J Am Oil Chem Soc (2010) 87:281–293
DOI 10.1007/s11746-009-1504-2

ORIGINAL PAPER

Lipase-Catalyzed Synthesis of Saccharide–Fatty Acid Esters Using Suspensions of Saccharide Crystals in Solvent-Free Media

Ran Ye · Sang-Hyun Pyo · Douglas G. Hayes

Received: 21 July 2009 / Revised: 15 October 2009 / Accepted: 24 October 2009 / Published online: 16 November 2009

Abstract Saccharide–fatty acid esters, important biobased and biodegradable emulsifiers in foods, cosmetics, and pharmaceuticals, were produced with high yields and productivity via immobilized *Rhizomucor miehei* lipase-catalyzed esterification in solvent-free systems at 65 °C. Preliminary experiments demonstrated high rates of reaction occurred in the presence of acetone near or above its boiling point, due to the formation of 10–200 μm suspensions of saccharide particles. Subsequently, a two-step process was developed to produce a solvent-free supersaturated solution of 1.5–2.0 wt% saccharide that remained stable for ≥10–12 h. The solvent-free suspensions were used in a bioreactor system at 65 °C, consisting of a reservoir open to the atmosphere that contained molecular sieves, a peristaltic pump, and a packed bed bioreactor, operated under continuous recirculation. At 10 h intervals, suspensions were re-formed by treating the substrate/product mixture with additional acyl acceptor and applying strong agitation. Using this system and approach, a product mixture containing 88% fructose oleate was formed, of which 92% was monoester, within 6 days. This equates to a productivity of 0.2 mmol h^{-1} g^{-1}, which is similar to values reported for synthesis in the presence of solvent.

Keywords Biobased surfactant · Biocatalysis · Bioreactor · Enzyme · Fructose–oleic acid esters · Lipase · Saccharide–fatty acid esters · Solvent-free · Sucrose–oleic acid esters

Introduction

Fatty acid esters of mono- and di-saccharides, biocompatible and biobased surfactants produced commercially by Mitsubishi-Kagaku (Tokyo, Japan) and Sisterna PV (Roosendaal, Netherlands), are effective emulsifiers found in a variety of food, cosmetic, and pharmaceutical products such as chocolate, toothpaste, lotions, shampoo, and lipstick [1–3]. They possess excellent antimicrobial activity as well [2, 4]. Although they are typically produced chemically at high pressure and temperature [5], biocatalytic synthesis (using lipases primarily) has received great interest due to enhanced sustainability: near-ambient pressure and temperature (leading to lower energy usage and CO_2 production), the absence of alkaline or acidic conditions (leading to lower amounts of waste products), and a more narrow product distribution, with mono- and di-esters formed selectively via the primary hydroxyl groups of the acyl acceptor [6, 7]. The major hurdle to overcome for lipase-catalyzed synthesis is the poor miscibility of the lipophilic acyl donor and hydrophilic acyl acceptor, leading to unacceptably slow reaction rates. Several different approaches have been employed to overcome this barrier (reviewed in [6–8]), with the most common approach being to employ immobilized thermophilic lipases with polar organic solvent or solvent mixtures near their boiling

Electronic supplementary material The online version of this article (doi:10.1007/s11746-009-1504-2) contains supplementary material, which is available to authorized users.

R. Ye · S.-H. Pyo · D. G. Hayes (✉)
Department of Biosystems Engineering and Soil Science, University of Tennessee, 2506 E.J. Chapman Dr., Knoxville, TN 37996-4531, USA
e-mail: dhayes1@utk.edu

Present Address:
S.-H. Pyo
Department of Biotechnology, Lund University, 221 00 Lund, Sweden

points or under reflux [e.g., *tert*-butyl or *tert*-amyl alcohol, methyl ethyl ketone, or acetone; or their mixtures with very polar solvents (octanol–water partition coefficients, or log P values, of $\leq$1), such as dimethylsulfoxide, DMSO, at <20 vol%). Great care must be employed to minimize their tendency to inactivate the biocatalyst (particularly very polar solvents) and accumulate water, which reduces yields [4, 9–11]. If such systems are operated correctly, partially solubilized acyl acceptor can be employed; and, the reaction medium's composition and temperature can be tuned to selectively precipitate out the monoester product [12, 13]. Recently, the use of ionic liquids [14, 15] and pressurized solvent systems near or above their critical points (e.g., supercritical CO_2/solvent mixtures) [16, 17] have shown enhanced rates of reaction.

Our group has focused upon employing low amounts of organic solvent, or completely solvent-free conditions, using the acyl donor as solvent, taking advantage of the enhanced miscibility of acyl donor and acceptor in the presence of the saccharide–fatty acid ester product. In stirred batch experiments operated at 65 °C with immobilized lipases and the fed-batch delivery of acyl acceptor in the presence or absence of *tert*-butanol (*t*-BuOH), 80–93% conversion into a monoester-enriched ester product was achieved in ~1 week [18, 19]. *t*-BuOH was completely evaporated away within 12–24 h, typically equivalent to ~25% conversion. Recently, the use of bioreactor systems containing packed-bed or stirred-tank bioreactors and a packed column of saccharide crystals mixed with silica gel, operated under solvent-free conditions and undergoing continuous recirculation, achieved 80–85% conversion [20, 21]. However, very low rates of reaction were achieved due to the ~1 order of magnitude lower concentration of acyl acceptor in the solvent-free media compared to the stirred batch reactions. Moreover, transport through the packed column yielded fructose concentrations at the saturation limit; in contrast, the liquid phase in the batch mode experiments contained suspensions of ~100 μm sized saccharide crystals [20, 21].

In this paper, the batch mode approach successfully employed previously by us was revisited using acetone, a solvent accepted for food processing, near or even above its boiling point temperature. During this investigation, it was discovered that the enhancement of reaction rate provided by acetone was due to the formation of 10–200 μm-sized suspensions, stable over several hours, as a result of the solvent's evaporation. Subsequently, stable suspensions of the same size range were formed under solvent-free conditions by a 2-step process: stirring for several minutes or hours, followed by sedimentations for 0.5–2 min. A solvent-free suspension was employed in a bioreactor system operated under continuous recirculation.

Materials and Methods

Materials

Technical grade oleic acid, 98% pure, as determined by HPLC [18], and Lipozyme®IM, lipase (EC 3.1.1.3) from *Rhizomucor miehei* immobilized onto macroporous anionic beads, or "RML," the latter a product manufactured by Novozymes Inc. (Franklinton, NC, USA), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fructose (>98% purity), sucrose (>99%), acetone (HPLC-grade), acetonitrile (HPLC-grade), and molecular sieves (Type 3A, 4–8 mesh, Grade 562) were obtained from Fisher Scientific (Pittsburgh, PA, USA). All materials were used without further purification. The saccharide crystals were ground into a fine powder using a mortar and pestle. Technical grade fructose–oleic acid ester (FOE), the reaction product and a component of the initial charge to the bioreactor system, was synthesized by the RML-catalyzed fed batch reaction protocol given in our modified from previous report [18], as described elsewhere [21]. The purity of FOE was 89.6% (and the remaining fraction being oleic acid), with its composition being 83.6% monoester (ME) and 16.4% diester (DE). This product was mixed with oleic acid to obtain the desired proportions of oleic acid and ester, and fed to the bioreactor system.

Stirred, Fed-batch Operation of Lipase-Catalyzed Esterification in the Presence of Acetone

For monitoring the effect of temperature at a constant lipase concentration, 5.7 g (20 mmol) oleic acid, 5.0 g acetone, 0.90 g (5 mmol) fructose, and 0.28 g RML (0.05 g per gram of oleic acid) were mixed together at 300 rpm in a 20 mL scintillation vial using a 4-position hot plate-magnetic stirrer (Super-Nuova from Barnstead, Dubuque, IA, USA) at a specified temperature. Additional batches of fructose, 0.45 g (2.5 mmol), were added on Days 1 and 3, Days 1, 3, 5, and 7, and Days 1, 3, 5, 7, and 11 of the time course for reactions at 24 °C, 45 °C, 55 °C, and 65 °C, respectively, to provide total amounts of fructose equal to 10, 15, and 17.5 mmol, respectively. The vials were opened to the atmosphere to allow for the free evaporation of water and acetone, with 95% of the latter removed within 7 h and over 99.9% within 24 h for the reaction at 65 °C. For a series of experiments for which the RML concentration varied and the temperature held constant at 65 °C, the reactions were operated identically, except that 0.9 g (5 mmol) fructose was added in fed-batch mode on Days 1, 3, and 5 (20 mmol fructose total).

