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ORIGINAL PAPER

Optimization of the Solvent-Free Lipase-Catalyzed Synthesis of Fructose-Oleic Acid Ester Through Programming of Water Removal

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Abstract Fructose oleate, an environmentally-friendly biobased surfactant, was prepared using solvent-free suspensions of saccharide in a mixture of acyl donor and monoester (the latter present at ≥5 wt% initially) continuously recirculated through a closed-loop packed bed bioreactor (PBBR)-based system at 53 °C, with the PBBR containing immobilized *Rhizomucor miehei* lipase (Lipozyme®IM, Novozymes, Franklinton, NC, USA). To replenish the acyl acceptor consumed during the time course of reaction, the medium was isolated, fructose added, and a suspension formed by rigorous stirring at 80 °C for 6 h followed by centrifugation to remove larger particles, with the placement of the acyl acceptor replenishment treatments during the time course of reaction were optimized. Water removal via free evaporation was augmented during the latter portion of the time course (using a molecular sieve packed column, N_2 bubbling, vacuum pressure, or a combination of the latter two), with an optimal performance achieved when initiating N_2 + vacuum ($2.16\,mg_{H_2O}\,h^{-1}$ removal rate) upon reaching 60% ester, to maintain the liquid-phase water content near 0.40 wt%. When employing the above-mentioned conditions, 92.6 wt% fructose oleate was produced within 132 h, yielding a productivity of $0.297\,mmol_{Ester}\,h^{-1}\,g^{-1}_{lipase}$.

Keywords Biobased surfactant · Biocatalysis · Bioreactor · Enzyme · Lipase · Saccharide–fatty acid esters · Solvent-free · Water activity (control of)

Introduction

Saccharide–fatty acid esters, biodegradable, environmental friendly nonionic biobased surfactants prepared from inexpensive renewable agricultural feedstocks, are employed as emulsifiers in foods, pharmaceuticals and cosmetics [1–3]. In addition, they possess antimicrobial activity, leading to their potential employment in food preservation [4, 5] and insecticides [6]. Traditionally, saccharide–fatty acid esters are produced by chemical methods under harsh conditions, for instance, high pressure and temperature, yielding undesirable byproducts and unsafe operation condition. In contrast, biocatalytic synthesis of saccharide–fatty acid esters employs near-ambient operation conditions and leads to a narrow product distribution [7]. However, the presence of significant hurdles hinders the application of biocatalysts for industrial utilization [8]. The major problem is the poor miscibility of polar and non-polar substrates, resulting in slow reaction rates. Although several methods have been successfully utilized to enhance the miscibility, particularly the employment of co-solvents: polar organic solvents [9–11] supercritical CO_2 [12, 13] and ionic liquids [14, 15], their use involves several disadvantages, including the high cost of solvents and/or their recovery or disposal, reduction of process safety, and the environmental impact of solvent utilization.

The aim of our group is to develop "green" approaches for the lipase-catalyzed synthesis of saccharide–fatty acid esters, utilizing the ester product to improve the dissolution of the saccharide into the acyl donor (oleic acid), the latter

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acting as substrate and solvent [16–18]. Recently, we have employed suspensions of saccharide crystals 10–200 μm in length dispersed in solvent-free media, formed by stirring the solid-phase saccharide and the liquid-phase media (acyl donor plus fatty acid ester) for several minutes, followed by sedimentation to remove larger particles [19]. Continuous recirculation of the saccharide-enriched solvent-free media through a closed-loop bioreactor system consisting of a reservoir open to the atmosphere (to allow for free evaporation of the reaction product, water), a peristaltic pump, and packed bed bioreactor (PBBR), has led to an enhanced reaction rate and yield: 88% conversion of acyl donor into fructose-oleic acid ester, or FOE (92 wt% monoester and 8 wt% diester) within 6 days, starting with a reaction medium containing 75 wt% oleic acid and 25 wt% FOE [19]. The suspension-based medium was retreated with additional fructose at 10 h intervals to replenish consumed acyl acceptor, requiring a temporary stoppage of the recirculation flow. The mole ratio of fructose to oleic acid fed to the bioreactor system throughout the entire time course of reaction was approximately 1:1. Molecular sieves (MS) were added to the reservoir upon achieving 60 wt% conversion of oleic acid to augment water removal via free evaporation, to increase the final yield upon the approach to thermodynamic equilibrium [19]. The primary objective of this study was to improve the performance of the bioreactor system through improving the control of the water concentration in reaction medium and optimizing the placement of the acyl acceptor replenishment (i.e., suspension formation) intervals during the time course of reaction. Different approaches for water removal were compared. Also, the water removal strategies were applied to stirred tank bioreactors (STBRs) to test their universality.

