Increased Rate of Lipase-Catalyzed Saccharide–Fatty Acid Esterification by Control of Reaction Medium

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ABSTRACT: Through proper selection of initial conditions and control of the reaction medium composition, a productivity rate over 10-fold higher than that previously reported was achieved for lipase-catalyzed fructose–oleic acid esterification. From a screening process, *tert*-butanol (*t*-BuOH) was selected as the most effective solvent for cosolubilizing fructose and oleic acid during the initial stage of the reaction. A *t*-BuOH concentration of 0.35–0.55 w/w produced the highest rate and extent of reaction at 60°C. Neither water addition nor removal applied to initial reaction materials improved the rate. Since both fructose–oleic acid mono- and diester promoted higher fructose solubility than either oleic acid or oleic acid/*t*-BuOH mixtures, *t*-BuOH was not needed during the latter stage of the reaction. Also, the presence of *t*-BuOH hindered the removal of water by free evaporation. Thus, complete removal of *t*-BuOH during the middle-to-latter stage of reaction was found to enhance the reaction rate. In addition, the introduction of fructose to the reactor in small batchwise increments accelerated the reaction. The monoester to diester ratio decreased during the initial and middle stages of the reaction owing to the disappearance of *t*-BuOH, but increased slightly during the later stages presumably because of the ester formation.

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KEY WORDS: Biosurfactants, fructose esters, lipases, saccharide esters.

The surfactant industry, with an annual production of 3 million tons, worth US$4 million, is facing increased consumer demand for biocompatible and biodegradable products (1–3). Saccharide–fatty acid (FA) esters, manufactured from inexpensive agricultural feedstocks (saccharide and FA), are highly biocompatible and biodegradable and have excellent surface-active properties (1,4,5). Sucrose–FA ester biosurfactants are currently manufactured at 5,000 metric tons per year (1).

Although chemical synthesis of saccharide–FA surfactants is currently the most economical choice, greater attention is being paid to enzyme (particularly lipase)-catalyzed synthesis as an ecologically safe, "green" alternative (1,2). Moreover, the mild conditions and high regioselectivity of biocatalytic reactions reduce the formation of by-products and impurities, thus improving product biocompatability (2). Biosurfactant esters have been produced *via* biocatalysis from a variety of monosaccharides (e.g., fructose, galactose, xylose, and glucose) and sugar alcohols (e.g., sorbitol) (2, and references therein). Recently, disaccharide esters, particularly those of sucrose, have received increased attention due to their higher solubility in water relative to monosaccharide esters (6–8).

The main problem with the enzymatic approach to synthesis of polyol esters is the design of reaction media and process conditions that simultaneously allows cosolubilization of the lipophilic fatty acyl and hydrophilic saccharide substrates, permits high lipase activity and stability, and contains low water levels, to facilitate esterification rather than hydrolysis. Medium design is especially challenging for reactions involving di- and polysaccharide substrates owing to their low solubility in organic solvents and high hygroscopicity. Because of the need for low water concentrations, lipase-catalyzed polyol-FA esterification is frequently hosted in nonaqueous media, particularly in organic solvent. However, for most of the solvents that yield the highest biocatalytic turnover, i.e., lipophilic (high log *P*) solvents (9), saccharide solubility is very poor. Methods employed to increase saccharide solubility in lipophilic solvent (with disadvantages listed in parentheses) include (2, and references therein) use of aqueous-nonaqueous two-phase liquid systems (slow reaction and mass transfer rates); use of saccharide ethers or glycopyranosides (ether linkages can be formed using glycosidases or organic chemistry; presence of an ether group changes properties of saccharide–FA esters); derivatization with isopropylidine, a known blocking agent for –OH groups, or complex formation using phenylboronic acid [expensive; requires additional process step(s) to remove derivatives from product]; suspension of silica gel-containing adsorbed fructose in acyl donor-containing liquid phase [difficult separation of silica gel and lipase solid phases (10)]; use of polar or polar/nonpolar cosolvent mixtures at reflux (safety issues involved with using refluxing solvents on a large scale).