For an additional series of experiments, different combinations of the reaction medium components at their initial concentrations (oleic acid, fructose, RML, and/or

acetone) were pre-incubated together at 55 °C for 24 h in closed scintillation vials. Subsequently, the mixtures were heated to 65 °C, the vials were uncapped to allow for free evaporation of water and acetone, and the missing medium components were added to initiate the reactions. The reactions' initial and operating conditions, including fed-batch programming of fructose, were conducted as described above for the variation of the RML concentration, except that 0.57 g of molecular sieves (0.1 g per gram of oleic acid + FOE) was added on Day 11. Small aliquots were taken to analyze fructose and FOE concentration during the time course.

Supersaturated Solutions of Fructose in Oleic Acid + Fructose–Oleic Acid Ester

The preparation followed a 2-step process. First, a slurry of fructose crystals and a mixture of oleic acid and FOE (typically, 0.5 g and 2.0 g, respectively) was formed and transferred to a 20 mL scintillation vial placed on the hot plate-magnetic stirring device described above, opened to the atmosphere, to allow for the free evaporation of water and acetone (when used). The stir rate, temperature, and duration were specified. Second, the slurry was centrifuged at a specified angular velocity and for a specified time to remove larger particles. The model Centrific™ table-top centrifuge and model AccuSpin™ microcentrifuge, both from Fisher, were used for angular velocities of $\leq$3,000 rpm and >3,000 rpm, respectively. The supernatant was collected and employed in enzymatic reactions, and/or was analyzed for fructose content, particle size, water content and absorbance at 1,000 nm. For experiments which analyzed the stability of the supersaturated solutions, the suspensions prepared as described above were allowed to settle without stirring applied at a controlled temperature.

Operation of a Packed-Bed Bioreactor System Undergoing Continuous Recirculation

The bioreactor system consisted of a 20 mL scintillation vial open to the atmosphere which served as reservoir, a peristaltic pump (BioLogic LP® from Bio-Rad, Hercules, CA, USA), and a packed bed bioreactor (PBBR; 50 mm L × 10 mm ID Omnifit® chromatography column packed with 0.25–1.0 g of RML). The frit restrictors contained in the endcaps of the Omnifit® column were not used, but were replaced by a small piece of a 100-denier, 156-mesh polyester net manufactured by SiamDutch Mosquito Netting Co. Ltd., Bangkok, Thailand, determined gravimetrically to possess an areal density of 29 g m^{-2}. C-FLEX® 1.6 mm ID tubing made of a styrene–ethylene–butylene modified block copolymer from Cole-Parmer (Vernon Hills, IL), was used to connect the reservoir to the pump, the pump to the PBBR, and the PBBR to the reservoir, to form a closed-loop system that underwent continuous recirculation. PharMed® BPT 1.6 mm ID tubing (Saint-Gobain Performance Plastics Corp., Akron, OH) was used within the peristaltic pump apparatus. The PBBR and associated tubing were placed within a convection oven to yield a constant temperature of 65 °C for the recirculating liquid phase. The reservoir's contents were maintained at 65 °C and stirred gently at 200 rpm using the above-mentioned hot plate/stirrer. Molecular sieves (0.10 g per gram of oleic acid + FOE) were added to the reservoir for some experiments after reaching 60–70% ester content to reduce the water content and enhance the degree of conversion.

The reaction was carried out using a supersaturated solution of fructose prepared by the method described above, by stirring a slurry or saccharide crystals (1.5 g) in an oleic acid/saccharide–oleic acid mixture (10 g) at 80 °C and 800 rpm for 6 h, followed by centrifugation at 800 rpm for 30 s, and then collecting the supernatant. The initial charge to the bioreactor systems consisted of a supersaturated solution formed using 25% FOE and 75% oleic acid. At 10-h intervals, the suspensions contained within the reservoir were re-treated as described above to incorporate additional saccharide. For the retreatment to occur, the bioreactor system was temporarily disabled by stopping the pump, the reservoir's contents removed to a separate container, 1.5 g of saccharide added, and the suspension reformed by the 2-step method given above and then returned to the reservoir.

Monitoring of Water, Oleic Acid, Ester, and Fructose Concentration

The water content for an aliquot of the reaction mixture, after being diluted with methanol, was analyzed by Karl–Fischer titration using a Coulometric KF Titrator (Denver Instrument Company, Aurora, CO). Quantitative analysis of oleic acid and its mono- and di-esters on a fructose-free basis was performed using a dual-pump system from Varian (Walnut Grove, CA) and a model Mark III evaporative light scattering detector from Alltech Associates, a division of WR Grace (Deerfield, IL) [21]. An analytical reversed phase (4.6 × 250 mm, pore diameter 5 μm) C_{18} column from Alltech was employed using separation conditions consisting of a column temperature of 25 °C and an isocratic solvent system, acetone/acetonitrile/acetic acid (45/45/10 v/v/v) at flow rate of 1.0 mL min^{-1}. Response factors were measured and employed to convert peak areas into concentrations.

To analyze the fructose content, 40 mg-sized aliquots of column effluent were subjected to liquid–liquid extraction

by the system of *n*-hexane and water (500 μL of each) [21]. The extraction was carried out 3× at 35 °C for 2 h using a thermomixer (Eppendorf AG, Germany). The aliquots from the pooled aqueous extraction solutions were diluted with acetonitrile to match the composition of the HPLC mobile phase to prevent peak broadening in the HPLC analysis. An analytical Prevail Carbohydrate ES column (4.6 × 250 mm, pore diameter 5 μm) from Alltech was employed using a column temperature of 25 °C and an isocratic solvent system, acetonitrile/deionized water (80/20 v/v) at flow rate of 1 mL min^{-1}. Standard curves for fructose concentration in an oleic acid/fructose oleate liquid phase versus peak area were obtained and found to be independent of the reaction mixture's composition.

Measurements of Absorbance and Particle Size for Suspensions

The absorbance of solutions between 500 and 1,000 nm was performed to provide a measure of turbidity using a model UV-1700 instrument from Shimadzu (Japan) and either a 1.0 or 0.2 cm pathlength quartz length cuvette by Hellma (Plainview, NY, USA), with all reported values normalized to a 1.0 cm pathlength. Absorbance values at 1,000 nm are reported herein since at this wavelength the values were often <1.0 units, meaning that the linear Beer-Lambert law is applicable. Differences of absorbance values between samples at this wavelength are representative in trend of differences at the other wavelengths. The particle size distribution of the dispersions present in the above-mentioned solutions was analyzed by Zeta potential Analyzer, Zeta PALS (Brookhaven Instruments Corporation, Holtsville, NY, USA).

Results and Discussion

Effect of Acetone on Lipase-Catalyzed Fructose–Oleic Acid Esterification Operated in Fed-Batch Mode

As described elsewhere [20] bioreactor systems employing a silica gel/fructose packed bed for delivery of acyl acceptor yielded conversions >85%, but slow rates of reaction compared to those operated using in fed-batch fructose addition in stirred batch mode [18]. The best results for fed-batch operation occurred when *t*-BuOH was present during the initial stage of the reaction (from 0 to ~20% conversion of oleic acid) [18]. Therefore, the latter approach was revisited with *t*-BuOH being replaced by acetone, a polar solvent acceptable for use in food processing and employed previously for lipase-catalyzed saccharide–fatty acid ester synthesis [11, 22, 23]. As depicted in Fig. 1a, the employment of acetone at moderate temperatures (24 °C and 45 °C) yielded poor results. However, when the temperature was just below or slightly above the boiling point temperature of acetone, 56 °C (55 °C and 65 °C, respectively), the rate of reaction was greatly accelerated. This occurred, despite the complete removal of acetone via evaporation within a few hours. The optimal RML concentration at 65 °C was shown to be approximately 5% (solvent-free basis; Fig. 1), which is comparable to the optimal concentration reported by us for batch-mode and solvent-free bioreactor operation [18, 20]. RML at a concentration >5% is not as effectively dispersed and thus does not yield a higher biocatalytic turnover. The time course of reaction achieved was highly repeatability, demonstrated by the small error bars and the overlapping of the time course of reaction for the reactions operated at 65 °C and RML concentrations above 0.050 g per gram of oleic acid, despite the employment of two different programs for the fed-batch addition of fructose (for the filled versus unfilled symbols; Fig. 1a). The fructose concentration in the liquid phase remained low throughout the time course, at 0.1–0.2 wt%, but increased slightly as a function of time, as a result of the fed-batch addition programming employed (Fig. 1b), leading to the product distribution in favor of DE (66% DE and 34% ME; Table 1). Moreover, the competition between fructose and ME as nucleophiles attacking the acyl-enzyme intermediate is skewed toward the latter due to its relatively large concentration. This result demonstrates that the selectivity toward ME or DE can be controlled through the acyl acceptor/acyl donor mole ratio. The water content of the liquid phase remained fairly high throughout the time course, near 0.6 wt% (Fig. 1b; Table 1).

