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Solvent-Free Lipase-Catalyzed Synthesis of Technical-Grade Sugar Esters and Evaluation of Their Physicochemical and Bioactive Properties

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Abstract: Technical-grade oleic acid esters of sucrose and fructose were prepared using solvent-free biocatalysis at 65 °C, without any downstream purification applied, and their physicochemical and bioactivity-related properties were evaluated and compared to a commercially available sucrose laurate emulsifier. To increase the conversion of sucrose and fructose oleate, prepared previously using solvent-free lipase-catalyzed esterification catalyzed by *Rhizomucor miehei* lipase (81% and 83% ester, respectively), the enzymatic reaction conditions was continued using $CaSO_4$ to control the reactor's air headspace and a lipase (from *Candida antarctica B*) with a hydrophobic immobilization matrix to provide an ultralow water activity, and high-pressure homogenation, to form metastable suspensions of 2.0–3.3 micron sized saccharide particles in liquid-phase reaction media. These measures led to increased ester content of 89% and 96% for reactions involving sucrose and fructose, respectively. The monoester content among the esters decreased from 90% to <70% due to differences in regioselectivity between the lipases. The resultant technical-grade sucrose and fructose lowered the surface tension to <30 mN/m, and possessed excellent emulsification capability and stability over 36 h using hexadecane and dodecane as oils, comparable to that of sucrose laurate and Tween® 80). The technical-grade sugar esters, particularly fructose oleate, more effectively inhibited gram-positive foodborne pathogens (*Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Bacillus subtilis*). Furthermore, all three sugar esters displayed antitumor activity, particularly the two sucrose esters. This study demonstrates the importance of controlling the biocatalysts' water activity to achieve high conversion, the impact of a lipase's regioselectivity in dictating product distribution, and the use of solvent-free biocatalysis to important biobased surfactants useful in foods, cosmetics, personal care products, and medicine.

Keywords: antimicrobial activity; antitumor activity; emulsification; high-pressure homogenation; lipase; sugar-fatty acid esters; surfactants

1. Introduction

Saccharide-fatty acid esters are biodegradable, biocompatible and environmentally-friendly biobased surfactants and emulsifiers [1–3]. Saccharide esters enriched in monoester have been widely

used as emulsifiers in the food, cosmetic and pharmaceutical industries, and as agents for drug delivery [4,5]. In addition, they possess antimicrobial, antitumor, and anti-human immunodeficiency virus (HIV) activity [6,7]. Moreover, they have been employed to form drug-delivery systems [4,5]. The conventional method for their chemical synthesis involves extreme reaction conditions; for instance, high temperature, the use of solvents such as dimethylformamide and dimethylsulfoxide, and alkali or acid catalysts [8]. In contrast, biocatalytic synthesis is operated under mild reaction conditions, thereby reducing the formation of undesirable byproducts and lowering energy consumption during processing [9–11]. However, the poor miscibility of hydrophobic acyl donors and hydrophilic acceptors leads to a slow reaction rate. Different means have been developed to enhance miscibility of acyl donors and acceptors for lipase catalyzed reactions, including, polar organic solvents such as *tert*-butanol [12], ionic liquids [13], and ultrasound irradiation [14]. However, these methods suffer from some deficiencies, for instance, the usage of expensive equipment and solvents, downstream separation challenges and costs, environmental issues, and safety concerns.

In our laboratory, we developed a sustainable approach to produce saccharide-fatty acid esters in solvent-free media, by forming metastable suspensions of 10–100 μm-sized saccharide particles at 1–2 wt %. The production of sugar esters further improves the maximum concentration and stability of the suspended saccharide particles [15–19]. We utilized the solvent-free suspension-based medium in a closed-loop bioreactor system operated under continuous recirculation at 53 °C to prepare sugar esters on a 30 gram scale. The system contained a peristaltic pump, a packed-bed bioreactor (PBBR) filled with immobilized *Rhizomucor miehei* lipase (RML), and a reservoir open to the atmosphere, which allowed for free evaporation of the co-product water during the first stage of the reaction. During the time course of reaction, additional acyl acceptor was added periodically by temporarily removing the solvent-free media from the bioreactor system, and adding saccharide particles under stirring. When reaching approximately 60 wt % ester, a stronger water removal method was introduced to increase the yield, e.g., the combination of N_2 bubbling and vacuum pressure or molecular sieves, which decreased the water concentration in the liquid phase from ~0.8 wt % to ~0.4 wt %. This approach generated a yield 69~92 wt % esters within 132 h. Recently, the combination of high-speed homogenization and high-intensity ultrasound was utilized to reduce the particle size of sucrose crystals five-fold, enhancing the rate and extent of reaction [20].