Recently, the importance of controlling or programming the reaction conditions has been addressed. For example, the rate of saccharide–FA esterification was accelerated by crystallizing the monoester product at subambient temperature by employing a solvent that enhanced and hindered the solubi-

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lization of saccharide and monoester, respectively (11). Alternatively, the composition of reaction medium can be programmed during the course of reaction to increase the rate and extent of esterification. Moreover, polyol solubility often increases greatly during the esterification reaction, even in the absence of solvent, due to the formation of mono- and diesters (ME and DE, respectively) (12,13). Very few investigations have taken advantage of this important result. The rate of enhancement of saccharide esterification through reaction medium control is the topic of this paper.

EXPERIMENTAL PROCEDURES

Immobilized lipases, from *Candida antarctica,* I-CAL (Chirazyme L-2, c.-f, C2, Lyo.) and *Rhizomucor miehei,* I-RML (Chirazyme L-9, c.-f, Lyo.), were generous gifts from Boehringer-Mannheim (Indianapolis, IN). Lipozyme IM (immobilized *R. miehei* lipase) was kindly donated by Novo-Nordisk (Danbury, CT). The immobilized *R. miehei* lipases from Boehringer-Mannheim and Novo-Nordisk differed only in their immobilization matrices and exhibited nearly identical activities in fructose–oleic acid esterification (data not shown). Therefore, the abbreviation I-RML will also be used to represent Lipozyme IM. Technical grade oleic acid (94%; containing linoleic, stearic, and palmitic acids as impurities) was purchased from Sigma-Aldrich (Milwaukee, WI). All other reagents were of high purity and used without further purification. Deionized water was used throughout.

All reactions were performed in stirred-batch mode in open reactor vessels to permit the free evaporation of water and solvent. Reactions were conducted at 60–70°C, i.e., the optimal temperature range reported for I-RML and I-CAL lipases employed in reactions involving saccharides (14–16). A typical reaction consisted of mixing 25 mmol fructose, 50 mmol oleic acid, 0.5 g immobilized lipase, and 13.4 g *tert*-butanol (*t*-BuOH, 46.9 wt% on a fructose-free basis) at 400 rev min^{-1} and 65°C. Small aliquots were removed periodically for analysis.

The depletion of substrates, formation of products, and solubility of fructose were monitored using a dual-pump high-performance liquid chromatography (HPLC) system from Rainin (Woburn, MA) with an analytical (4.6 mm × 25 cm) reversed-phase C_{18} column (Microsorb, Rainin) oven-controlled at 25°C, and an evaporative light-scattering detector (Alltech, Deerfield, IL). An isocratic solvent system consisting of acetone/acetonitrile/acetic acid (45:45:10, vol/vol/vol), was employed at 1.0 mL min^{-1}. Identification of peaks as fructose, free fatty acid, ME, or DE were based on the technical literature (14) and standards. Water content was determined using Karl-Fischer titration.

RESULTS AND DISCUSSION

Selection of solvent and initial conditions. The solubility of fructose in pure oleic acid is extremely low (Fig. 1), hence the need to employ a solvent that will cosolubilize oleic acid and fructose. Several polar solvents known to permit biocatalysis by I-RML and I-CAL—toluene (log P = 2.5), isopropyl ether (log P = 1.9), *t*-BuOH (log P = 1.3), acetone (log P = –0.23), acetonitrile (log P = –0.33), and 1,4-dioxane (log P = –1.1)—were examined for their ability to solubilize fructose. Solubility in toluene and isopropyl ether was very poor. Of the remaining solvents, dioxane and *t*-BuOH yielded the highest solubility. *t*-BuOH was chosen over dioxane because of its lower volatility and cost.

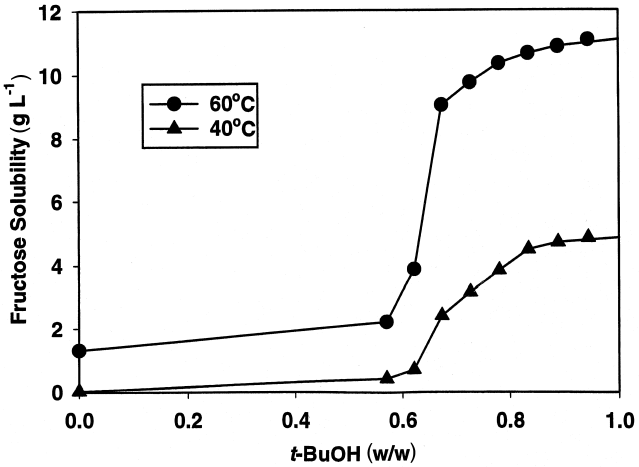

FIG. 1. Solubility of fructose in oleic acid–*tert*-butanol (*t*-BuOH) mixtures as a function of the *t*-BuOH weight fraction.