Table 1 compares the performance of the acetone-based reactions of Fig. 1 to other approaches employed by our group [18, 20, 21]. As demonstrated, the initial rate of reaction for a PBBR-based system was a factor of ~2–3 smaller than the batch-mode reactions, due to the ~5–10-fold lower fructose concentrations. The rate of reaction achieved using acetone was slightly higher than obtained for batch-mode using *t*-BuOH or in the absence of solvent. The conversion, 88% (after the addition of molecular sieves) is comparable to that achieved using *t*-BuOH, 90% (93% when controlling the system's water activity via calcium sulfate) [18].

To help further understand the underlying cause for the enhancement of reaction rate by acetone, different combination of the starting materials (substrates and RML) were pre-incubated with acetone in a closed vessel for 24 h at 55 °C. Subsequently, the mixture's contents were transferred to a vessel open to the atmosphere (to allow for free evaporation of water and acetone) and heated to 65 °C. The ingredients not present in the pre-incubation mixture were then added to initiate the reaction. When RML was pre-

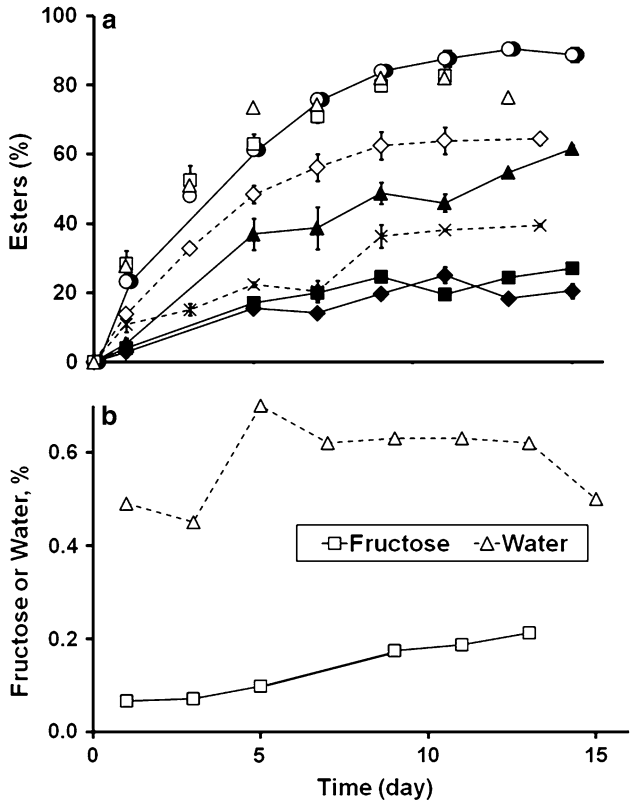

Fig. 1 Effect of temperature and lipase concentration on the RML-catalyzed esterification of fructose and oleic acid in the presence of acetone. **a** Percent conversion and **b** water and fructose concentration (65 °C, 5% RML reaction). **a** Effect of temperature (*filled diamonds* 24 °C, *filled squares* 45 °C, *filled triangles* 55 °C, *filled circles* 65 °C) at a constant RML concentration, 0.05 g per gram of oleic acid. Effect of RML concentration (*crosses* 0.01, *open diamonds* 0.025, *open circles* 0.050, *open squares* 0.075, *open triangles* 0.10 g per gram of oleic acid) at 65 °C. Initial conditions (with volumes referenced to 25 °C): 6.37 mL (4.8 g) acetone, 6.37 mL (5.7 g, 20 mmol) oleic acid, and fructose (0.9 g, 5 mmol). Fructose (1.8–3.6 g, 10–20 mmol, total) added in fed-batch mode as described in the experimental section. *Error bars* reflect the standard deviation of measurements for 2 replicates of the biochemical reaction

incubated, either in the presence or absence of acyl donor or acceptor substrate, the rate of reaction was relatively slow; but, the production of FOE continued steadily (Fig. 2). However, when the substrates were pre-incubated with acetone in the absence of RML, the time course of reaction was significantly faster, being nearly slightly higher that displayed in Fig. 1a (e.g., for day 3, ~60 vs. ~50% conversion into FOE). Therefore it can be concluded that the enhancement by acetone is due to the improved dispersion of fructose in solvent-free media (oleic acid + FOE), not to a change in RML's inherent activity. On the contrary, it is probable that during the pre-incubation acetone desorbed water from RML, lowering RML's water content below the manufacturer's suggested level, 10%, hence yielding a lower rate.

On the Formation of Stable Suspensions of Saccharide in Solvent-Free Media

As noted previously by us, the low fructose concentration observed for solvent-free FOE/oleic acid liquid phase that exits from a packed crystalline fructose/silica gel column in bioreactor systems corresponded to the solubilization limit, with particles of size >10 μm (dynamic light scattering, DLS) and absorbance in the visible spectral region (500–1,000 nm) being below detection limits; in contrast, stirred batch systems yielded ~100 μm sized particles and significantly large absorbance [21]. Employment of suspended saccharide particles, or supersaturated solutions, for the synthesis of lipase-catalyzed saccharide–fatty acid esters has led to greatly enhanced rates of reactions when conducted in organic solvents [10, 24] and ionic liquids [15, 25, 26]. To determine whether the presence of acetone near or above its boiling point led to the formation of suspensions (similar to the forced evaporation of water to make suspensions in ionic liquids [15]), the liquid phase formed at 65 °C as per Figs. 1 and 2 at three different FOE/oleic acid ratios were analyzed for their apparent fructose concentration by HPLC, the average size of particles (DLS), and for the relative mass density of particles via absorbance in the visible region, with the values at 1,000 nm reported herein. In addition, the same measurements were performed for controls which shared the same compositions as the samples except for the absence of acetone. Samples were mixed via magnetic stirring at 800 rpm for nearly 1 day, then subjected to centrifugation at either 3,000 rpm or 12,000 rpm for 2 min. These conditions are comparable to those employed previously except for differences in the mixing device (vortex mixer employed previously) and duration (2 days previously) [21].

As demonstrated in Fig. 3, as the FOE concentration increased and centrifugation angular velocity decreased, the fructose concentration, absorbance, and average particle size increased. The Fig. 3 data are reasonably close to those obtained previously [21], with the variance attributed to minor difference in mixing conditions between the two data sets. The presence of acetone led to slight but significant increases of all three measurements, compared to the controls. Therefore, it is concluded that suspensions formed during the reactions performed in the presence of acetone (Figs. 1, 2); and, the presence of acetone undergoing evaporation increased the concentration of suspended fructose, but only led to a slight increase in the average size of the particles. Of note, the trends for the fructose concentration and absorbance are very similar, demonstrating the latter, a measurement easily obtained, can be used to assess saccharide concentration for one sample relative to another.

Table 1 Comparison of bioreactor systems for the rate and percent conversion of RML-catalyzed esterification of fructose and oleic acid at 65 °C that employ free evaporation for removal of water and solvent, unless noted otherwise

Bioreactor system	RML ($g\ g^{-1} \times 100\%$)[a]	Fructose wt% @ 25 (50) % FOE	Initial rate (% FOE day^{-1})	% FOE before (after) MS addition	% H_2O before (after) MS addition	ME/FOE ($g\ g^{-1}$)[b]
Stirred batch mode, *t*-BuOH[c] [18]	3.5	1.5 (2.7)	9.6	90 (93)	1.0 (N/A[d])	0.85
Stirred batch mode, Acetone[c,e]	5.0	0.07 (0.07)	16.7	84 (88)	0.6 (0.6)	0.34
Stirred batch mode, Solvent-free[c] [20, 21]	7.5	0.07 (0.18)	13.3	74 (80)	0.8 (0.5)	0.35
PBBR/Desorption column[f] [20, 21]	5.0	0.01 (0.02)[g]	5	83 (84)[h]	0.7 (0.3)[h]	0.76
PBBR system using supersaturated fructose solution[f,i]	7.5	1.5 (1.9)	15	(88)[j]	(0.8)[j]	0.92

FOE fructose oleate esters, *ME* fructose–oleic acid monoester, *MS* molecular sieves (0.10 g per gram of oleic acid + FOE), *PBBR* packed-bed bioreactor, *t-BuOH tert*-butanol

