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ORIGINAL PAPER

Desorption of Fructose from a Packed Column to an Oleic Acid/Fructose Oleate Mixture for Employment in a Bioreactor System

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Abstract Adsorption isotherms for a packed column consisting of fructose and silica gel in contact with a liquid phase consisting of oleic acid and fructose mono- and di-oleate at 65 °C were determined to assist the column's utilization in a bioreactor system for the solvent-free lipase-catalyzed synthesis of saccharide ester biosurfactants. The liquid-phase fructose content was very low, ranging from 0.005 to 0.1% (w/w), with the higher values obtained for a higher fructose concentration in the column and a higher ester content in the liquid phase. The desorption behavior of fructose followed the Freundlich isotherm. An increase in the liquid phase space velocity increased the initial rate of fructose desorption linearly. Dynamic light scattering measurements demonstrate solutions obtained by mechanical stirring of crystalline fructose and oleic acid/fructose oleate contained 50–250 μm sized aggregates, even after centrifugation at 12,000 rpm, but that the aggregates were absent from the desorption column effluent stream. Therefore, employment of a desorption column will lead to a low saccharide concentration in the liquid phase, but will reduce suspension-induced fouling of lipase in a bioreactor downstream of the column, which could improve the retention of lipase activity.

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Introduction

Saccharide–fatty acid esters are biodegradable and biocompatible surfactants or emulsifiers currently employed in the food, cosmetics, and pharmaceutics industries [1, 2]. Several different approaches have been employed for their production. Chemical methods require costly and environmentally unsafe conditions such as high temperature, high pressure, alkaline pH, and use of organic solvents [3–5]. In contrast, employment of biocatalytic synthesis using immobilized lipase occurs under environmentally-friendly operating conditions and leads to a very narrow product distribution of typically 1–3 mono- and di-ester species [5–7]. However, there are several problems with the biocatalytic approach, particularly the very poor miscibility of polar saccharide and nonpolar fatty acid substrates, leading to a slow rate of reaction [5–7]. Most organic solvents that improve miscibility are incompatible with food processing. Non-solubilized saccharide promotes fouling and aggregation of immobilized lipase, leading to inactivation and a low reaction rate [8, 9].

In a previous study by our group, the solubility of saccharide in a mixture of fatty acid substrate and ester product was reported in the form of a ternary phase diagram, to assist in determining conditions to operate the lipase-catalyzed solventless synthesis of saccharide–fatty acid ester at 65 °C; moreover, the presence of the ester greatly increased the solubility to an extent that a co-solvent would not be required [10]. Conversions of

80–90% were achieved for immobilized *Rhizomucor miehei*-catalyzed esterification of oleic acid and saccharide (fructose and sucrose) at 65 °C using near-stoichiometric amounts of substrates in batch mode. A solvent, *tert*-butanol, was employed only during the initial period to enhance fructose solubility, and was allowed to evaporate away completely upon reaching ~25% conversion. The time course of the reaction was effectively simulated by a mathematical model based on the Ping-Pong Bi Bi enzyme kinetic mechanism. Immobilized lipase exhibited excellent activity retention, with no loss of activity during the three successive batches, equating to 24 days of operational time [10]. A simple bioreactor system consisting of a desorption column containing crystalline fructose in series with a packed-bed bioreactor containing immobilized lipase was constructed and then employed to produce fructose oleic acid ester under solventless conditions [11]. The purpose of the desorption column was to provide, in theory, a liquid-phase saccharide concentration at saturation for the bioreactor inlet stream. A liquid phase consisting initially of 30% fructose oleic acid ester and 70% oleic acid was continuously recirculated through the desorption and bioreactor columns. Although the liquid phase ester content reached 81%, the reaction rate was significantly slower than observed in batch phase reactions. Subsequent mathematical modeling and analysis demonstrated the source of the reduced rate was the low liquid-phase concentration of fructose relative to the solubility limit predicted by the triangular phase diagrams [10].