Fructose solubility is nearly 10-fold greater in *t*-BuOH than in oleic acid (Fig. 1). However, the inclusion of 30% oleic acid in *t*-BuOH did not lower the fructose solubility significantly (Fig. 1). A mixture of 67.4% *t*-BuOH/32.6% oleic acid at 60°C permitted a fructose/oleic acid mole ratio of 0.053:1, which is a 23-fold increase compared to pure oleic acid at 60°C. The solubility of fructose increased linearly with product (ME and DE) ester content (Fig. 2). At 60°C, the presence of fructose–oleic acids ME and DE yielded a higher fructose solubility than pure *t*-BuOH. For example, a mixture of 40% (ME + DE)/60% oleic acid solubilized fructose at 66.8 g/L (0.128 mole fructose/mole of acyl groups, 0.180 mole fructose/mole of free oleic acid) at 60°C (Fig. 2). The mixtures of fructose, oleic acid, ME, and DE appeared to the eye to be monophasic; however, we cannot verify whether a true mixture or a "melt" is formed. It is apparent that the presence of ME/DE greatly decreased the amount of solid-phase fructose and improved the contact between free fatty acid (FFA) and fructose. It is believed that ME and DE increased solubility because of the increase in medium polarity that occurs when FFA groups are replaced by polyol esters and/or by the surfactant capabilities of ME and DE.

The effect of the initial *t*-BuOH weight fraction on the time course of esterification was examined between 0.10 and 0.70 w/w on a fructose-free basis. The optimal *t*-BuOH range for initial reaction rate and overall conversion coincided, occurring between 0.35 and 0.55 w/w (Fig. 3). This range differs from that for fructose solubility; moreover, solubility in-

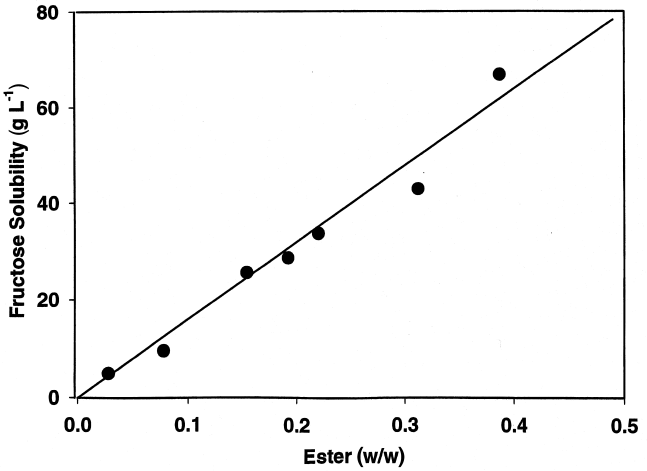

FIG. 2. Solubility of fructose as a function of weight fraction mono- plus diester in a mixture of oleic acid, mono-, and diester at 65°C. On an oleic acid-free basis, the ester mixture consisted of 96% monoester and 4% diester in all cases.