[a] Per gram of oleic acid + FOE

[b] After addition of MS

[c] Fructose added in fed-batch mode

[d] Water content not measured; however, $a_w = 0.311$ and <0.1 prior to and after storage in a closed desiccator that contained $CaSO_4$, respectively

[e] Depicted in Fig. 1

[f] Bioreactor system undergoing continuous recirculation

[g] 0.04 (0.07) = maximum value possible [3]

[h] No MS added, tabulated values reflect absence versus presence of a MS column within the system (0.10 g per gram of oleic acid + FOE)

[i] Depicted in Fig. 8

[j] MS added during the middle of the time course

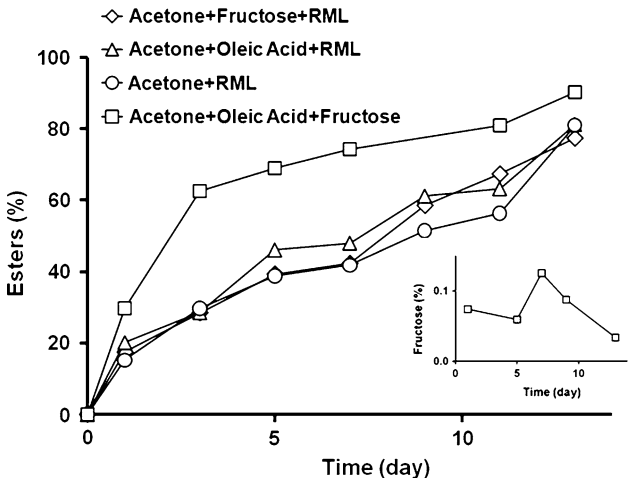

Fig. 2 Effect of pre-incubation (24 h) on the time course of RML-catalyzed esterification of fructose and oleic acid in the presence of acetone at 65 °C. *Legend* indicates medium components pre-incubated at 55 °C in a closed vessel. RML concentration: 0.05 g per gram of oleic acid. Other reaction conditions equal those given in Fig. 1a. *Inset* Fructose concentration versus time for experiment employing pre-incubation of acetone, oleic acid, and fructose

The effect of several parameters involved with the two-step suspension formation process was examined for FOE/oleic acid 25/75 g/g. Figure 4 depicts the effect of the mass ratio of acetone to oleic acid + FOE and the stirring time, with the stir rate (800 rpm), temperature (65 °C), and centrifugation conditions (3,000 rpm, 2 min) held constant. The stir times were selected to allow for nearly 100% loss of acetone via free evaporation. As illustrated, the absorbance increased linearly with the stirring time and the proportion of acetone up to a limit of 6 h (3:1 acetone/oleic acid + FOE mass ratio). When the ratio was increased from 3:1 to 5:1, no further increase of absorbance was detected, indicating a plateau for fructose concentration was achieved. For a second series of experiments that shared the same stirring times but employed solvent-free conditions, the absorbances were slightly below those of suspensions prepared using acetone, with the difference disappearing as the stirring time approached 8 h (Fig. 4). In conclusion, the concentration of suspended fructose increases linearly with stirring time; and, if a longer stir time is employed, acetone does not provide any enhancement and therefore is not needed, under the conditions employed for Fig. 4.

Figure 5 plots the effect of stir rate for a fixed stirring time (3 h) under similar conditions as those listed for Fig. 4. The results suggest the concentration of suspended fructose increased linearly with stir rate, with a two-fold increase achieved when the stir rate was increased from 200 to 800 rpm. In addition, temperature slightly increased the liquid-phase fructose concentration between 65 °C and 85 °C in a linear fashion, from 0.40 to 0.55% (Fig. S1 of the ESM). Figure 6 illustrates that the concentration,

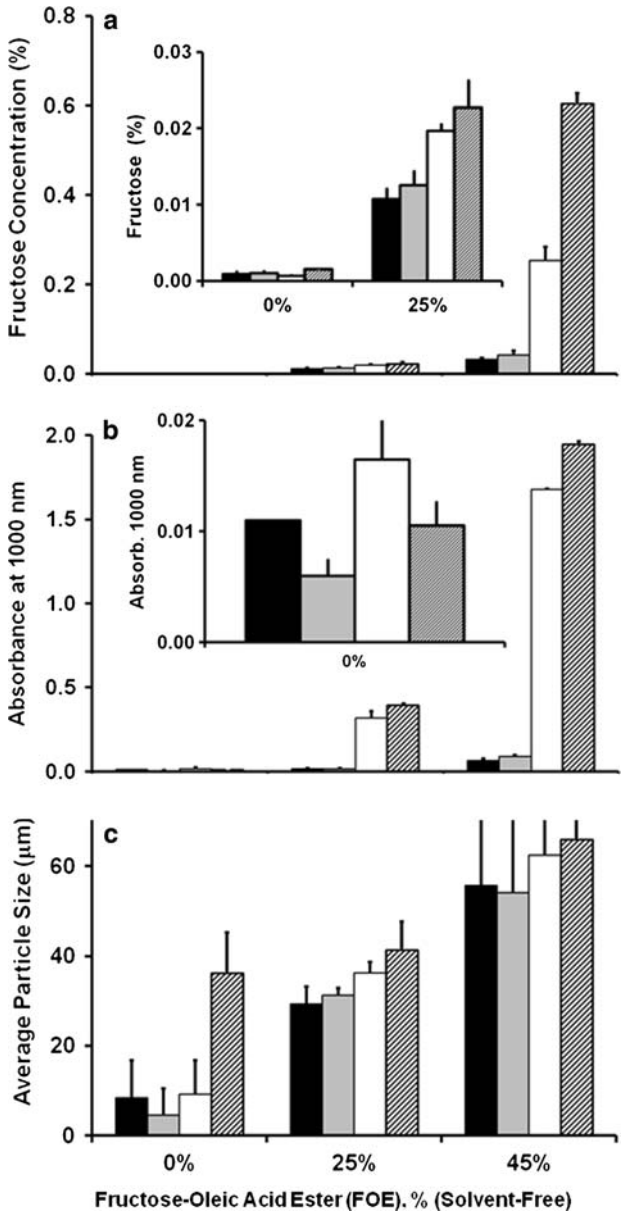

Fig. 3 Effect of acetone, wt% fructose–oleic acid ester (FOE; solvent-free basis), and sedimentation angular velocity on **a** the apparent solubility, **b** the absorbance at 1,000 nm, and **c** the average particle size of the suspensions of fructose in oleic acid/FOE mixtures at 65 °C. *Black bars* 12,000 rpm, no acetone; *gray bars* 12,000 rpm, acetone; *white bars* 3,000 rpm, no acetone; *striped bars* 3,000 rpm, acetone. *Insets* for **a** and **b** employ smaller *y*-axis ranges to provide a more precise view of the low concentrations and absorbances, respectively, for the 0 and 25% FOE data. Method: 6.0 g of FOE/oleic acid mixed with 1.5 g fructose and either 0.0 or 6.0 g acetone for 20 h at 800 rpm in a 20-mL scintillation vial opened to the atmosphere. After centrifugation for 2.0 min, the supernatant was collected and analyzed

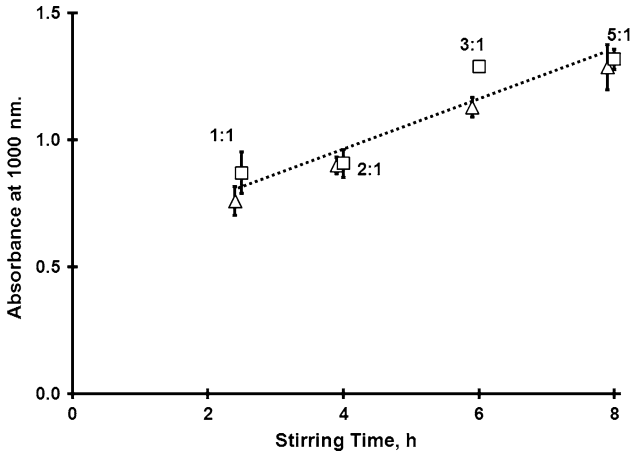

Fig. 4 Effect of stirring time on the absorbance at 1,000 nm for a suspension of fructose in solvent-free media (*open triangles*) and in the presence of acetone (*open squares*). Conditions: mixing of 0.5 g fructose, 2 g oleic acid/FOE 75/25 w/w, and acetone at a mass ratio per oleic acid/FOE as indicated in the figure; stirring at 800 rpm, 65 °C, and the indicated time, followed by centrifugation at 3,000 rpm for 2 min