Experimental

Materials

Technical grade oleic acid, 90% pure, 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). The latter possessed 140 U g^{-1}, where 1 U refers to the amount of enzyme which releases 1 μmol stearic acid min^{-1} from tristearin at pH 8.0 and 70 °C). Fructose (>98% purity), acetone (HPLC-grade), acetonitrile (HPLC-grade), and molecular sieves (MS; 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, the reaction product and a component of the initial charge to the bioreactor system, was synthesized by the RML-catalyzed reaction protocol given in our previous reports [7, 19]. The reaction product consisted of 89.6 wt% ester, or FOE (of which 83.6 wt% was monoester and 16.4 wt% diester), and 10.4 wt% oleic acid. This product was mixed with oleic acid to obtain the desired proportions of oleic acid and ester, and fed to the bioreactor system.

Methods

Formation of Suspensions of fructose in oleic acid/ fructose-oleic acid ester mixtures

A suspension of saccharide crystals in solvent-free media was formed by mixing 1.5 g fructose crystals and 10 g of an oleic acid/FOE mixture in a 20-mL scintillation vial open to the atmosphere on a magnetic stirrer/hotplate ("Super-Nuova," Barnstead, Dubuque, IA, USA) operated at 70–85 °C and 800 rpm (radius of 1.5 cm) for 6 h. The slurry was centrifuged at 800 rpm (radius of 5 cm) for 0.5–1.0 min, with the supernatant collected.

Operation of the Packed-Bed Bioreactor System Undergoing Continuous Recirculation

The bioreactor system included three major components connected in series, forming a closed-loop system that underwent continuous recirculation: a 20-mL scintillation vial open to the atmosphere serving as a reservoir, a peristaltic pump (BioLogic LP® from Bio-Rad, Hercules, CA, USA), and a packed bed bioreactor (PBBR; 50 × 10 mm ID Omnifit® chromatography column packed with 7.5 g of RML, or equivalently, 0.75 g_{RML} g^{-1} of oleic acid + FOE). The PBBR and its associated tubing were placed in an oven at constant temperature, 53 °C. The reservoir was kept at 65 °C and stirred at 200 rpm using the stirrer/hot plate referred to above. The system underwent continuous recirculation at 0.50 mL min^{-1}, a flow rate found to be optimal in our previous work [19]. Other details of the bioreactor system are described in our previous paper [19].

At specified intervals (typically 10 h), the suspensions contained within the reservoir were re-treated as described above through introduction of additional saccharide (1.5 g), followed by stirring at 70–85 °C and sedimentation. At the beginning of the process to reform the suspensions, recirculation through the bioreactor system was temporarily paused by stopping the pump; and the reaction medium was recovered from the reservoir. After the fructose addition, stirring and sedimentation steps were completed, the suspension-based reaction medium was returned to the reservoir and the pumps re-initiated. MS were incorporated into a packed column (MSC) inserted between the reservoir

and pump for several experiments. To prepare the MSC columns, typically 1.2 g of MS were packed into a 50 × 10 mm ID Omnifit® chromatography column, providing a ratio of 12 g MS g^{-1} of reaction medium.

Operation of the Stirred Bioreactor System Under Batch Mode

The stirred-tank bioreactor (STBR) consisted of a 20 mL scintillation vial open to the atmosphere, placed on the hot plate/stirrer described above and maintained at 65 °C and stirred at 300 rpm (radius of 1.5 cm) for the effective dispersion of RML particles. The reaction medium was retreated at 10-h intervals to replenish consumed acyl acceptor (and re-form suspensions) as described above, except that RML particles were removed via microfiltration prior to the addition of fructose and subsequent stirring at 75–80 °C. Molecular sieves were added to the STBR upon reaching 60% FOE and dispersed in the liquid-phase media via magnetic stirring.

Water Removal via Vacuum Pressure and N_2 (g) Bubbling

A Wheaton Celstir spinner double-side arm flask (50 mL) with a tight screw cap was placed on a hot plate/stirrer at 80 °C and stirred gently at 200 rpm (radius = 2.5 cm). The right side of the flask was connected to a N_2 (g) source and a flow rate meter when needed; and, the left side was interfaced with a vacuum pump and a vacuum meter when needed. Subsequently, the solvent-free suspension-based medium was placed in the flask. After water control treatment, the reaction medium containing fructose suspensions was transferred to the bioreactor system.

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

The water content for the reaction mixture was analyzed using a coulometric Karl-Fischer Titrator (Denver Instrument Co., Aurora, CO, USA) after its dilution with methanol. The relative amounts of oleic acid and its mono- and di-esters formed with fructose were determined 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 WR Grace (Deerfield, IL, USA) [18]. An analytical reversed phase (4.6 × 250 mm, pore diameter 5 μm) C_{18} column from Alltech was employed using an isocratic solvent system, acetone/acetonitrile/acetic acid (45/45/10, v/v/v) at a flow rate of 1.0 mL min^{-1} at a column temperature of 25 °C. Response factors were measured and employed to convert peak areas into concentrations [7]. Chromatograms of mono- and di-ester species obtained by us agree with those reported in Ref [18].