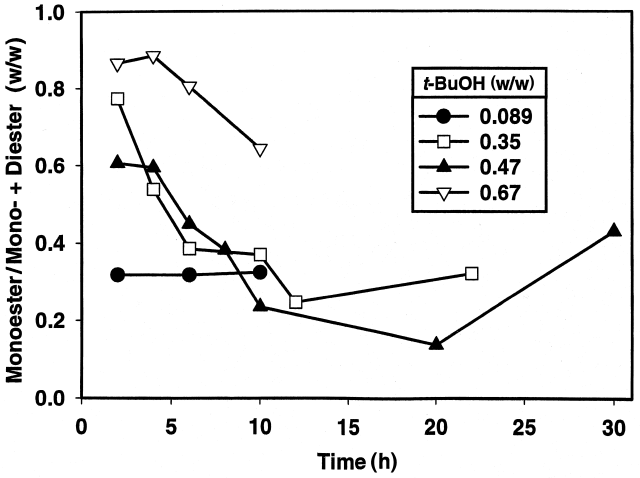

FIG. 4. Effect of initial weight fraction *t*-BuOH on distribution of ester products. Initial conditions as in Figure 3 with Chirazyme L-2, c.-f, Lyo. employed as lipase. See Figure 1 for abbreviation.

creased sharply only when the percentage *t*-BuOH was above 0.55 w/w (Fig. 1). The trends of both figures may be explained by changes in the microstructure and physicochemical properties of the *t*-BuOH/FFA binary mixture. It is well known that thermodynamic and mass transport properties can change quite dramatically with composition in binary mixtures when one of the two components self-associates, e.g., through hydrogen bonding (17). For example, at low weight fractions of the self-associating species (e.g., an alcohol), the self-associating species forms aggregates, or clusters, in a continuous phase consisting of mostly nonassociating species (17). Cluster formation breaks down as the fraction of self-associating species is increased when the continuous phase becomes a binary mixture. When the *t*-BuOH weight fraction is below 0.55 w/w, it is speculated that clusters of *t*-BuOH and fructose occur in a continuous phase of FFA, and that above 0.55 w/w, cluster formation is not present (Fig. 1). The presence of clusters would suggest the formation of an interface on a microscopic scale between a continuous FFA phase and a dispersed *t*-BuOH/fructose phase. This would accelerate the reaction since lipases act at or near interfaces.

For enzyme-catalyzed esterification of polyols, the presence of a polar solvent is known to promote the formation of the more polar product, ME, relative to DE (14,18). Results from our experiments support this trend (Fig. 4). The fraction of ME in the esters decreased with time until about 10 h (Fig. 4). The decrease was due to the evaporation of *t*-BuOH, the majority of which occurred during the first 24 h (Table 1). After 10 h, the ME fraction increased slightly with time, presumably due to the increase in medium polarity, because the

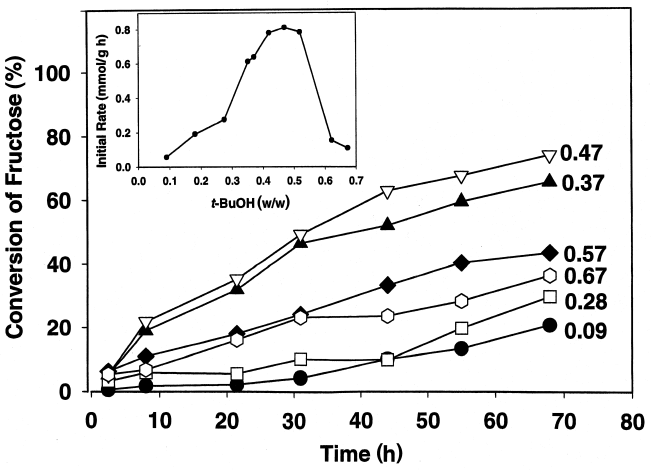

FIG. 3. Effect of initial weight fraction *t*-BuOH (on a fructose-free basis) on the time course of fructose ester synthesis. Inset: Effect of weight fraction *t*-BuOH on the initial productivity of fructose esters. Initial conditions: 65°C, 50 mmol oleic acid, 25 mmol fructose, and 0.5 g I-RML (immobilized *R. miehei* lipase, Lipozyme IM; Novo Nordisk, Danbury, CT) for percentage conversion vs. time, and Chirazyme L-2, c.-f, C2, Lyo. (Boehringer-Mannheim, Indianapolis, IN) for productivity rate vs. weight fraction *t*-BuOH (inset). Evaporation rate data for *t*-BuOH contained in Table 1. For abbreviation see Figure 1.