To improve the performance of the bioreactor system derived previously [11], the performance of the fructose desorption column requires further investigation. In this report, silica gel was employed as a mechanical support for saccharide in the desorption column, which was chosen based on its high polarity, hence large capacity for saccharide loading, its cost, and its abundance. To understand desorption characteristics, we need to obtain equilibrium isotherm data, and relate to isotherm models obtained from thermodynamics theory [12, 13]. Generally, this approach has been effectively used to evaluate and design chromatographic separation processes [14–17]. In this paper, evaluation and modeling of fructose distribution between a packed desorption column containing silica gel as adsorbent and a liquid phase consisting of oleic acid, and fructose oleic acid ester mixture at different ratios was conducted. The desorption behavior of fructose followed the Freundlich isotherm. This information will be useful in the design of bioreactors for the synthesis of saccharide–fatty acid esters, and to further understand partitioning of saccharides between a solid phase and a lipid-based liquid phase ester.

Theory

Initial characterization is focused upon the Freundlich isotherm [17]:

$$C_{FS} = aC_F^N \quad (1)$$

where C_F and C_{FS} refer to liquid phase and solid phase concentration of fructose, respectively, and a and N are parameters of the isotherm equation.

The parameters a and N were determined for the system fructose/oleic acid/fructose oleic acid ester as a function of the solvent composition, i.e., the weight fraction of fructose oleic acid ester and the ratio of fructose to silica gel in the solid phase, at 65 °C. The power law relationship of Eq. (1) translates into a straight-line relationship when C_{FS} is plotted versus C_F using log–log coordinates, with N and log(a) provided by the slope and y-intercept, respectively. The plots described above were prepared for each of a series of experiments that shared the same solute-free liquid phase composition but employed different C_{FS} values.

Materials and Methods

Materials

Technical grade oleic acid, 98% pure, as determined by HPLC [10], and Lipozyme®IM, lipase (EC 3.1.1.3) from *Rhizomucor miehei* immobilized onto macroporous anioic beads were purchased from Sigma-Aldrich (St Louis, MO, USA). Fructose (98% purity), *tert*-butanol and acetonitrile (HPLC-grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Silica gel with particle size 32–63 μm was purchased from Selecto Scientific (Suwanee, GA). All materials were used without further purification.

Fructose oleic acid ester was synthesized by the lipase catalyzed fed batch reaction modified from previous report [10]. The initial stage of reaction was conducted in a 1 L vessel placed on the hot plate opened to the atmosphere, consisting 285 g (1.0 mol) oleic acid, 18 g (0.1 mol) fructose, 250 g *tert*-butanol and 10 g immobilized lipase at 65 °C. *tert*-Butanol and the reaction product, water, were allowed to freely evaporate during the time course of reaction. Fructose was added in 0.1 mol batchwise increments every two days for 20 days, thus resulting in a stoichiometric feed for the reaction. Small aliquots taken from reaction mixtures were periodically collected and analyzed using HPLC. The reaction medium was treated by addition of 500 mL *n*-hexane, followed by filtration under reduced pressure. After evaporation and vacuum drying of added solvents, the purity of fructose oleic acid ester was 77.4%, with the mono and di-ester present at a ratio of

8.3–1.7 (g/g). This product was mixed with oleic acid to obtain the desired proportions of oleic acid and ester.

Operation of Fructose Desorption Column

The fructose desorption column was prepared using an Omnifit® chromatography column (Omnifit, UK) with dimension 100 mm × 10 mm ID, equating to a bed volume of 5.50 mL, and a total column volume between plungers at the inlet and outlet of 5.62 mL. The columns were packed with a mixture of grinded fructose and silica gel at 30–70 wt% fructose. Void volume fraction was determined by measuring the fluid density and the weight difference between the liquid-free and liquid-filled packed column. Values are provided in Fig. 1 as a function of the mass fraction of fructose. The packed column and a vial with 6.0 g liquid phase solution were enclosed inside of a convection oven (Isotemp® Economy Lab Incubator, Fisher Scientific) controlled so that the liquid phase contents of the system were at 65 °C.

The columns were first equilibrated by recirculating 6.0 g oleic acid at 0.1 mL min^{-1} for 4 h. Then 6.0 g of an oleic acid/ester mixture was introduced into the column at a flow rate between 0.05 and 0.2 mL min^{-1}. The solution was then continuously recirculated though the desorption column and reservoir by a BioLogic LP® peristaltic pump (Bio-Rad, Hercules, CA). Small aliquots were taken from the column's effluent stream for compositional analysis by HPLC.

Quantitative Chemical Analysis Using HPLC

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, USA) and a model Mark III evaporative light scattering detector from Alltech Associates, a division of W.R. Grace (Deerfield, IL, USA). An analytical reversed phase C_{18} column (4.6 × 250 mm, pore diameter 5 μm) 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 mL min^{-1}. Response factors were measured and employed to convert peak areas into concentrations.