To analyze the fructose content, 40-mg aliquots of column effluent were subjected to liquid–liquid extraction using *n*-hexane and water (500 μL of each) [18]. 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 with a column temperature of 25 °C and using an isocratic solvent system, acetonitrile/deionized water (80/20, v/v) at a 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 [18].

Results and Discussion

Effect of Molecular Sieve Concentration on Production of Fructose-Oleic Acid Esters Using a Packed-Bed Bioreactor System

Although free evaporation typically removes most of the by-product water when operating reactions take place at 60 °C, a significant amount can accumulate in the liquid and biocatalyst phases during the time course of esterification, leading to a lower reaction rate and yield, particularly when ≥50% conversion of the acyl donor is achieved [8, 17]. However, excessive removal of water from the enzymes' microenvironment can lead to inactivation since water is essential for maintaining the three-dimensional conformations of enzymes [8, 16].

To determine the optimal water content in the reaction medium, MS was added at different concentrations during the time course of FOE synthesis using suspensions of fructose crystals in solvent-free media at 53 °C. Molecular sieves were introduced in the form of a MSC inserted into the PBBR-based bioreactor system, as described in "Experimental", at 70 h during the time course of reaction, corresponding to 60 wt% FOE in the liquid-phase medium (fructose-free basis). Previously, it was shown that when water was removed before achievement of 50 wt% FOE, the reaction rate decreased, presumably due to the desorption of water from the immobilized lipase by the water-depleted liquid phase [16]. Isotherms that relate the water concentration of the liquid-phase reaction medium and RML are reported elsewhere [8, 17]. The employment of a MSC lessens shear-induced breakage of the MS particles that would occur by adding MS to a stirred vessel. Shear-degraded MS particles are not well dispersed in the liquid phase and therefore do not effectively adsorb

water from it [10]. In addition, due to their adsorption onto immobilized lipase, shear-degraded MS particles may remove water from the enzyme's microenvironment [7].

After the MSC's introduction at 70 h, the production of FOE increased measurably (Fig. 1a). The maximum FOE concentration (89.9 wt%) was achieved when employing an MSC containing 12 wt% MS (i.e., 0.12 g MS g^{-1} of reaction mixture), corresponding to a residence time of 6.21 min per pass. Greater or lesser amounts of MS led to lower production of FOE (Fig. 1). The amount of water removed was directly proportional to the MS mass in the MSC column, as shown when the water concentrations decrease at 70 h in Fig. 1b, is plotted against the molecular sieve concentration (Figure S1 of the Electronic Supplementary Materials). Therefore, the water content achieved using a 12 wt% MSC, 0.42 ± 0.05 wt% (Fig. 1b) appears to be optimal. A lower amount, 7 wt% MS, reduced the water content moderately, from 0.78 to 0.61 wt%, and slightly decreased the yield of FOE. Conversely, MS concentrations >12% lowered the yield of final products. Specifically, 16 and 20 wt% MSC lowered the water concentration below the optimal (0.24–0.09 wt% in Fig. 1b, respectively), leading to excessive removal of water from the biocatalyst and to the loss of activity [20]. MS themselves have no negative or irreversible effect on enzyme activity [20]. Others have also reported optimal MS levels for biocatalytic reactions in apolar media [10, 21]. The change of concentration of FOE (Fig. 1a), water (Fig. 1b), and fructose (not shown) versus time is nearly identical for all experiments plotted in Fig. 1 up to the addition of the MSC (0–70 h), and agrees with time course data using the same bioreactor system and conditions reported previously [19], demonstrating good repeatability of the bioreactor performance.