The Food and Agriculture Organization (FAO) of the European Union specifies that sucrose esters contain no solvents, and fatty acid and sucrose at amounts <3 wt % and <5 wt %, respectively [21]. Therefore, in order to meet the FAO requirements without the need for downstream purification, the yield for the authors' solvent-free enzymatic approach needs to be increased. Therefore, the first objective of this work is to increase the conversion of lipase-catalyzed synthesis of fructose and sucrose oleate (*i.e.*, to meet the FOA requirements) by introducing three new approaches during the latter stage of reaction (*i.e.*, upon achieving ~80%–85% conversion using the approach described above). First, high-pressure homogenation (HPH) will be employed to provide μm sized suspensions of saccharide particles. Second, $CaSO_4$ will be used to further reduce the water activity of the bioreactor through its control of the water activity for the reactor's air headspace. Third, immobilized *Candida antarctica* lipase B (CALB) will be employed rather than RML because the former possesses a lower water content due to its more hydrophobic immobilization matrix, polyacrylate [22]. The lower water content of the enzyme's microenvironment is anticipated to increase the equilibrium conversion. The second objective is to test the hypothesis that the sugar esters produced from the approach described above will perform well as an emulsifier and as antimicrobial and antitumor agents without the need of further purification.

2. Results and Discussion

2.1. Lipase-Catalyzed Synthesis of Technical-Grade Sucrose and Fructose Oleate

In order to improve the final yield of sugar fatty ester and the rate during the latter stage of the reaction, three strategies were implemented: the replacement of RML with CALB, use of $CaSO_4$

to lower the water activity of the bioreactor's air headspace, and HPH to reduce the particle size of suspended saccharide, hence increasing the concentration of saccharide in the reaction medium. HPH reduced the particle sizes of saccharide crystals in the solvent-free suspension media from 91.5 ± 2.8 μm to 2.1 ± 0.6 μm for fructose oleate and 98.7 ± 2.1 μm to 3.3 ± 0.8 μm for sucrose oleate (data not shown), a 6–7-fold reduction in particle size than that produced by high-speed homogenation plus ultrasound [20]. Figure 1 depicts the time course of sucrose and fructose-oleic acid esterification after implementation of the three strategies. The conversion of fructose and sucrose oleate increased from approximately 83% to 96% and from 80% to 89%, respectively, in a four-day period. The productivity for ester production for the reactions depicted in Figure 1A–D were 0.035 mmol· h^{-1}· $g_{lipase}{}^{-1}$ and 0.025 mmol· h^{-1}· $g_{lipase}{}^{-1}$ for fructose and sucrose oleate, respectively. The former productivity is one order of magnitude higher than that obtained during the final stage of reaction (~82% to ~88% ester) in our previous publications, 0.0014–0.0017 mmol· h^{-1}· $g_{lipase}{}^{-1}$ [15,16], and comparable to the productivity of lipase-catalyzed synthesis of sucrose palmitate in the presence of a 2-methyl-2-butanol/dimethylsulfoxide mixture [23]. The presence of $CaSO_4$ reduced the water concentration from 0.49% to 0.09% for fructose oleate and from 0.51% to 0.12% for sucrose oleate (Figure 1B). In our previous studies, when reaching ~60% conversion, molecular sieves were introduced to the esterification reaction to decrease the water concentration from ~0.92% to ~0.41% [16]. Although a large amount of molecular sieves further decreased the water concentration to approximately 0.10 wt % in the cited study, a concern with this approach is that debris, which commonly forms from shear-induced abrasion of the molecular sieves is likely to adsorb onto the immobilized lipase, thereby reducing the biocatalytic activity [16]. The saccharide concentration in the liquid phase of the reaction medium decreased during the time course of reaction due to its consumption by the reaction; however, saccharide was not completely consumed (Figure 1C). It appears that the ultra-low water concentration did not negatively affect CALB activity, in agreement with the results in literature [20,24]. As shown in Figure 1D, the proportion of monoester decreased during the time course of both reactions, from 90% to 60%–70%. The decrease is attributable to differences in the inherent regioselectivity between CALB and RML [25,26], the latter used to prepare the initial reaction medium. An additional factor is the lower water concentration for CALB due to its more hydrophobic immobilization matrix, which results in the more hydrophobic microenvironment for the enzyme, thereby enhancing the transformation of monoester into diester [20,27]. Upon completion of the biochemical reactions, the only purification enacted was the removal of CALB by sedimentation.