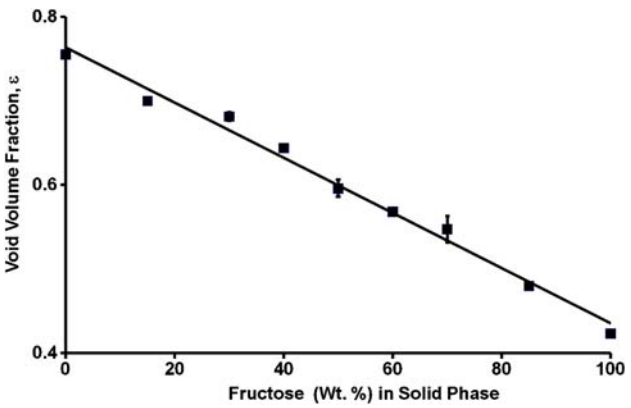

Fig. 1 Effect of the mass fraction of fructose in the desorption column on the void volume fraction. *Error bars* reflect standard deviation

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 (100 μL of each). The extraction was carried out at 35 °C for 2 h using a thermomixer (Eppendorf AG, Germany). The hexane phase was discarded and the aqueous phase was recovered and then subjected to two additional extraction stages. 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 mm × 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 liquid phase composition.

Detection of Undissolved Fructose Crystals

Two samples were prepared for each of three different fructose oleate/oleic acid mixtures by mixing of 1.0 g of the liquid phase with 0.2 g fructose at 65 °C for 48 h using the thermomixer described above, with one sample centrifuged for 2 min at 12,000 rpm and the other at 3,000 rpm using a model accuSpin™ Micro R microcentrifuge (Fisher Scientific, Pittsburgh, PA, USA). In addition, for each of the three liquid-phase compositions investigated, column effluent was collected after several hours of continuous recirculation through the silica gel-based packed columns as discussed above that contained 50 wt% fructose.

The nine prepared samples were analyzed for their fructose content employing the HPLC-based method discussed above and for the presence of aggregates by absorbance and dynamic light scattering. The absorbance of solutions between 500 and 1,000 nm was ascertained to provide a measure of turbidity using a model UV-1700 instrument from Shimadzu (Japan) and 1.0 cm path length quartz cuvettes. Absorbance values at 1,000 nm are reported since at this wavelength the values are <1.0 units, meaning that the linear Beer–Lambert law is applicable. Differences in absorbance values between samples at this

wavelength are representative in the trend of differences at the other wavelengths. The particle size distribution of the dispersions present in the above-mentioned solutions was analyzed by dynamic light scattering using a Zeta potential Analyzer, Zeta PALS (Brookhaven Instruments Corporation, Holtsville, NY, USA).

Adsorption Isotherm Data for Suspension of Silica Gel in Liquid Phase

Silica gel (0.5, 1.0, 5.0, or 10.0 mg) was added to 1.0 g of a mixture of fructose oleate/oleic acid (25/75 w/w) that contained fructose at a concentration near saturation (obtained from a liquid mixture that was continuously recirculated through a packed bed consisting of ground fructose/silica gel 70/30 w/w) in a 2-mL microcentrifuge tube and mixed at 65 °C for 24 h using the thermomixer described above. Subsequently, the mixture was centrifuged for 10 min at 12,000 rpm using the microcentrifuge described above. The initial and final fructose concentrations of the liquid phase were determined using the liquid–liquid extraction plus HPLC analysis protocol described above. The solid-phase fructose concentration was determined from a mass balance. Three replicates of the series of adsorption isotherm experiments described above were performed.

Thin-Layer Chromatographic Analysis

To determine the relative strength of adsorption of the adsorbate, fructose, and liquid-phase components, fructose and fructose-oleic acid mono- and di-esters, standards were analyzed using thin-layer chromatography (TLC) with an analytical plate coated with 60A silica (SELECTO SCIENTIFIC, Atlanta, GA, USA) and an acetonitrile/acetone/acetic acid 45/45/10 v/v/v solvent system. The plates were detected by charring, through treatment with 40 wt% sulfuric acid, followed by incubation in an oven.