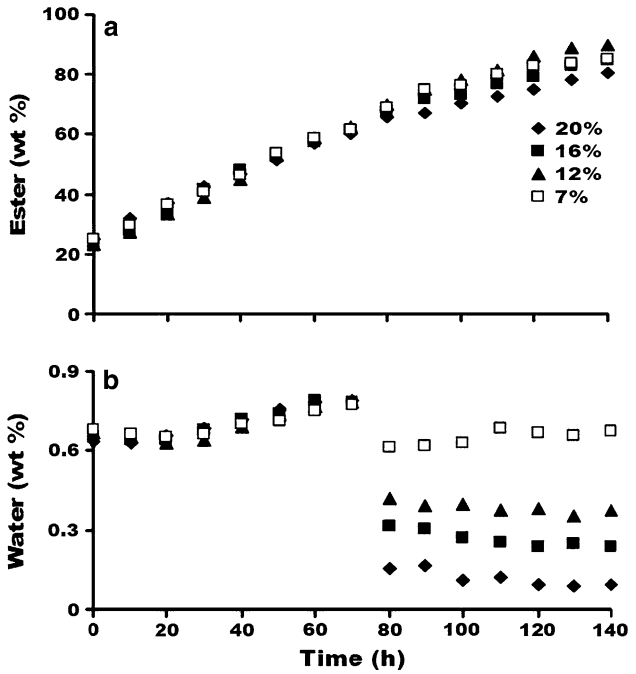

Fig. 1 Effect of molecular sieve concentration (g per g oleic acid + fructose–oleic acid ester, or FOE, given in legend) on the liquid-phase concentration of **a** FOE and **b** water for the solvent-free immobilized *Rhizomucor miehei* lipase- (RML-) catalyzed synthesis of FOE using a bioreactor system containing a packed-bed bioreactor (PBBR; 0.075 g RML g^{-1} of oleic acid + FOE), a molecular sieve column (MSC), and a reservoir undergoing continuous recirculation at 0.50 mL min^{-1} at 53 °C utilizing a suspension of fructose crystals in solvent-free media. The initial medium consisted of oleic acid/FOE 75/25, w/w. MSC was integrated into the system at 70 h during the time course of the reaction. The liquid-phase medium in the reservoir was retreated at 10.0 h intervals to replenish the consumed acyl acceptor by adding fructose, stirring at 80 °C and 800 rpm for 6 h, followed by centrifugation at 800 rpm for 1 min. The initial reaction medium contained 1.45 wt% fructose, equivalent to a ratio of 0.030 mol of fructose per mole of oleic acid

Effect of Timing for Incorporating the Molecular Sieve Column into the Packed-Bed Bioreactor System

As described previously by us [7, 16], removal of water produced during solvent-free lipase-catalyzed saccharide–fatty acid esterification at 45–65 °C has been achieved primarily through free evaporation, supplemented by MS or saturated salt solutions, the latter to control the water activity of the air headspace through employing salts yielding low water activity, such as LiCl. The latter two methods have been employed during the final stages of the reaction (FOE content >60 wt%) to increase the rate and conversion [7, 16, 17]. However, as described above, removal of water from either the liquid phase or the immobilized lipase during the initial reaction period, via dessication of the materials prior to the start of the reaction or by employing vacuum pressure, led to a decreased reaction rate [16]. Therefore, determining the appropriate time for introducing MS for the production of saccharide–fatty acid esters is important for optimization. Figure 2 compares different introduction times for incorporating the MSC (12 wt% MS, or equivalently, a residence time of 6.21 min per pass for the MSC) into the PBBR-based bioreactor system. The maximum liquid-phase FOE concentration (89.1 wt%) was obtained when incorporating the MSC at 70 h, equivalent to 58.6 wt% FOE (Fig. 2a). The 70-h incorporation time produced a liquid phase water concentration of 0.42 wt% (Fig. 2b), which agrees with optimal water concentration obtained in Fig. 1b. Introduction of MS into the reaction during the early phase (at 30 or 50 h, equivalent to 39.5 and 52.9 wt% ester in the liquid phase, respectively) also achieved a water concentration of 0.42 wt%; but, the rate of reaction was reduced, presumably due to extraction of water from RML, leading to a suboptimal water activity and hence a reduced rate (Fig. 2). In contrast, deployment of MS during the latter stage of the reaction (≥90 h) minimally reduced the liquid-phase water concentration 0.88 wt%, which therefore led to a lower

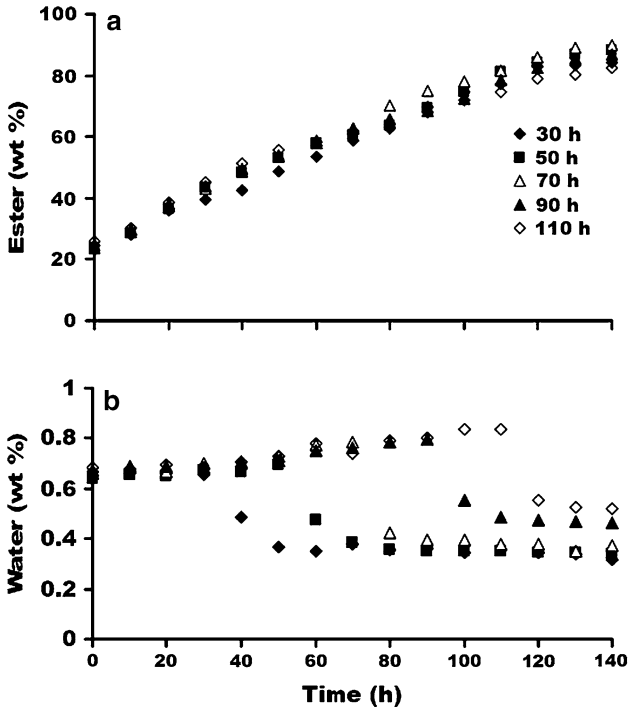

Fig. 2 Effect of introduction time for molecular sieve column (given in legend) on the RML-catalyzed esterification of fructose and oleic acid employing a suspension of fructose crystals in solvent-free media and a bioreactor system containing a packed-bed bioreactor undergoing continuous recirculation. Other conditions are the same as those of Fig. 1 with employment of 0.12 g molecular sieves per g of FOE + oleic acid