2.2. Composition and Surface Activity

Compositional profiles and surface activity-related properties for technical-grade fructose and sucrose oleate, prepared as described above, and for two commercially available surfactants, sucrose laurate and Tween® 80, serving as positive and negative controls, respectively, are given in Table 1. Sucrose laurate was highly pure, containing only 0.1% free fatty acid and no detectable saccharide; in contrast, fructose and sucrose oleate contained 3.4% and 10.0% free fatty acid, respectively, and 0.5%–1.0% saccharide. Sucrose laurate was more highly enriched in monoesters, 86% among the esters, compared to 65%–69% for the technical-grade enzymatic products, therefore yielding a slightly lower hydrophilic–lipophilic balance (HLB) value for sucrose oleate (10.1) compared to sucrose laurate (11.2). The HLB of fructose oleate is only 7.2, due to the presence of only one saccharide unit per molecule. Plot of surfactant concentration, C (log scale), *vs.* surface tension, γ, for the three sugar esters resembled plots frequently encountered for surfactants: a linear decrease of γ *vs.* log C at low C, and a constant, minimal γ at high C (Figure 2). Parameters derived from the Figure 2 plots—the critical micellar concentration (CMC), γ (at C = CMC), the surface excess (Γ), and the surface area per surfactant (A_s) are given in Table 1 [28–30]. The surface tension for sucrose laurate achieved by us 19.7 mN/m, is lower than values reported for the same ester in the literature, 35–40 mN/m [31–33]; also, the CMC reported in Table 1 (0.63 g/L) is higher than reported values, 0.1–0.2 g/L [24,31,33], with the lower temperature used in our studies (22 °C) compared to those used in the cited reports (25–37 °C) being at least partially attributable to the latter difference [34]. Compared to sucrose laurate, technical-grade

sucrose oleate yielded a slightly higher γ (29.6 mN/m) and a higher CMC, the latter trend reflecting the lower purity and polarity for sucrose oleate [31,34]. For fructose oleate, its γ value (27.9 mN/m) is comparable to those reported in the literature for fructose caprate, laurate and myristate [30,32]. In summary, the technical-grade sugar esters prepared using solvent-free enzymology are effective surfactants, able to lower the surface tension to a similar extent as commercially available and more highly purified sugar esters.