Results and Discussion

Effect of the Composition of the Desorption Column and Recirculating Fluid on the Desorption of Fructose

The time course of fructose desorption from the fructose/silica gel adsorbent to oleic acid/fructose oleate liquid mixture at 65 °C is depicted in Fig. 2 as a function of liquid and solid phase composition. The ordinate represents the fructose concentration of the column effluent. The fructose content of the liquid phase was very low, within the range of 0.005–0.1% (w/w). The concentration of fructose in column effluent was increased by employing a solid phase containing a higher fructose content and higher proportion of oleic acid ester in the liquid phase. Moreover, an increase of fructose in the solid phase from 30 to 70% increased the fructose concentration in the liquid phase 2-fold and the rate of mass transport of fructose to the liquid phase (Fig. 2a). This trend contrasts the decrease of the void volume fraction with an increase of fructose in the solid phase indicated in Fig. 1, which would provide a lower transport rate. However, a further increase of the solid-phase fructose concentration beyond 70% fructose did not increase the desorption rate or amount (data not shown). Similarly, an increase of ester content in the liquid phase increased the liquid-phase fructose content nearly 10-fold (Fig. 2b). Saturation of the liquid-phase fructose concentration appeared to be reached within 5 hr of recirculation time.

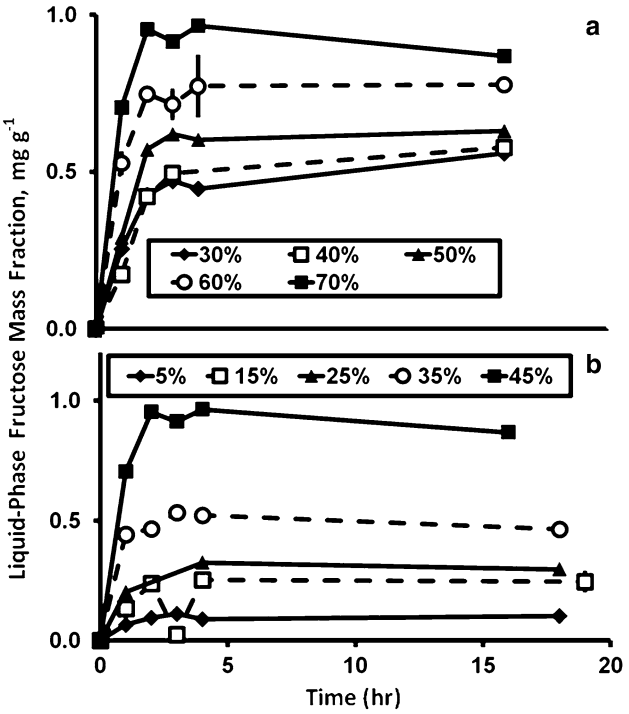

Fig. 2 Effect of the composition of **a** the desorption column and **b** the recirculating fluid on the time course of fructose uptake by the liquid phases recirculated through a fructose/silica gel desorption column at 65 °C. **a** Liquid phase concentration of oleic acid:ester 55:45 g/g, solid phase composition (wt%) given in the legend. **b** The desorption column consisted of a fructose:silica gel 70:30 g/g and a liquid phase composition (wt% ester) given in the legend

Fructose Desorption Isotherm and Modeling

The equilibrium relationship between the solid- and liquid-phase fructose concentration is depicted in Fig. 3. The data employed in this figure were taken from Fig. 2 (with two additional data collected for the fructose oleate/oleic acid 25/75 w/w liquid phase), using the average liquid-phase

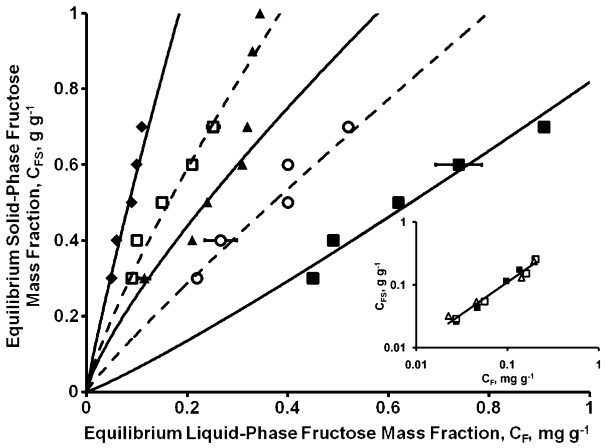

Fig. 3 Fructose adsorption isotherm at 65 °C. Symbols and liquid phase composition (% ester) indicated by the legend given in Fig. 2b. Curves represent Freundlich isotherm model applied to the data. Inset: adsorption isotherm data obtained for a suspension of silica gel in oleic acid/fructose-oleic acid ester 25/75 g/g that contained fructose at near saturation levels at 65 °C, plotted in log–log coordinates. Marker types in the inset differentiate the three separate series of experiments conducted