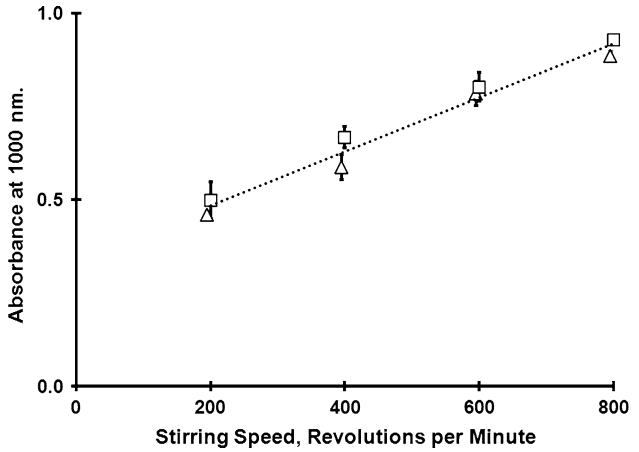

Fig. 5 Effect of stirring rate on the absorbance at 1,000 nm for a suspension of fructose in solvent-free media (*open triangles*) and in the presence of acetone (*open squares*). Conditions: mixing of 0.5 g fructose, 2 g oleic acid/FOE 75/25 w/w, and 2 g acetone at 65 °C and the indicated stir rate for 3 h, followed by centrifugation at 3,000 rpm for 2 min

absorbance, and average particle size decreased linearly as the angular velocity and centrifugation time increased, with the highest concentration achieved using small values, 800 rpm and 1.0 min, respectively, resulting in the formation of 200-µm sized particles. The decrease of all three measurements with centrifugation time and speed is due to the sedimentative removal of large fructose particles.

Figure 7 demonstrates that the suspensions are reasonably stable over a 12 h time period, with a decrease in fructose concentration being within 20%. The suspensions formed in the absence of acetone yielded the greatest stability. Therefore, a metastable solution of ~10–100 µm-sized fructose crystals suspended in liquid-phase media can be formed for solvent-free mixtures of FOE and oleic acid.

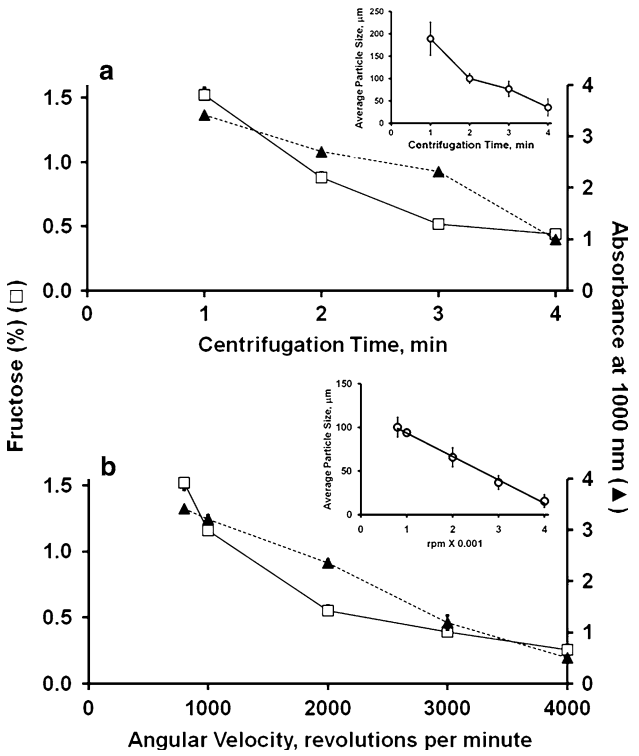

Fig. 6 Effect of centrifugation parameters on the apparent fructose concentration, absorbance at 1,000 nm, and particle size (*insets*) for a suspension of fructose in a solventless medium. Suspension prepared by mixing fructose (1.25 g) with 5 g of a mixture of oleic acid/FOE 75:25 w/w at 85 °C for 6.0 h under stirring at 800 rpm. **a** Centrifugation time varied at a constant angular velocity (800 rpm). **b** Angular velocity varied at a constant centrifugation time (1 min; 2 min for particle size)

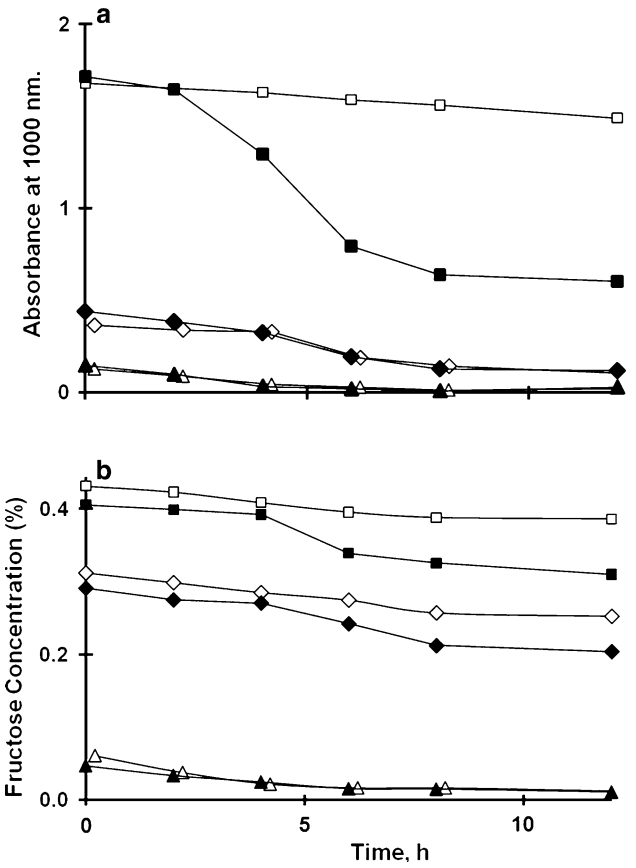

Fig. 7 Change of **a** absorbance at 1,000 nm and **b** apparent fructose concentration of liquid phase for a suspension of fructose crystals and fructose–oleic acid esters (FOE)/oleic acid prepared in the presence or absence of acetone (*filled* and *open symbols*, respectively) at 65 °C. FOE concentration: 0.0% (*filled triangles*, *open triangles*), 25% (*filled diamonds*, *open diamonds*), 45% (*filled squares*, *open squares*). Suspension prepared by mixing either 0 or 6 g acetone, 2 g FOE/oleic acid, and 0.5 g fructose at 85 °C for 3 h (100% evaporation of acetone), and centrifuging at 3,000 rpm for 2 min

Similarly, suspensions of saccharides in ionic liquids are reported to be stable over several hours [15].

The solubilization of 1.5% fructose (Fig. 4) and sucrose (as described below) for a supersaturated solvent-free media containing 25% saccharide–oleic acid ester at 65 °C, or equivalently, 22.6 and 18 g L^{-1}, respectively, represents a 60- and 1,500-fold increase in the concentration relative to a saturated solution under the same conditions [21]. The concentrations are comparable to those obtained for a supersaturated *t*-BuOH solution of glucose (1.2%; 9 g L^{-1}; 300-fold higher concentration than saturation [10]) and supersaturated solutions of fructose formed by nonpolar alkyl imidazolium–boron tetrafluoride ionic liquids (18–28 g L^{-1}, ~1.5–2.3%) [15], and significantly higher than the concentrations achieved by dissolution into acetone of sugar alcohols (~0.4%, 3 g L^{-1}) [27], fructose (~0.06%, ~0.5 g L^{-1}) [28], and glucose (~0.04%, ~0.3 g L^{-1}) [10], respectively. However, they are 3- and 10-fold lower than the solubility of sucrose in a DMSO/*tert*-amyl alcohol (80/20 v/v) mixture (8.5%; 68 g L^{-1}) [24] and in supersaturated solutions of fructose in polar alkyl imidazolium trifluoromethanesulfonate ionic liquids (130–230 g L^{-1}, ~11–19%) [15], respectively.

Saccharide–Fatty Acid Synthesis Using a Bioreactor System and Solvent-Free Suspensions

A simple bioreactor system consisting of a reservoir open to the atmosphere, a peristaltic pump, and a packed-bed bioreactor (PBBR) containing RML was configured. The liquid phase, initially consisting of a suspension of fructose crystals in a mixture of oleic acid/FOE 75/25 w/w, was continuously recirculated through the system at a constant flow rate and maintained at 65 °C. At 10-h intervals, the system was stopped and the reservoir's liquid phase isolated and treated with additional fructose to form a new suspension. The suspensions were stable throughout the 10-h period for which they were used. For instance, the fructose concentration for a suspension in oleic acid/FOE

75/25 w/w decreased linearly, from 1.7% initially, to 1.5% over a 12-h period (Fig S2 of ESM). Figure 8a depicts the effect of the PBBR's volume, reported as a fraction of the liquid-phase mass, at a constant flow rate, Q, of 0.3 mL min^{-1} for two different suspension concentrations: 0.08 and 1.7%, prepared by two different sets of centrifugation conditions: 800 rpm, 30 s and 3,000 rpm, 2 min, respectively. As anticipated, the rate of reaction was slower for the suspension possessing the lower fructose concentration (8.4% conversion day^{-1}, compared to 15% day^{-1} for the higher suspension concentration); but, the initial rate was only ~2-fold lower, incommensurately small compared to the ~20-fold difference in fructose concentration (Fig. 8a). Since the higher suspension concentration yielded a higher rate of reaction, it was employed in subsequent experiments.