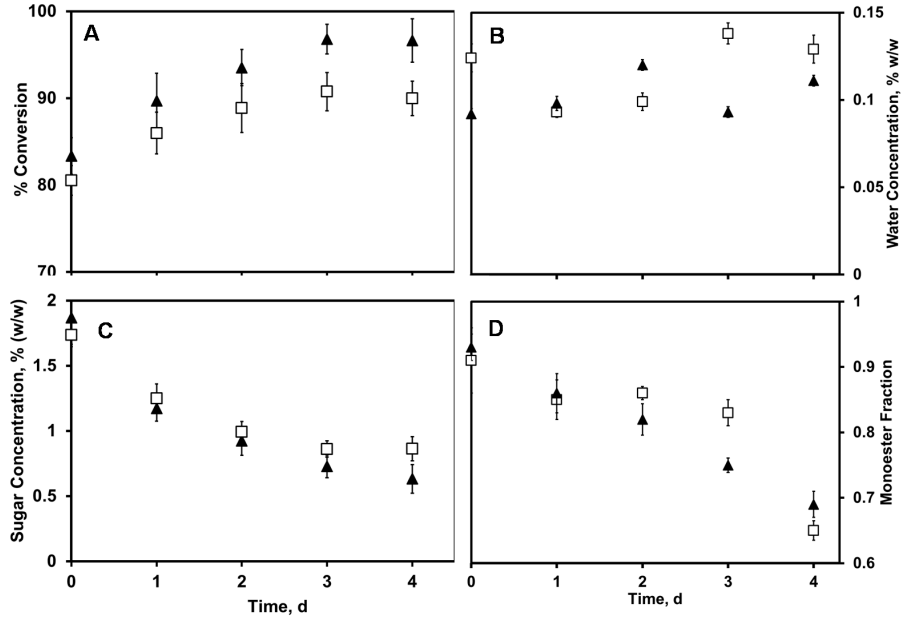

Figure 1. Time courses of the lipase-catalyzed synthesis of fructose oleate (triangle) and sucrose oleate (square): (**A**) conversion of oleic acid; (**B**) water concentration; (**C**) saccharide concentration; and (**D**) fraction of monoester among all esters. Reaction conditions: 20 g of a solvent-free suspension of saccharide particles in solvent-free reaction media (prepared via *Rhizomucor miehei* lipase (RML)-catalyzed esterification), prepared via high-pressure homogenation (HPH), was mixed with 2 g of *Candida antarctica* lipase B (CALB). The reaction was conducted in a closed stirred system using $CaSO_4$ to lower the water activity of the bioreactor head space at 65 °C and a stir rate of 350 rpm.

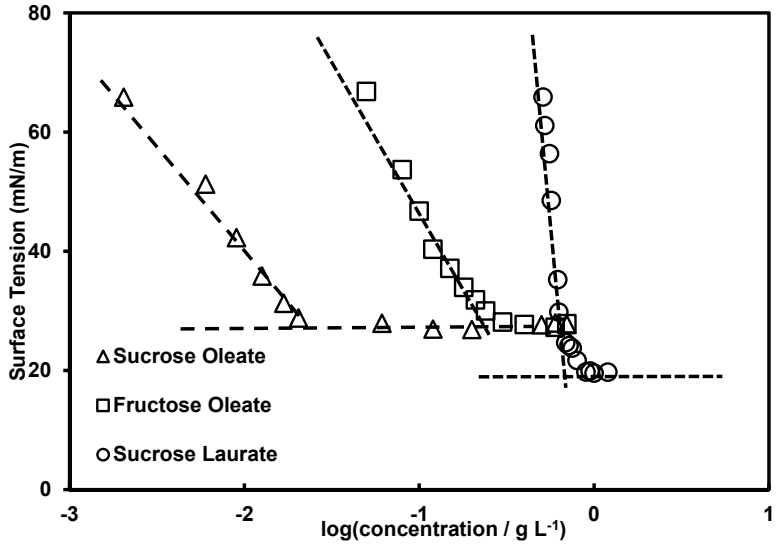

Figure 2. Surface tension (γ) at 22 °C *vs.* concentration (*C*) for sugar esters: sucrose oleate (Δ), fructose oleate (▫), and sucrose laurate (○).Intersection of the dashed lines represent determination of the critical micellar concentration (CMC).

Table 1. Comparison of chemical and surfactant-related properties of sucrose oleate and fructose oleate, prepared from the enzymatic reaction, *versus* sucrose laurate and Tween® 80[1], commercial emulsifiers, at 22 °C.