fructose concentration after 5 h of recirculation and the initial solid-phase fructose concentration from Fig. 2 as the abscissa and ordinate of Fig. 3, respectively. This assumes that equilibrium is approached through 5.0 h of recirculation and neglects the small percentage loss of fructose from the solid phase resulting from desorption. Each plot within Fig. 3 represents a series of experiments employing a common solute-free liquid-phase composition, with the composition of the solid phase varied between experiments. Figure 3 demonstrates that the liquid-phase fructose concentration increased several-fold upon an increase of the liquid-phase ester content, in agreement with previous results [10, 18], and the slope of the isotherms decreased with the ester concentration. The values of liquid-phase fructose content given in Figs. 2 and 3 are several-fold lower than the values reported previously by our group [10]. The underlying cause of this discrepancy is discussed below.

For each plot displayed in Fig. 3, the data points are described by a single curve. Moreover, the Freundlich isotherm (see "Theory" section) modeled the data quite well for data obtained for solid phase material containing fructose at 70% or less (Fig. 3). Note for the 25% fructose oleate liquid phase data of Fig. 3 that solid-phase fructose concentrations above 70% did not follow the Freundlich isotherm, probably reflecting the pure dissolution of fructose crystals into the liquid phase; therefore, the Freundlich isotherm model was applied only to data obtained using 70% fructose or less in the solid phase.

To confirm the applicability of the Freundlich isotherm for describing the adsorption of fructose using the packed column-derived results given in Figs. 2 and 3, a separate series of experiments was performed for which silica gel was suspended in a fructose oleate/oleic acid 25/75 g/g liquid-phase system which contained fructose concentrations near saturation at 65 °C. Under these conditions, the occurrence of dissolution of fructose crystals into the liquid phase, an alternative hypothesis to describe the transport of fructose from the packed columns to the liquid phase rather than desorption, will be negligible. As shown in the Fig. 3 inset, the Freundlich model describe the three sets of data obtained very well, with the values of log(a) and N obtained from linear regression being 2.0 ± 0.1 g/g and 0.96 ± 0.04, respectively. The fit of the Langmuir isotherm to this data provided an inferior fit compared to the Freundlich isotherm (analysis not shown).

Values of log(a) and N for the isotherms, obtained from the log–log type plots as defined in the "Theory" section, are listed in Table 1. These values are similar to the values reported above for the experiments for which silica gel was suspended into liquid phase, further suggesting that the dissolution of fructose crystals as a competing mechanism to adsorption was negligible for data obtained using 70 wt% fructose or less in the solid phase. In general it appears that the log(a) remained constant and N slightly increased in a linear fashion as the mass fraction of fructose monooleate in the liquid phase was increased.

TLC was employed to determine the relative adsorption of fructose and the liquid-phase main components, to assess if the adsorption isotherm modeling approach required modification to include the competitive adsorption of fructose monooleate and oleic acid. Under the TLC conditions employed, fructose, fructose monooleate, and oleic acid possessed R_f values of 0.58, 0.92, and 0.96, respectively, indicating that fructose binds strongly to silica gel and the binding of monooleate and oleic acid is negligibly small.

Table 1 Value of the Freundlich isotherm parameters obtained from linear regression of log–log plots of data given in Fig. 3 at 65 °C

	5% FOE[b]	15% FOE	25% FOE	35% FOE	45% FOE
log(a), g/g[a]	2.66 ± 0.82	2.76 ± 0.27	2.53 ± 0.45	2.81 ± 0.41	2.63 ± 0.13
N[a]	0.73 ± 0.20	0.81 ± 0.07	0.78 ± 0.12	0.91 ± 0.12	0.92 ± 0.04

[a] Parameters defined in Eq. (1) using C_F and C_{FS} in units of g/g; error bars represent standard deviation