Figure 8a shows that the rate of the reaction increased with the volume (mass) of RML contained within the PBBR, hence with the average residence time of the liquid phase in the PBBR. Two experiments that used 0.075 g_{RML} per gram of oleic acid + FOE, with one employing molecular sieves (added at 70 h) yielded nearly identical time courses, indicating the high repeatability of the experimental results. The time course for all reactions was linear for 2 days, up to ~60% conversion for the 0.75 g_{RML} per gram experiments; thereafter, the reaction rate slowed, presumably due to the relatively high (0.7%) water content (Fig. 8b). The addition of molecular sieves reduced the water content to ~0.5% (Fig. 8b), leading to a slight increase in the level of FOE (Fig. 8a). Optimization of the bioreactor system's water activity, to improve the rate of reaction and yield in the latter stage of the time course, is currently under investigation.

Figure 8b plots the fructose concentrations in the reservoir component of the bioreactor system both at the beginning and end of a 10-h interval, moreover, before and after the liquid phase is retreated with additional fructose and the suspensions reformed. The figure illustrates for the first 60 h of the time course, the fructose concentration at the end of the 10-h cycles for the reactions conducted with 0.075 g_{RML} per gram of oleic acid + FOE is nearly zero, which suggests the reaction rate is limited by the depletion of acyl acceptor substrate. Because the rate slows at times >60 h (Fig. 8a), the residual fructose concentration "before retreatment" is significantly higher, ~0.3%. In general, the time course of fructose utilization expressed in Fig. 8b, moreover, the distance between the "before retreatment" and "after retreatment" data versus reaction time, is consistent with the time course of ester synthesis (Fig. 8a). The increase in fructose concentration "after retreatment" versus time is consistent with the increased polarity of the liquid phase due to the formation of FOE, Fig. 3, as described in previous investigations [18–20]. The

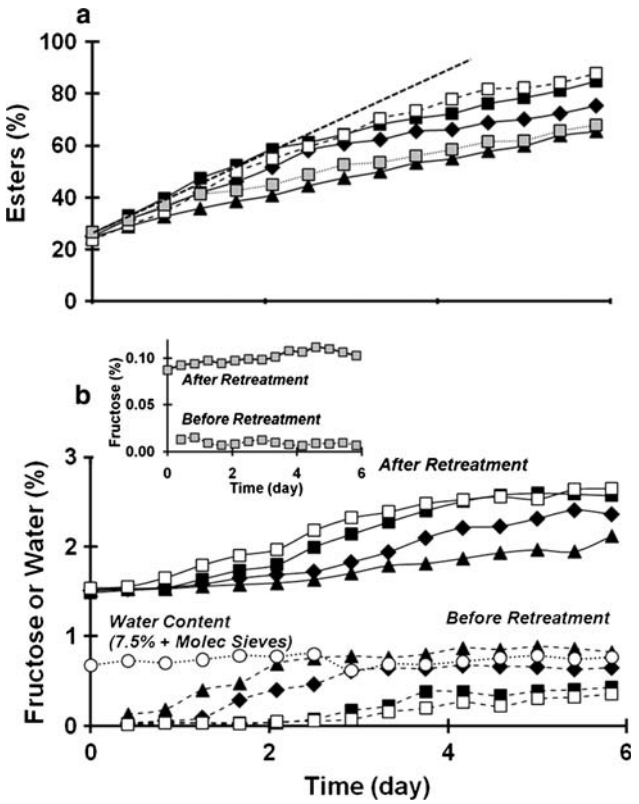

Fig. 8 Effect of bioreactor volume on the change of **a** fructose–oleic acid esters and **b** fructose concentration before (*dashed lines*) and after (*solid lines*) retreatment for solvent-free RML-catalyzed fructose–oleic acid ester synthesis utilizing a suspension of fructose crystals and a bioreactor system containing a packed-bed bioreactor (PBBR) undergoing continuous recirculation at 0.30 mL min^{-1} and 65 °C. Concentrations depicted are those of the reservoir. Mass of PBBR per mass unit of reaction medium × 100%: *filled triangle* 2.5%, *filled diamonds* 5.0%, *filled squares* 7.5%; *open squares* 7.5%, with molecular sieves (10.0 wt%) added to the system's reservoir on Day 3. Suspension of fructose reformed at 10-h intervals by recovering solution in reservoir, adding additional fructose, stirring at 80 °C and 800 rpm for 6 h, followed by centrifugation at 800 rpm for 30 s, and collecting supernatant. *Open circles* water content for experiment using 7.5% RML with 10% molecular sieves (**b**). *Shaded squares* in **a** and inset of **b**: 7.5% RML, using a suspension lower in fructose concentration, formed as indicated above, except that centrifugation occurred at 3,000 rpm for 2 min. *Dashed line* in **a** represents the determination of the initial rate of reaction

high fructose concentration depicted in Fig. 8b, 1.5–2.5%, drives the selective formation of ME versus DE, with 80–92% of the FOE product being ME (Fig S3 of ESM).

To determine if the experiments described by Fig. 8 were operated at an optimal average residence time, additional experiments were performed in which the initial charge to the bioreactor system (10 g of oleic acid/FOE 75/25 w/w) and PBBR volume (3.46 mL; 0.75 g_{RML}) were held constant and Q varied (between 0.3 and 1.2 mL min^{-1}). The initial rate for this series of reactions, determined from plotting the time course data between 0 and 40 h (Fig S4 of ESM) and those depicted in Fig. 8, for

which the PBBR volume was varied, are plotted as a function of residence time per pass through the bioreactor system, estimated to be the volume of the PBBR divided by Q (Fig. 9). The maximal initial rate, 15.5–16.0% conversion of oleyl acyl groups per day, was achieved when employing a residence time of 10 min or greater, or equivalently $Q \leq 0.3$ mL min^{-1} for a PBBR containing 0.075 g per gram of oleic acid + fructose. At residence times below 6 min, the initial rate increased with residence time in a linear fashion, consistent with classical Michaelis–Menten kinetics and observed in batch-mode reactions by us [18].

In our group's previous batch mode work, other acyl acceptors were successfully employed as replacements for fructose, particularly sucrose, xylose, and glucose [18]. The rates of reaction were a factor of 2–5 lower than achieved for fructose, attributable mainly to the inherent ability of the acyl acceptors' primary hydroxyl groups to penetrate into the active site of the enzyme and nucleophilicly attack the acyl-enzyme intermediate, consistent with the technical literature [7]. However, when employing bioreactor systems using packed silica gel/saccharide crystal columns for delivery of the saccharide, the rate of reaction for other saccharides was unacceptably slow, by over an order of magnitude compared to fructose (unpublished data). The underlying reason for the slow rate was the very low concentration of the saccharides in the solvent-free medium. As described above, employment of the packed columns produced saturated solutions but not suspensions, evidenced by the absence of micron-sized particles [21]. Compared to the concentration of fructose provided by the packed columns, which varied from 0.01 to 0.1% as the fraction of ester was increased from 5 to 65%, the concentration of xylose and glucose varied from 0.005 to 0.04% and 0.002–0.01%, respectively; and, the sucrose concentration remained below 0. 002% for sucrose–oleate concentrations ≤0.85 (Fig S5 of the ESM). When using suspensions and the reservoir-PBBR bioreactor systems employed for the experiments of Figs. 8 and 9 for sucrose oleate synthesis, the time course was similar to that obtained for preparation of fructose oleate (Fig. 10). The difference between the rates of reaction for fructose and sucrose (11.8 and 15–16% conversion of acyl groups per day, respectively, a 27% difference), is comparable to difference between acyl acceptors obtained using suspensions in batch mode [18]. The sucrose concentration using the solvent-free bioreactor system was ~2% throughout the time course, comparable to that obtained for fructose (Figs. 3, 10). The product distribution consisted of 80–90% ME and 10–20% DE throughout the time course (Fig S3 of the ESM).