Property	Fructose Oleate	Sucrose Oleate	Sucrose Laurate	Tween® 80 [1–3]
Free fatty acid, wt %	3.4	10.0	0.1	ND
Saccharide, wt %	0.63	0.86	<0.10	ND
Moisture, wt %	0.11	0.13	0.05	ND
Monoester among the esters, wt %	69	65	86	ND
Hydrophilic–Lipophilic Balance (Griffin HLB) [4]	7.2	10.1	11.2	15.0
Density, g/mL	934	952	689	1070
Critical Micellar Concentration (CMC), g/L	0.18	0.021	0.63	0.014
Surface tension (γ) at C = CMC, mN/m)	27.9	29.6	19.7	38.0
Surface excess (Γ), mol· $m^{-2} \times 10^6$	1.91	0.849	8.19	0.74
Specific surface area (A_s), nm^2	0.87	1.96	0.20	2.24

[1] Polyoxyethylene (20) sorbitan monooleate; [2] properties Taken from references [28,29]; [3] ND refers to "not determined"; [4] Hydrophilic–lipophilic balance; calculated by the following formula [30]: HLB = 20 × Hydrophilic group molecular weight/Total surfactant molecular weight.

2.3. Composition and Surface Activity

The literature demonstrates that sugar esters are useful emulsifiers of oil in water [30,35]. Figure 3 compares the ability of the technical-grade sugar esters and controls to emulsify hexadecane and dodecane in water, and the stability of the emulsions. The figure shows that all of the sugar esters exhibited excellent emulsification ability and stability over 36 h for both oils, indicating that the enzymatic preparation produced an effective foam-stabilizer and emulsifier agent. The absorbance values for emulsified hexadecane were slightly greater than those for dodecane, indicating the degree of emulsification was higher for the former oil, due to its higher lipophilicity. Sucrose laurate generated the highest degree of emulsification for both oils, probably reflecting its higher monoester content among the sugar esters. In contrast, Tween® 80, a common nonionic surfactant widely used in foods, cosmetics and pharmaceutics, serving as a negative control, produced the lowest absorbance values, due to its higher polarity (higher HLB, Table 1). Emulsification was slightly higher for fructose oleate than for sucrose oleate, perhaps reflecting the former's higher purity. Except for the emulsification of dodecane by sucrose oleate, which underwent a 26.9% decrease of absorbance in 36 h (perhaps due to its lower purity), the percent decrease for all other surfactant/oil combinations was only 10.9%–18.1%. Our results contrast the report in the literature that an increase of diester fraction lowers the emulsion stability [30]. In summary, the technical-grade sugar esters prepared from solvent-free biocatalysis in the absence of purification performed well as emulsifiers.

2.4. Antimicrobial Activity

In the literature, the antimicrobial activity of sugar fatty acid esters have been evaluated, including sucrose monolaurate [36–38], lactose monolaurate [37,38], fructose dilaurate [36], and 6′-*O*-lauroylmaltose [39]. However, the antimicrobial properties of fructose and sucrose oleate have not been reported. As displayed in Figure 4, all three sugar esters inhibited the growth of gram-positive food-borne pathogens. Fructose oleate displayed higher antimicrobial activity against *Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Bacillus subtilis* than that of the two sucrose esters. However, the sucrose esters more strongly inhibited the growth of *Staphylococcus aureus* than fructose oleate. In addition, the sugar esters prevented growth of *Pediococcus pentosaceus* and *Bacillus subtilis* to an increasing extent as the concentration of sugar esters was increased; however, when the concentration exceeded 0.5 mg/mL, no significant improvement of microbial inhibition by *Lactobacillus platarum* was observed. In summary, sugar esters prepared via solvent-free biocatalysis had equal or superior antimicrobial activity compared to commercially available sucrose laurate.