increase of FOE yield, Fig. 2a, b. The time courses of reaction depicted in Fig. 2 up to the introduction of the MSC are very similar to each other and agree with Fig. 1 and those reported previously by us [19], further demonstrating the good repeatability of the PBBR-based system's performance.

Effect of Bioreactor Type on Production of Fructose Oleate

Recently, in a side-by-side comparison for the solvent-free lipase-catalyzed synthesis of saccharide–fatty acid ester using saccharide concentrations equivalent to saturation (i.e., in the absence of suspensions, at significantly lower saccharide concentrations), the rate and extent of the reaction was higher for PBBRs compared to STBRs, [7]. The two typical bioreactor types are compared in Fig. 3 for bioreactor systems employing solvent-free suspensions. Molecular sieves were introduced into the stirred vessels of both bioreactor systems (the reservoir of the PBBR system and the STBR itself) at 70 h with identical concentrations. The highest productivity ($0.193\ \mathrm{mmol_{FOE}\ h^{-1}\ g_{lipase}^{-1}}$) and yield (90.2 wt% FOE in the liquid phase) were achieved by the PBBR system, with the productivity and yield achieved in the STBR being only $0.171\ \mathrm{mmol_{FOE}\ h^{-1}\ g_{lipase}^{-1}}$ and 82.5 wt% FOE, respectively. The addition of MS decreased the water content more effectively for the PBBR system (0.42 wt%) compared to the STBR-based system (0.82 wt%). These values are in strong agreement with our previously published results, which utilized much lower saccharide concentrations for the two bioreactor types [7]. Surprisingly, the MS particles did not undergo breakage as much as anticipated. However, an adsorbed saccharide multilayer was observed to form on the MS particles in the STBR during the later phase of the reaction, leading to a poorer dispersion of RML and MS particles and hindering the ability of MS particles to adsorb water. The adsorbed saccharide multilayer may possibly result in a higher water concentration in the –STBR liquid phase through water desorption from the atmosphere [7], thus reducing the yield of FOE.

Comparison of Water Removal Methods

There are several approaches that have been applied for removal of water during lipase-catalyzed esterification: MS [10, 11, 22, 23], silica gel [7], azeotropic distillation [24, 25], salt hydrate pairs [26, 27], dry air or N_2 [28, 29], vacuum pressure [30, 31], free evaporation [11, 17, 32], and pervaporation [32–34]. However, few publications have investigated and compared various water removal

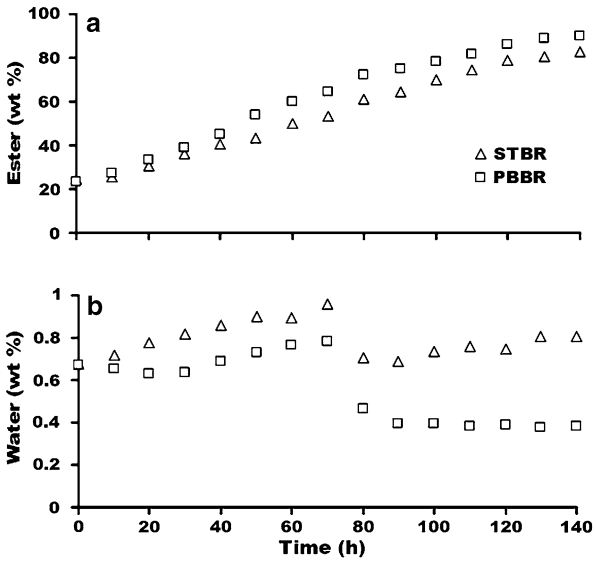

Fig. 3 Effect of bioreactor type on RML-catalyzed fructose–oleic acid ester synthesis utilizing solvent-free suspensions of fructose crystals. *Triangles* a stirred tank bioreactor (STBR) open to the atmosphere, operated at 65 °C and 300 rpm (radius of 1.5 cm) and *squares* the packed-bed bioreactor (PBBR) system described for Fig. 1 undergoing continuous recirculation at 53 °C (except for the absence of a MSC). Molecular sieves (13 wt%) were introduced into the STBR and the reservoir of the PBBR system at the onset of 60 wt% FOE in the liquid-phase medium. For both bioreactors, liquid-phase media in the reservoir was retreated at 10.0 h intervals to replenish consumed acyl acceptor