[b] The content (%, w/w) of fructose oleic acid esters (FOE) in oleic acid

Effect of Recirculation Rate

The mass transport of fructose to the liquid phase is controlled by the relative velocity of the liquid phase moving through the desorption column. Employing 35% fructose oleic acid ester/65% oleic acid as the liquid phase and 50% fructose/50% silica gel as the desorbent at 65 °C, the effect of velocity was investigated using recirculation rates, Q, of 0.05, 0.1, and 0.2 mL min^{-1}. The fructose concentrations obtained after 18 h of recirculation, were similar, within the error limits of the measurements (0.045–0.050%, Fig. 4). However, the transport rate increased as the recirculation rate was increased. The inset of Fig. 4 plots the initial rate of fructose desorption from the column as a function of space velocity, SV, defined to be

$$\mathrm{SV} = Q/\varepsilon \mathrm{V} \tag{2}$$

where ε and V refer to the void volume fraction and the column volume, respectively. SV values corresponding to Q values of 0.05, 0.10, and 0.20 mL min^{-1} were calculated to be 0.015, 0.030, and 0.060 min^{-1}, respectively. As depicted in the inset of Fig. 4, the mass transport rate of fructose increased linearly with SV, at an amount equal to 6.85 mg g^{-1} h^{-1} per inverse minute.

Detection of Undissolved Fructose Crystals

Generally, fructose, a polar molecule, is poorly miscible with nonpolar fatty acid such as oleic acid; but, the miscibility is increased in the presence of fructose oleic acid ester as depicted in Fig. 2, and as described previously by our group [10, 18]. The fructose concentrations measured in the desorption column effluents are nearly an order of magnitude lower than those reported previously by us [10], for which the samples analyzed were prepared by stirring suspensions of fructose crystals in oleic acid/fructose ester mixture for several hours, followed by centrifugation at 3,000 rpm to remove unsolubilized fructose. To further understand the underlying reason for the difference, column effluent samples obtained from the desorption experiments described above that employed a fructose/silica gel 1/1 g/g packed column were compared with samples of corresponding liquid phase composition prepared as described in the previous investigation [10], except that centrifugation using each 3,000 and 12,000 rpm were employed. The three samples prepared for a given liquid-phase composition were analyzed for their apparent solubility, the absorbance at 1,000 nm, which reflects the optical turbidity attributable to undissolved particles, and by dynamic light scattering to detect the presence and size of micron-sized aggregates.

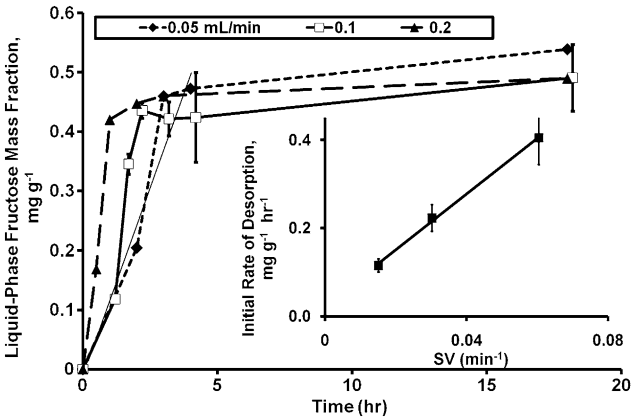

Fig. 4 Effect of recirculation rate of the liquid phase on the desorption of fructose from fructose/silica gel column. Conditions consisted of 35% fructose oleic acid ester/65% oleic acid in the liquid phase and 50% fructose/50% silica in the desorption column at 65 °C. Inset: space velocity (SV) versus slopes of initial period from main graphs

The apparent solubility of the samples prepared according to previous methods [10] were higher than those obtained from the column effluent, as expected, with the apparent solubility decreasing as the centrifugation rate was increased (Fig. 5a). Samples of desorption column effluent were relatively clear in appearance to the naked eye; in agreement, they yielded the lowest absorbance values at 1,000 nm. In contrast, samples prepared by mechanical mixing of fructose with oleic acid/fructose oleate followed by centrifugation were slightly cloudy in appearance, and samples obtained by 3 K centrifugation were cloudier than those treated by 12 K centrifugation. In agreement, trends for absorbance values are consistent with the above-mentioned observations (Fig. 5b). Furthermore, the apparent size of particles obtained by light scattering analysis were negligibly small for samples obtained from the desorption column (Fig. 5c), with the detection limit for the light scattering apparatus being 0.5 nm, as provided by the manufacturer.