Table 1 compares the performance of the PBBR bioreactor scheme employing a supersaturated solution of saccharide suspensions in solvent-free media to other completely or nearly solvent-free media for the RML-catalyzed synthesis of fructose–oleic acid esters. The initial rate obtained (15.5–16.0% conversion per day), fructose concentration (1.5% for 25% FOE/75% oleic acid), and ME fraction among the esters (0.92) are in strong agreement with those obtained using stirred batch systems, where suspensions were also probably present. The final conversion, 88% (occurring when molecular sieves were added to the bioreactor system's reservoir) is a little lower than batch mode results (90–93%) due to the relatively high water content, 0.8%.

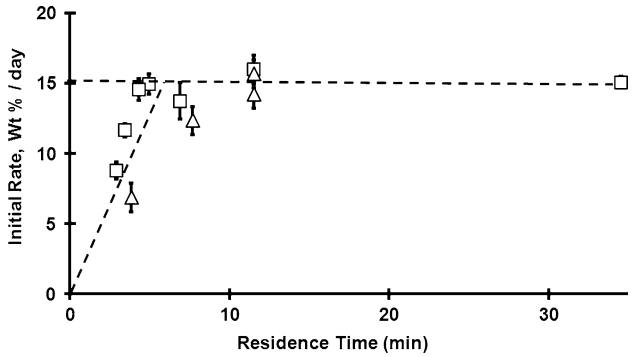

Fig. 9 Effect of residence time in packed bed bioreactor (PBBR) per pass through the bioreactor system on the initial rate of esterification (wt% FOE/day) for a series of experiments where the volumetric flow rate, Q, was varied and the volume of the PBBR, V_{PBBR}, was held constant (*open squares*; 0.75 g RML, equal to 3.46 mL; data plotted in Fig S4 of ESM) and V_{PBBR} was varied for $Q = 0.3$ mL min^{-1} (*open triangles*; data plotted in Fig. 8). The initial charge to the bioreactor system was 10 g (75% oleic acid and 25% FOE). *Error bars* reflect the standard error of the slope of a straight-line fit applied to %esters versus time during the initial period of the time course

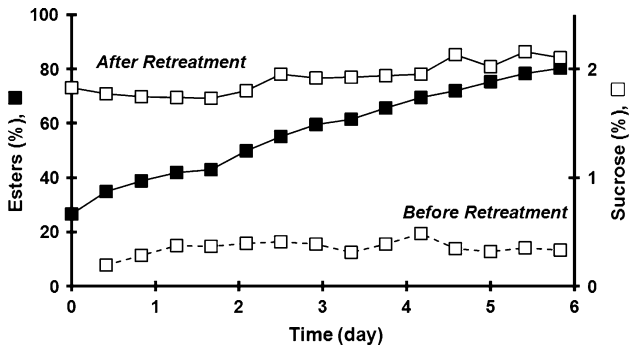

Fig. 10 Effect of bioreactor volume on the change of esters and saccharide concentration before (*dashed lines*) and after (*solid lines*) retreatment for solvent-free RML-catalyzed sucrose–oleic acid ester synthesis utilizing a suspension of sucrose crystals and a bioreactor system containing a packed-bed bioreactor (PBBR; 0.075 g RML per gram of oleic acid + sucrose oleate) undergoing continuous recirculation at 0.30 mL/min and 65 °C. Suspension of sucrose reformed at 10.0 h intervals by recovering solution in reservoir, adding additional sucrose, stirring at 80 °C and 800 rpm for 6 h, followed by centrifugation at 800 rpm for 1 min, and collecting supernatant

The conversion achieved and overall duration of the reaction, 80% FOE in 110 h and 88% FOE in 144 h (or 73 and 84% conversion of the oleic acid fed to the bioreactor system, respectively), according to Fig. 8 (starting at 25% conversion), are slower than many literature examples that employed solvents: 70% conversion within 24 h for fructose palmitate synthesis in the presence of methyl ethyl ketone [11], 25% conversion within 4 h for a supercritical fluid CO_2/acetone mixture [16], and ~40% conversion in 12 h, 90% conversion in 100 h, for glucose–laurate synthesis using a suspension of glucose in the polar ionic liquid 1-butyl, 3-methyl imidazolium trifluoromethanesulfonate that was 10-fold higher in saccharide concentration [15]. (In subsequent work, it was shown that the stability of lipase in the latter ionic liquid was poor; however, when it was mixed with an equal proportion of the less polar ionic liquid 1-butyl-3-methyl imidazolium bis(trifluoromethanesulfonyl)imide, the stability was greatly improved: loss of 10% activity after 5 reuses and the time course of reaction was ~20% slower [25]. However, the decrease of rate obtained by using the ionic liquid mixture can be compensated for by employing ultrasonic mixing [26].)

However, when comparisons are made on the basis of productivity, the solvent-free approach described herein performs more favorably. Table 2 compares the productivity achieved in this report versus those calculated from the literature to achieve ~70–75% conversion of acyl donor, the latter criterion chosen to provide a common basis. The solvent-free approach of Fig. 8 yielded a productivity of 0.24 and 0.21 mmol of acyl groups converted to fructose and sucrose esters, respectively, per hour. (If the time required to retreat the fructose suspensions are included in the calculations, the productivity and productivity per mass of lipase in Table 2 will be lowered by 37.5%. However, one can envision a scaled-up process for which suspensions using an acyl donor/saccharide ester mixture obtained from a previous run can be prepared simultaneously with the operation of the bioreactor system which will be ready for use in the subsequent run for the bioreactor system, thus minimizing down time for the bioreactor system.) This productivity is nearly 10-fold higher than that achieved using ionic liquids [15] and is comparable to productivities achieved using solvents—methyl ethyl ketone, *tert*-butanol, and acetone—which ranged between 0.2 and 0.65 mmol g^{-1} h^{-1} (Table 2). A direct comparison cannot be made due to the differences between lipase types and substrates. However, in making such a comparison, it is important to note that the solvent-free approach has many inherent advantages. First, the absence of solvent reduces material costs and makes the process very sustainable and environmentally friendly. Second, the downstream purification is greatly simplified and is thus less expensive. Moreover, using the bioreactor system employed for Figs. 8–10, the technical grade product residing in the reservoir at the end of the operational cycle requires no further purification. In contrast, a solvent recovery and reuse step would be needed for the other examples. In terms of scale (ester production rate, mmol h^{-1}), the solvent-free systems are among the largest, with the exception of Ref. [12] (Table 2).

Conclusions

Through batch experiments conducted in the presence of acetone in open vessels, it was found that the rate of lipase-

Table 2 Comparison of productivity for the achievement of 70–75% conversion into saccharide–fatty acid esters using lipases and near-stoichiometric substrate feeds

Reaction	Conditions	% Conversion	Reaction time (h)	Ester production rate (mmol h^{-1})	Productivity (mmol h^{-1} g^{-1})
Glucose + Lauric Acid, CAL [10]	*t*-BuOH, 60 °C	72	50	6.0×10^{-2}	2.0×10^{-1}
Sucrose + Vinyl Palmitate, CAL [24][a]	*t*-PentOH/DMSO, 60 °C	77	120	6.4×10^{-3} [b]	2.6×10^{-2}
Fructose + Palmitic Acid, CAL [11]	Acetone (or MEK), 40 °C	70	24	1.5×10^{-2}	6.5×10^{-1}
Glucose + Stearic Acid, CAL [12]	MEK, 60 °C	79	48	8.3	4.1×10^{-1}
Glucose + Lauric Acid, CAL [15]	Ionic liquid, 50 °C	75	50	1.7×10^{-3}	3.3×10^{-2}
Fructose + Oleic Acid, RML[c]	Solvent-free, 65 °C	73	110	1.8×10^{-2}	2.4×10^{-1}
Sucrose + Oleic Acid, RML[d]	Solvent-free, 65 °C	72	120	1.0×10^{-2}	2.1×10^{-1}

CAL immobilized *Candida antarctica* B lipase (Novozym 435, Novozymes Inc., Franklinton, NC, USA), *RML* immobilized *Rhizomucor miehei* lipase (Lipozyme IM, Novozymes Inc.), *DMSO* dimethyl sulfoxide, *MEK* methyl ethyl ketone, *t-BuOH tert*-butanol (2-methyl 2-propanol), *t-PentOH tert*-pentanol or -amyl alcohol (2-methyl 2-butanol)

[a] 3:1 mole ratio of acyl donor to acyl acceptor

[b] Assumes volume size was 10 mL, since total volume was not given; per mass of lipase for productivity