methods for lipase-catalyzed reactions in solvent-free media. Figure 4 illustrates the effect of four different water removal treatments: vacuum pressure (67.7 kPa), N_2 bubbling (2.5 L min^{-1} at standard pressure and temperature, 101.3 kPa and 273.15 K, respectively), MS (20 wt% overall), and a combination of vacuum pressure and N_2 bubbling, applied to a suspension of fructose crystals in oleic acid/FOE 75/25, w/w at 80 °C versus time. When comparing the performance between 1 and 21 h, the methods listed in their order of effectiveness to remove water consists of: vacuum + N_2 bubbling (2.16 $mg_{H_2O}\,h^{-1}$) > molecular sieves (1.42 $mg_{H_2O}\,h^{-1}$) > N_2 bubbling (0.68 $mg_{H_2O}\,h^{-1}$) > vacuum (0.23 $mg_{H_2O}\,h^{-1}$). Hence, the combination of vacuum and nitrogen was selected to enhance water removal from the reaction medium to optimize the performance of the bioreactor in subsequent experiments. In addition, air and/or N_2 bubbling yields relatively high convective mass transfer rates for water with a minimal influence on enzyme activity and stability [28]. However, the combination of vacuum and N_2 lowered the water content from 0.67 to 0.24 wt% in 20 h, and to 0.16 wt% in 140 h (Fig. 4), far below the optimal level, 0.42 ± 0.05 wt% (Fig. 1), suggesting that great care must be employed when using this approach to prevent excessive removal of water.

Employment of Lower Initial Ester Concentrations

In our previous work [19] and up to this point in this paper, we utilized 25% FOE/75% oleic acid, w/w (on a fructose-free basis) as the initial liquid-phase reaction medium. It was unclear to us if liquid-phase media containing lower concentrations of ester would form metastable suspensions possessing a significantly high saccharide concentration, to allow the rate of reaction to be reasonably high. Figure 5 shows that the initial reaction rate (approximately 5 wt% increase of FOE per 10-h period up to 40 h) did not change appreciably on lowering the initial FOE concentration from 25 wt% to 5%, indicating that oleic acid/FOE 95/5, w/w could serve as the initial reaction medium, requiring 40 h (i.e., 4 cycles) of additional reaction time. Moreover, the fructose concentrations were reasonably high for all the experiments depicted: 0.69 wt% initially, increasing to 0.79, 1.18, and 1.49 wt% after the second, third, and fourth 10 h cycles, respectively, with the last value strongly agreeing with the initial fructose concentration for 25 wt% FOE/75 wt% oleic acid as the initial medium, 1.45 wt% (e.g., Fig. 1). However, FOE concentrations <5 wt%, for instance, 3 and 0 wt%, led to poor initial reaction rates due to the low concentration of saccharide suspensions achievable (unpublished data).

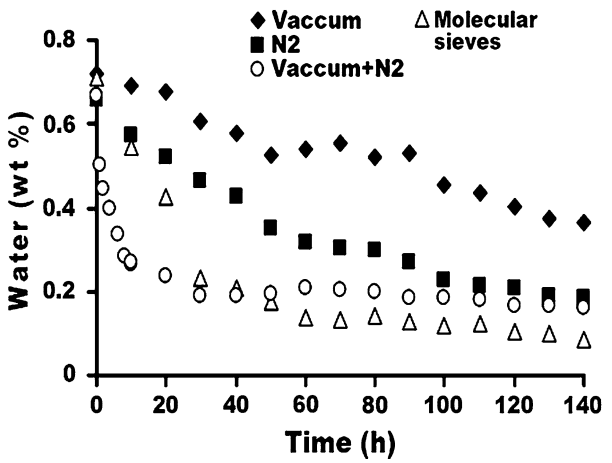

Fig. 4 Effect of water removal methods on the water concentration for a suspension of fructose crystals in FOE/oleic acid 25/75, w/w at 80 °C. Suspension formed by mixing 1 g of fructose with 10 g FOE/oleic acid. Water removal method: *diamonds* vacuum, 67.7 kPa; *squares* 2.56 L of bubbling of N_2 gas min^{-1}; *triangles* 10 wt% molecular sieves introduced initially into the reservoir; at 10 h, the original molecular sieves were removed and fresh molecular sieves (10 wt%) were added; *circles* Combination of vacuum and bubbling of N_2 gas

Optimal Placement of the Acyl Acceptor Replenishment Treatments During the Time Course of Reaction

For the PBBR-based bioreactor system employed in our previous work [19] and shown in Figs. 1, 2, 3 and 5 of this paper, the treatment of the reaction medium with additional saccharide (and subsequent formation of metastable suspensions) occurred at 10 h intervals during the time course of reaction. However, it was observed that the saccharide was nearly 100% consumed before the end of the 10 h intervals were reached, particularly during the initial period of the reaction where the rate is highest [19]. Therefore, the