TABLE 1
Percent Evaporation of *tert*-Butanol (*t*-BuOH) During the Time Course of Reaction as a Function of the Initial Weight Fraction of *t*-BuOH[a,b]

t-BuOH (w/w)	t_{100}[c] (h)	Evaporation of *t*-BuOH (%)	
		10 h	24 h
0.67	60.6	16.5	39.6
0.57	39.6	25.3	60.7
0.47	26.5	37.8	90.7
0.37	17.5	57.0	100.0
0.28	11.6	86.1	100.0
0.090	2.95	100.0	100.0

[a]Based on the measured evaporation rate of 0.47 g *t*-BuOH/h at 65°C, which was independent of the relative amounts of oleic acid, fructose, and *t*-BuOH.
[b]Applicable to the data in Figures 3, 4, and 7.
[c]Time required for 100% evaporation of *t*-BuOH.

covalent binding of fructose transformed a FFA into a more polar molecule.

During the course of the reaction, produced water accumulated in the system. Although the addition of water (e.g., 0.5–5 w/w) to either the solvent or to the solid-phase fructose dramatically increased fructose solubility, the rate and extent of esterification were diminished (data not shown). The difficulty in removing water from the reactor is due in part to the hygroscopicity of fructose. Moreover, attempts were made to control the water activity (a_w) of fructose prior to its addition to the reactor through storage over saturated salt solutions (19). However, unlike fatty acids, apolar solvents, and lipases, water content equilibrium was not reached. Instead, even when stored over a low-a_w agent (LiCl, $a_w = 0.11$), the water content of fructose continually increased. A similar occurrence has been reported for glycerol (20).

Since increasing the initial water content did not improve the reaction rate, we next examined the effect of water removal. Storage of all reaction materials—solvent, substrates, and lipase—over dessicant ($CaSO_4$) for 48 h substantially reduced their moisture content (Table 2). The effect of dehydrating each I-RML, fructose, and oleic acid as a pretreatment on the rate of esterification was measured. The results clearly demonstrate that the dehydration of lipase reduced the rate relative to a control experiment, where no pretreatment of reaction materials was applied (Fig. 5). Halling and co-workers reported a similar loss in activity for I-RML when its water molecules were stripped (19). However, as esterification proceded, the gap in conversion between the I-RML dehydration and control experiment decreased, presumably due to the rehydration of the dessicated I-RML by water (Fig. 5). In contrast to I-RML dehydration, water removal from the substrates had little or no effect on the time course of esterification (Fig. 5). When all reaction materials were dehydrated prior to reaction, the resultant time course matched that of the dehydrated I-RML, indicating the importance of the I-RML water content for maintaining productivity. Hence, in all subsequent experiments, reaction materials were employed without any moisture removal or addition applied.

Programming of reaction conditions. Owing to the large increase in fructose solubility in the presence of ME and DE (Fig. 2), it was hypothesized that a solvent was not necessary during the later stages of the reaction. Figure 6 demonstrates, in agreement, that the complete removal of *t*-BuOH increased

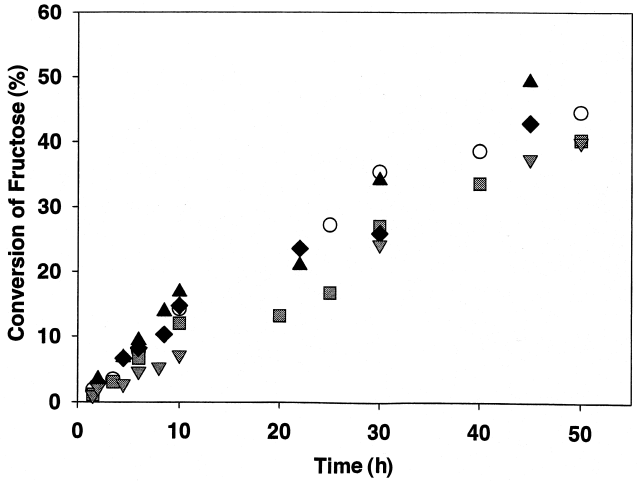

FIG. 5. Effect of dehydration on the percentage conversion of fructose. Initial conditions: 12.5 mmol of fructose, 25.0 mmol oleic acid, 0.25 g I-RML (immobilized *R. miehei* lipase, Chirazyme L-9 c.-f., C2, Lyo.; Boehringer-Mannheim, Indianapolis, IN), 6.9 g (46.9 wt%, fructose-free basis) *t*-BuOH, 65°C. Water contents of starting materials given in Table 2. (■) All reaction materials (fructose, oleic acid, *t*-BuOH, and lipase), (▲) oleic acid, (◆) fructose, (▼) lipase stored over dessicant, (○) no dehydration treatment. See Figure 1 for abbreviation.

the reaction rate at the later stages (beyond 20 h). The time at which complete solvent removal was applied also strongly affected the reaction rate, with the greatest enhancement occurring at the approach of 20 h (Fig. 6).