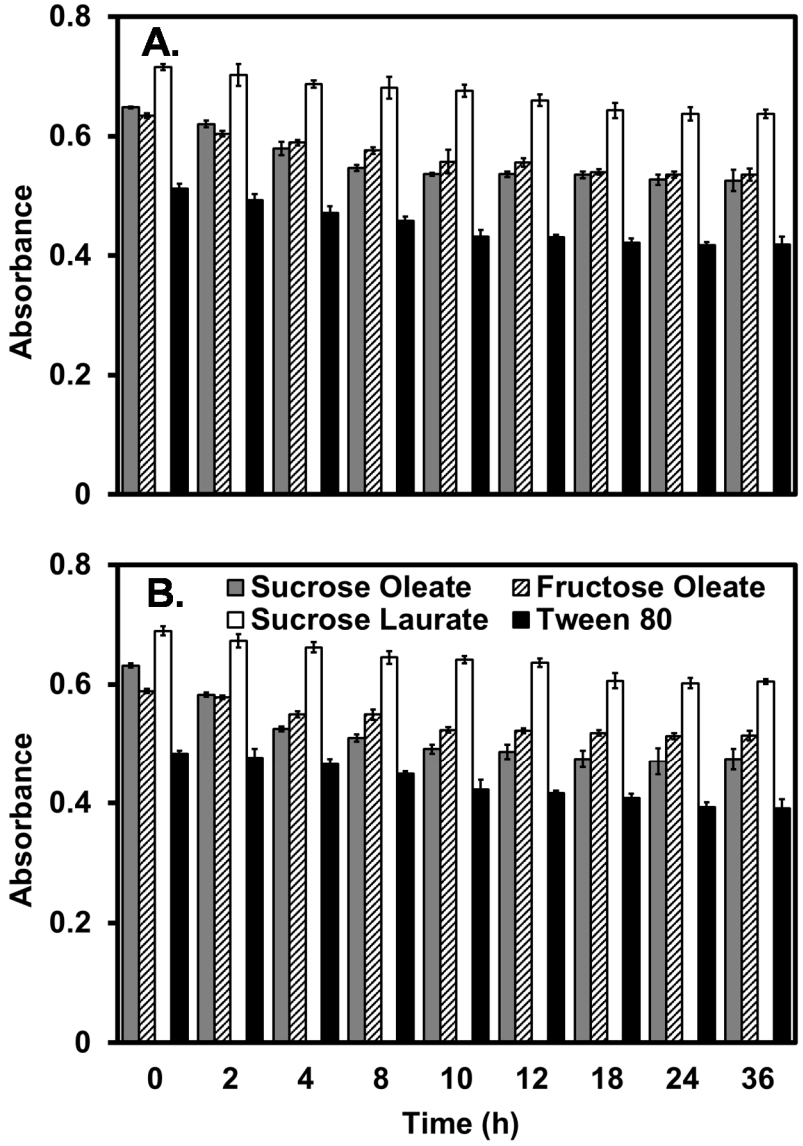

Figure 3. Emulsification of (**A**) hexadecane and (**B**) dodecane by sugar esters: sucrose oleate (gray); fructose oleate (slash); sucrose laurate (white); and Tween® 80 (black; a control) at 22 °C, evaluated over a 36 h period. Aqueous solutions of 2 g/L sugar ester were vigorously mixed with oil, then allowed to settle in the absence of stirring for 20 min. The turbidity of the sample was measured via absorbance at 600 nm.

The antimicrobial mechanism of sugar esters still remains unknown. One hypothesis is that sugar esters disrupt of the cell membrane [40]. The change of cellular morphology would alter the membranes' permeability, thereby yielding a selective loss of glycolytic intermediates and inducing autolytic processes, which would result in cell death [39,41]. The inhibitory activity of sugar esters is highly associated with the acyl acceptor, the chain length and double bonds of acyl donor (fatty acids), and the degree of esterification [37].