[c] Reaction displayed in Fig. 8

[d] Reaction displayed in Fig. 10

catalyzed fructose–oleic acid esterification was greatly enhanced when the reaction was performed above the solvent's boiling point temperature, suggesting that the enhancement was not directly related to the solvent's ability to co-solubilize acyl donor and acceptor, since the acetone evaporated away completely within a few hours. It was subsequently discovered that the enhancement was attributable to the formation of supersaturated solutions of 10–200 μm-sized suspensions of crystalline saccharide in the liquid-phase mixture of acyl donor and monoester-enriched product. Suspensions of 1.5–2.0% saccharide in mixtures of oleic acid/saccharide-ester 75/25 g/g that are stable for at least 10–12 h were formed nearly equally well in the presence of acetone or in solvent-free systems via a 2-step process: rapid stirring of the contents ($\sim$800 rpm) at an elevated temperature (e.g., 80 °C) for a significantly long period of time ($\geq$6 h), followed by centrifugation (e.g., 800 rpm for 0.5–1.0 min) to remove the larger particles. The suspensions were employed in a solvent-free bioreactor system undergoing continuous recirculation at 65 °C that contained in series a reservoir open to the atmosphere, a peristaltic pump, and a packed-bed bioreactor. The substrate/product liquid-phase mixture was removed from the bioreactor system at 10-h intervals so that the suspensions could be reformed by adding additional acyl acceptor under the vigorous stirring and centrifugation conditions noted above. Molecular sieves were added to the reservoir during the time course to remove the product water, thus improving the equilibrium conversion. Using this approach, within 6 days the liquid phase consisted of 88% esters, of which 92% were monoester and 8% diester, for fructose as acyl donor, with a similar yield, time course, and product distribution obtained using sucrose. The technical-grade product was then collected from the reservoir without the need for downstream purification. The productivity rate, 0.21–0.24 mmol h^{-1} g^{-1}, is comparable to the values obtained in the literature that employed acetone, methyl ethyl ketone, or *tert*-butanol.

Acknowledgments Financial support was kindly supplied by the US Department of Agriculture, Grant 2006-35504-17262. We also thank Dr. Svetlana Zivanovic and Ms. Jennifer Marie Ham for their assistance in the analysis of particle size distribution.

References

1. Choplin L, Sadtler V, Marchal P, Sfayhi D, Ghoul M, Engasser JM (2006) Phase behavior and rheological properties of enzymatically synthesized trehalose decanoate aqueous solutions. J Colloid Interface Sci 294:187–193
2. Otomo N (2009) Basic properties of sucrose fatty acid esters and their application. In: Hayes DG, Kitamoto D, Solaiman DKY, Ashby RD (eds) Biobased surfactants and detergents: synthesis, properties, and applications. AOCS Press, Champaign, pp 275–298
3. Glatter O, Orthaber D, Stradner A, Scherf G, Fanun M, Garti N, Clement V, Leser ME (2001) Sugar–ester nonionic microemulsion: structural characterization. J Colloid Interface Sci 241:215–225
4. Ferrer M, Soliveri J, Plou FJ, Lopez-Cortes N, Reyes-Duarte D, Christensen M, Copa-Patino JL, Ballesteros A (2005) Synthesis of sugar esters in solvent mixtures by lipases from *Thermomyces lanuginosus* and *Candida antarctica* B, and their antimicrobial properties. Enzyme Microb Technol 36:391–398
5. Feuge RO, Zeringue HJ Jr, Weiss TJ, Brown M (1970) Preparation of sucrose esters by interesterification. J Am Oil Chem Soc 47:56–60
6. Ballesteros A, Plou FJ, Alcalde M, Ferrer M, Garcia-Arellano H, Reyes-Duarte D, Ghazi I (2007) Enzymatic synthesis of sugar esters and oligosaccharides from renewable resources. In: Patel RN (ed) Biocatalysis in the Pharmaceutical and Biotechnology Industries. CRC Press, Boca Raton, pp 463–488
7. Pyo S-H, Hayes DG (2009) Synthesis of saccharide–fatty acid ester biosurfactants catalyzed by lipase. In: Hayes DG, Kitamoto D, Solaiman DKY, Ashby RD (eds) Biobased surfactants and detergents: synthesis, properties, and applications. AOCS Press, Champaign, pp 323–350
8. Kennedy JF, Kumar H, Panesar PS, Marwaha SS, Goyal R, Parmar A, Kaur S (2006) Enzyme-catalyzed regioselective synthesis of sugar esters and related compounds. J Chem Technol Biotechnol 81:866–876
9. Yan Y, Bornscheuer UT, Schmid RD (2002) Efficient water removal in lipase-catalyzed esterifications using a low-boiling-point azeotrope. Biotechnol Bioeng 78:31–34
10. Flores MV, Naraghi K, Engasser J-M, Halling PJ (2002) Influence of glucose solubility and dissolution rate on the kinetics of lipase catalyzed synthesis of glucose laurate in 2-methyl 2-butanol. Biotechnol Bioeng 78:814–820
11. Sabeder S, Habulin M, Knez Z (2006) Lipase-catalyzed synthesis of fatty acid fructose esters. J Food Eng 77:880–886
12. Yan Y, Bornscheuer UT, Stadler G, Lutz-Wahl S, Otto RT, Reuss M, Schmid RD (2001) Regioselective lipase-catalyzed synthesis of glucose ester on a preparative scale. Eur J Lipid Sci Technol 103:583–587
13. Cao L, Bornscheuer UT, Schmid RD (1999) Lipase-catalyzed solid-phase synthesis of sugar esters. Influence of immobilization on productivity and stability of the enzyme. J Mol Catal B Enzym 6:279–285
14. Ganske F, Bornscheuer UT (2005) Lipase-catalyzed glucose fatty acid ester synthesis in ionic liquids. Org Lett 7:3097–3098
15. Lee SH, Dang DT, Ha SH, Chang W-J, Koo Y-M (2007) Lipase-catalyzed synthesis of fatty acid sugar esters using extremely supersaturated sugar solution in ionic liquids. Biotechnol Bioeng 99:1–8
16. Tai HP, Brunner G (2009) Sugar fatty acid ester synthesis in high-pressure acetone–CO_2 system. J Supercrit Fluids 48:36–40
17. Iwamoto N, Shima M, Adachi S (2008) Synthesis of xylitoyl fatty acid monoesters by immobilized lipase in subcritical acetone. Biochem Eng J 38:16–21
18. Dang HT, Obiri O, Hayes DG (2005) Feed batch addition of saccharide during saccharide–fatty acid esterification catalyzed by immobilized lipase: time course, water activity, and kinetic model. J Am Oil Chem Soc 82:487–493
19. Zhang X, Hayes DG (1999) Increased rate of lipase-catalyzed saccharide–fatty acid esterification by control of reaction medium. J Am Oil Chem Soc 76:1495–1500
20. Pyo S-H, Hayes DG (2009) Designs of bioreactor systems for solvent-free lipase-catalyzed synthesis of fructose–oleic acid esters. J Am Oil Chem Soc 86:521–529

21. Pyo S-H, Hayes DG (2008) Desorption of fructose from a packed column to an oleic acid/fructose oleate mixture for employment in a bioreactor system. J Am Oil Chem Soc 85:1033–1040
22. Adachi S, Kobayashi T (2005) Synthesis of esters by immobilized-lipase-catalyzed condensation reaction of sugars and fatty acids in water–miscible organic solvent. J Biosci Bioeng 99:87–94
23. Arcos JA, Hill CG Jr, Otero C (2001) Kinetics of the lipase-catalyzed synthesis of glucose esters in acetone. Biotechnol Bioeng 73:104–110
24. Reyes-Duarte D, Lopez-Cortes N, Ferrer M, Plou F, Ballesteros A (2005) Parameters affecting productivity in the lipase-catalysed synthesis of sucrose palmitate. Biocatal Biotransform 23:19–27
25. Lee SH, Ha SH, Hiep NM, Chang W-J, Koo Y-M (2008) Lipase-catalyzed synthesis of glucose fatty acid ester using ionic liquid mixtures. J Biotechnol 133:486–489
26. Lee SH, Nguyen HM, Koo Y-M, Ha SH (2008) Ultrasound-enhanced lipase activity in the synthesis of sugar ester using ionic liquids. Process Biochem 43:1009–1012
27. Piao J, Adachi S (2004) Enzymatic preparation of fatty acid esters of sugar alcohols by condensation in acetone using a packed-bed reactor with immobilized *Candida antarctica* lipase. Biocatal Biotransformation 22:269–274
28. Degn P, Zimmermann W (2001) Optimization of carbohydrate fatty acid ester synthesis in organic media by a lipase from *Candida antarctica*. Biotechnol Bioeng 74:483–491