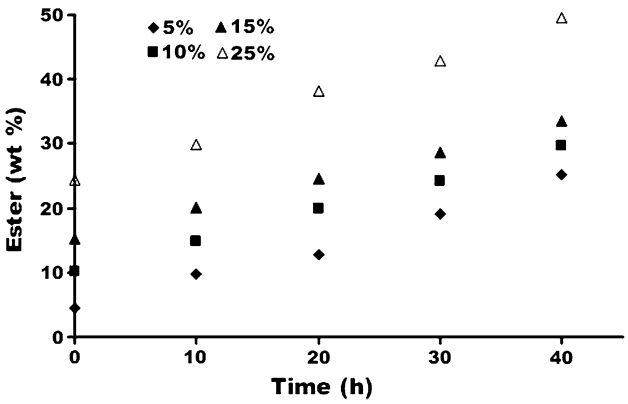

Fig. 5 Effect of the initial ester content of the solvent-free reaction medium (g per g oleic acid + FOE, given in legend) on the initial rate of RML-catalyzed esterification of fructose and oleic acid utilizing a PBBR undergoing continuous recirculation at 53 °C. Other conditions are the same as those in Fig. 1

placement of the saccharide replenishment treatments during the time course of the reaction can be further optimized. Figure 6 explores the time course of reaction between each saccharide replenishment treatment using the PBBR-based bioreactor system for an initial reaction medium that contained 5 wt% FOE (as per Fig. 5). For the first, second, and third intervals between treatments, it was clear that the formation of FOE was nearly complete at 3.0 h since the conversion slows with longer durations (dashed arrows). Therefore, separation of saccharide replenishment treatments by 3.0 h is sufficient for the initial portion of the time course of the reaction. Likewise, the 4th–6th intervals between treatments can be shortened to 4.0 h, the 7th interval to 6.0 h, and the 8th interval to 9.0 h (Fig. 6). In sum, the total reaction time is decreased by 44 h when the schedule of the saccharide replenishment treatments during the time course of the reaction is optimized.

Operation of a Packed-Bed Bioreactor System Using Solvent-Free Suspensions Under Optimal Conditions

With the goal being to optimize the performance of the PBBR-based bioreactor system for synthesis of the solvent-free lipase-catalyzed saccharide–fatty acid esters, the conditions employed for Fig. 1 were modified to take into account the knowledge learned on controlling the methodology and timing of water removal (vacuum pressure, 67.7 kPa + N_2 bubbling, 2.6 L min^{-1}, introduced upon reaching 60% esters, as per Fig. 2), bioreactor type (with PBBR chosen, based on Fig. 3), the interval time between saccharide replenishment treatments (programmed as described in Fig. 6), and lower initial amount of FOE, 5 wt% (as per Fig. 5). As shown in Fig. 7a, optimal conditions yielded 92.6 wt% FOE within 5.5 days. The FOE product consisted of 90 wt% monoester and 10 wt% diester, consistent with our previous results (Fig. S2 of ESM) [7, 17, 19]. The control experiment, employing 10-h intervals between saccharide replenishment treatments and only free evaporation for water removal, yielded <60 wt% ester over the same time period. In other words, the average FOE production rate, 9.13 wt% conversion of oleic acid into ester per day for the control was increased to 15.9 wt% conversion per day under optimal conditions. Figure 7b illustrates that the water concentration of the optimized process was maintained at approximately 0.4 wt%, in agreement with the optimal water content depicted in Fig. 1. It is much lower than the control experiment's water concentration, 0.9 wt%, which reduced the extent of the reaction. During the time course of reaction, the fructose concentration obtained after retreatment of the suspension medium with additional fructose, increased steadily, from 0.68 up to 2.52 wt%, due to the increased concentration of FOE (Fig. S3 of ESM). The stoppage of the reaction was not due to the depletion of acyl donor or acceptor substrate, but most probably to thermodynamic equilibrium. Upon completion of the reaction at 132 h, the reaction mixture consisted of 9.26 g of FOE, 0.74 g (2.6 mmol) of oleic acid and 0.25 g (1.4 mmol) of fructose; therefore, sufficient amounts of substrates were present to enable further esterification.