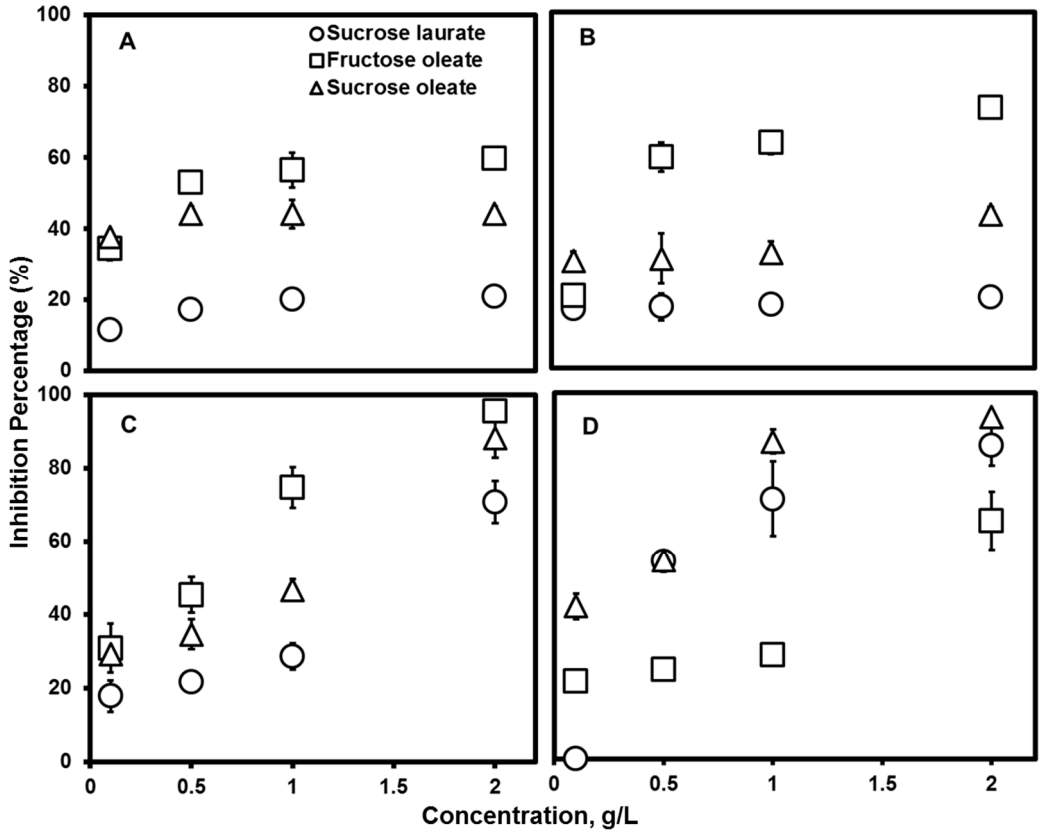

Figure 4. Antimicrobial activities of sugar fatty acid esters: sucrose oleate (○), fructose oleate (▫), and sucrose laurate (Δ) against: (**A**) *Lactobacillus plantarum*; (**B**) *Pediococcus pentosaceus*; (**C**) *Bacillus subtilis*; and (**D**) *Staphylococcus aureus*.

2.5. Antitumor Activity

As plotted in Figure 5, the antitumor activity of sugar esters increased with the increase of the concentration, with the viability decreased to <50% with the addition of either of the two sucrose esters at 1.2 g/L. No significant difference between sucrose oleate and sucrose laurate on the inhibition of tumor cell growth and proliferation were detected in this study. Kato *et al.* revealed that the presence of sucrose oleate and sucrose laurate allowed for the inhibition of the tumor cells [42]. It was proposed that the lipid chain of sugar fatty acid esters could attack the cell, giving rise to the esters' antitumor activity [42]. Ferrer *et al.* stated that the enzymatically-synthesized 6″-*O*-palmitoylmaltotriose could suppress two human hepatocarcinoma cancer cell lines, Hep-G_2 and HeLa, derived from a human hepatoma [43]. Of interest, at the same concentration, the antitumor activities of fructose oleate are inferior to those of the sucrose esters. The difference may be attributable to the esters' degree of glycosylation, since disaccharides are often more effective inhibitors of tumor hepatocytes cells than monosaccharides [43]. In addition, another study showed that the antitumor activity of sugar esters formed from long-chain fatty acids (e.g., stearic, palmitic and myristic acid) were superior to those formed from medium-chain fatty acids (e.g., laurate and caprylate acid) [44].