In addition, the increased reaction rate induced by solvent removal (Fig. 6) is related to the greater accumulation of water in the reaction medium when *t*-BuOH is present, according to Karl-Fischer titration analysis (data not shown). The same event may also explain the reduced rate occurring for the higher levels of *t*-BuOH during the later stages of the

TABLE 2
Water Content of Reaction Medium Materials (w/w %) When Stored over Dessicant for 48 h and in the Absence of Dehydration

Material	No dehydration[a]	Dehydrated[a]
I-RML[b]	6.94 ± 0.35	4.78 ± 0.24
Oleic acid	0.0264 ± 0.0034	0.0155 ± 0.0018
Fructose	2.26 ± 0.80	0.34 ± 0.02
tert-Butanol	0.0406 ± 0.0212	0.0158 ± 0.0156

[a]95% confidence intervals given.
[b]Immobilized *R. miehei* lipase (Chirazyme L-9 c.-f., C2, Lyo.; Boehringer-Mannheim, Indianapolis, IN).

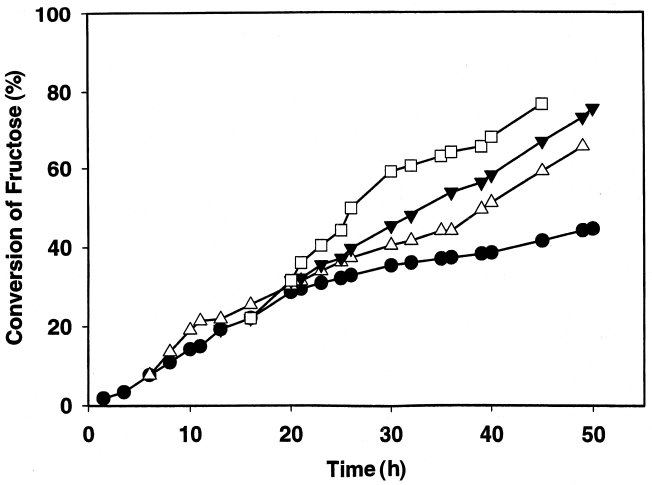

FIG. 6. Effect of solvent removal on the percentage conversion of fructose. Initial conditions: 12.5 mmol of fructose, 25 mmol oleic acid, 0.25 g I-RML, 6.7 g (0.469 w/w, fructose-free basis) *t*-BuOH, 60°C. (●) Control experiment; removal of solvent (and water) by free evaporation only; complete removal of solvent *via* rotary evaporation at: (△) 6 h, (▼) 13 h, and (□) 16 h. See Figure 1 for abbreviation.