The higher the content of fructose oleic acid ester, the larger the size measured, perhaps reflecting the role of fructose ester as an emulsifier (Fig. 5c). And the size of samples prepared by 12 K centrifugation was smaller than those prepared by 3 K centrifugation, in agreement with the lower absorbance observed for the former (Fig. 5c). Therefore, the apparent fructose solubility values reported previously by our group [10] do not reflect true solubility measurements since a significant amount of fructose was suspended and not dissolved, while the fructose concentration of the column effluent obtained in this work are more reflective of true solubility.

The several-fold lower saccharide solubility obtained for fatty acid/saccharide ester liquid-phase mixtures via transport of the liquid phase through a column of silica gel-supported saccharide (and also through a bed of pure

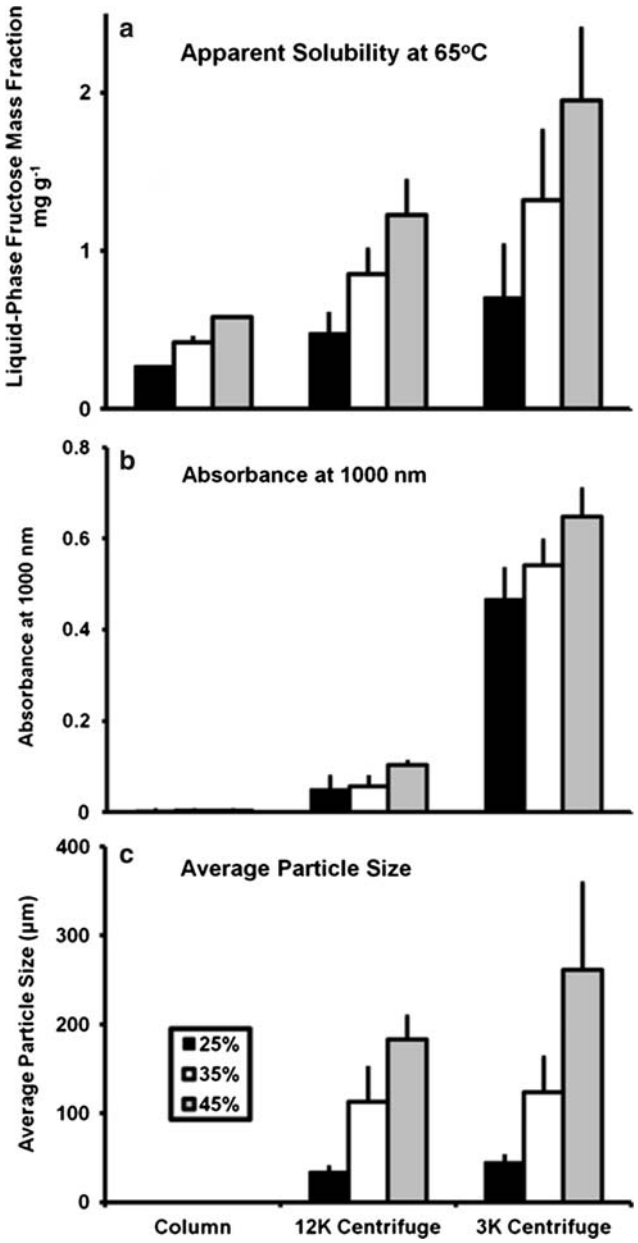

Fig. 5 Apparent solubility (**a**), absorbance at 1,000 nm (**b**), and particle size (**c**) of column effluent (obtained using a packed column of fructose/silica gel 1:1 g/g) and samples prepared by the mixing of crystalline fructose into liquid phase, followed by centrifugation to remove unsolubilized fructose, as a function of ester proportion (provided in legend) and angular velocity of centrifugation

saccharide crystals [11]), compared to solutions prepared by mechanical mixing of saccharide suspensions in the liquid phase, has two important ramifications for the design of bioreactor systems to conduct the solvent-free lipase-catalyzed synthesis of saccharide–fatty acid biosurfactants. First, the lower concentrations of fructose in systems that introduce the saccharide substrate via passage through a column may produce lower reaction rates due to the lower liquid-phase saccharide concentrations. Second, the absence of micron-sized aggregates in the liquid phase may reduce the extent of fouling onto the immobilized enzyme and lead to an improved enzyme activity retention. Currently the authors are investigating different bioreactor systems designs to shed light on the importance of the above-mentioned ramifications.

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