptive Control of Water in Esterification with Immobilized Enzymes: I. Batch Reactor Behavior, *Biotechnol. Bioeng. 60*:434–444 (1998).
24. Zhang, X., S. Adachi, Y. Watanabe, T. Kobayashi, and R. Matsuno, Prediction of the Equilibrium Conversion for the Synthesis of Acyl Hexose Through Lipase-Catalyzed Condensation in Water-Miscible Solvent in the Presence of Molecular Sieve, *Biotechnol. Prog. 19*:293–297 (2003).
25. Condoret, J.-S., S. Vankan, X. Joulia, and A. Marty, Prediction of Water Adsorption Curves for Heterogeneous Biocatalysis in Organic and Supercritical Solvents, *Chem. Eng. Sci. 52*:213–220 (1996).

[Received October 20, 2004; accepted May 27, 2005]