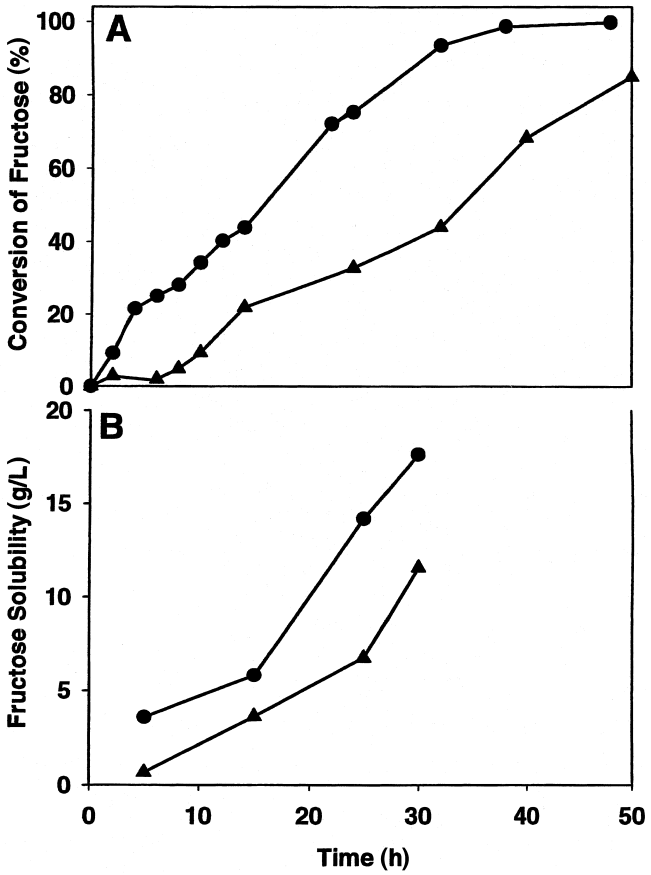

FIG. 7. Effect of the stepwise addition of fructose and solvent removal on (A) the percentage conversion of fatty acid and on (B) the solubility of fructose. Both reactions employed 0.5 g I-CAL (immobilized *Candida antarctica* lipase, Chirazyme L-2, c.-f, C2, Lyo.; Boehringer-Mannheim, Indianapolis, IN), a stirring rate of 450 rev min^{-1} and a reaction temperature of 65°C. (●) Fructose was added to 50 mmol oleic acid and 13.4 g (46.9 wt%, fructose-free basis) *t*-BuOH in 5-mmol increments for each 2-h period until the net amount of fructose added was 25 mmol. The solvent underwent free evaporation up to 10 h, at which time the reaction was stopped, *t*-BuOH was removed completely by rotary evaporation, and the reaction continued. (▲) 25 mmol of fructose was added at time zero to 50 mmol of oleic acid and 13.4 g (46.9 wt%, fructose-free basis) *t*-BuOH; solvent freely evaporated away throughout. Evaporation rate data for *t*-BuOH contained in Table 1. See Figure 1 for abbreviation.

reaction in Figure 3. In agreement, water removal through the use of salt-hydrate pairs was reported to greatly improve the rate and extent of lipase-catalyzed sucrose esterification (8). Moreover, the presence of a small excess-water phase has been demonstrated to irreversibly inactivate I-RML (21). In our experiment, water molecules were allowed to exit the reactor system through free evaporation at 65°C. Alternatively, water removal can be achieved through the employment of a moisture trap/solvent reflux system (6,14,22) or a hygroscopic solid-phase adsorbent (21).

Owing to the hygroscopicity and high viscosity of fructose, it was believed that ester synthesis would increase if a low level of fructose was maintained in the reaction medium. To support this hypothesis, the consumption of fructose was found to be accelerated at lower fructose/oleic acid ratios (data not shown), in agreement with others (15,23). However, the reduction of fructose mass did not lower the oleic acid consumption rate significantly, except when the conversion of limiting reactant approached 50% (data not shown). In order to maintain low fructose levels throughout the reaction and reach high product concentrations in the reaction medium, fructose was added in small batchwise increments (5 mmol) at 2-h intervals up to 10 h. The motivation for this approach was the stepwise addition of hydrogen peroxide during lipase-catalyzed peroxidation to prevent enzyme inactivation (24). For fructose–oleic acid esterification, this approach slightly increased the rate of reaction (data not shown). When stepwise fructose addition was applied along with complete solvent removal at 10 h, over 80% conversion was achieved in a 24-h period (Fig. 7). The formation of ME and DE paralleled the increase of fructose solubility during the course of reaction (Fig. 7B), as expected, based on the solubility data (Fig. 2).

The productivity rate demonstrated in Figure 7 is 1.4–1.6 mmol fructose h^{-1} $g_{I\text{-}RML}^{-1}$ for the achievement of 90% conversion of fructose. This value is higher by a factor of 10–100 than reported for lipase-catalyzed saccharide–fatty acid esterification (11), indicating that the rate and extent of this reaction are greatly increased by selectively controlling the solvent and fructose concentrations during the process.

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