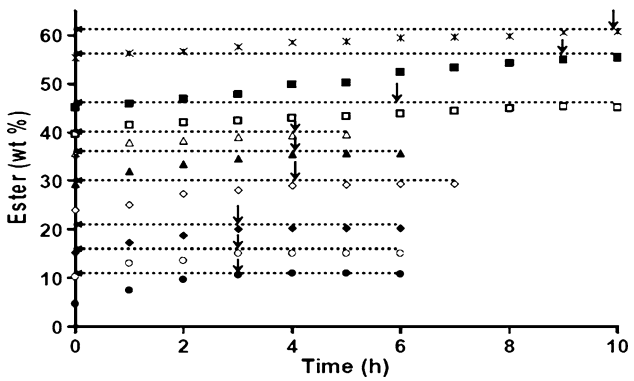

Fig. 6 Determination of the minimum interval times between saccharide replenishment (suspension formation) treatments for the solvent-free RML-catalyzed esterification of fructose and oleic acid utilizing a packed-bed bioreactor (PBBR) undergoing continuous recirculation at 53 °C. *Dashed arrows* indicate the occurrence of saccharide replenishment treatments using the procedure described in Fig. 1. The time at which conversion of the acyl acceptor concentration is nearly complete is indicated by *downward pointing arrows*. Other conditions are given in Fig. 5

Conclusions

The performance of the solvent-free immobilized *R. miehei* lipase-catalyzed synthesis of saccharide–fatty acid employing suspensions of crystalline saccharide in a packed bed bioreactor system operated under continuous recirculation, reported previously by us [19], was optimized in this study. The experimental results indicated that an additional water removal approach (to augment removal via free evaporation) should be introduced into the bioreactor system upon reaching approximately 60 wt% ester, with the goal being to maintain a liquid-phase water concentration of approximately 0.4 wt%. In addition, the combination of nitrogen gas bubbling + vacuum pressure was found to be the most efficient water removal method, followed by the use of molecular sieves, both of which were superior to either N_2 or vacuum applied as the sole method. For the comparison of bioreactor types, results demonstrated that the rate and yield of ester were significantly higher using a PBBR compared to a STBR, with the former enabling more efficient water removal. Optimization of the interval time between saccharide replenishment treatments led to a

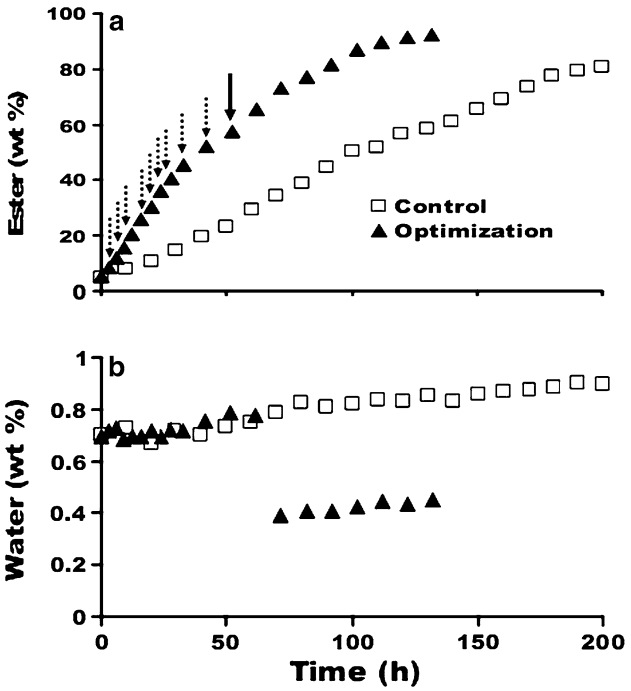

Fig. 7 Optimization of interval time for between saccharide replenishment treatments and removal of water for the solvent-free RML-catalyzed synthesis of fructose oleate using the packed-bed bioreactor system described in Fig. 1. **a** Production of FOE, **b** Water control of liquid phase. "Optimization": water removal via 67.7 kPa vacuum and 2.56 L min^{-1} N_2 (g) was introduced upon reaching approximately 60% ester in the liquid phase (indicated by *downward pointing arrows*; timing of saccharide replenishment treatments indicated by dashed arrows. "Control" experiment: vacuum plus N_2 bubbling were not incorporated interval time was employed; interval time between saccharide replenishment treatments was 10.0 h. Other conditions were the same as those described in Fig. 1, except saccharide replenishment treatment conditions varied slightly: stirring was carried out at 70 and 85 °C for "optimized" and "control", respectively, and the centrifugation time was 30 s

decrease of the reaction time by 44 h; and, the initial ester concentration of the metastable solvent-free suspensions could be reduced from 25 to 5 wt% without a concurrent decrease in reaction rate. By incorporating the improvements described above, a yield of 92.6 wt% fructose oleate was obtained in the PBBR-based bioreactor system within 132 h, increasing the productivity ($0.297\ mmol_{FOE}\ h^{-1}\ g^{-1}_{lipase}$), more than a twofold increase compared to a control experiment (0.129 mmol h^{-1} g^{-1}), which employed 10-h interval times between saccharide replenishment treatments and did not include N_2 bubbling plus vacuum to augment water removal via free evaporation.

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