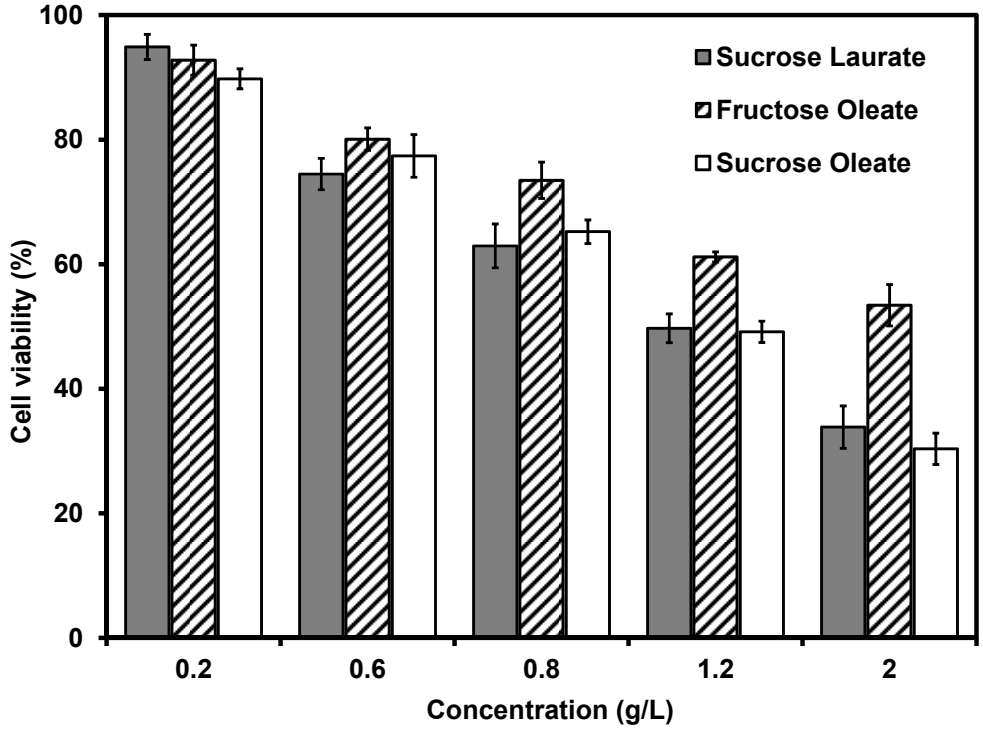

Figure 5. Antitumor activities of sugar fatty acid esters on ascitic tumor cells of Ehrlich carcinoma: sucrose laurate (black); fructose oleate (slash); and sucrose oleate (white).

3. Experimental Section

3.1. Materials and Methods

Novozym® 435, Lipase B from *Candida antarctica* (CALB) immobilized onto macroporous acrylic resin beads 10,000 propyl laurate unit (PLU)/g, where 1 PLU refers to the amount of enzyme which forms 1 μmol/min of propyl laurate during esterification at 60 °C, was kindly donated by Novozymes (Franklinton, NC, USA). Sucrose and fructose (>98% purity), acetone (high-performance liquid chromatography (HPLC)-grade), acetonitrile (HPLC-grade), methanol (HPLC grade, 0.04 *w*/*w* water content) and molecular sieves (Type 3A, 4–8 mesh, Grade 562) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Sucrose laurate was purchased from Modernist Pantry (purity > 99%, Portsmouth, NH, USA). Tween® 80, polyoxyethylene (20) sorbitan monooleate, was obtained from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was used throughout and all materials were used without further purification. Crude grades of sugar esters—sucrose oleate, consisting of 80.5 wt % ester (of which 92.5% is monoester and 7.5% diester), and 19.5 wt % oleic acid, and sucrose oleate, containing 83.4 wt % ester (91.2% monoester, 8.8% diester) and 16.6 wt % oleic acid—were synthesized under solvent-free conditions, using a bioreactor system, operated under continuous recirculation, with a PBBR containing RML [15]. Microorganisms and the Ehrlich ascites tumor cell line were purchased from American Type Culture Collection (Manassas, VA, USA).

3.2. Formation of Supersaturated Solutions of Size-Reduced Sucrose Using High Pressure Homogenization

Initially, sucrose and fructose crystals were carefully ground into a fine powder using a mortar and pestle, followed by milling at a rate of 3700 rpm and a radius of 4 cm (3383 L, Thomas Wiley® Mini-Mill, Thomas Scientific, Swedesboro, NJ, USA). The suspension of sucrose crystals in solvent-free media was formed by mixing 20.0 g sucrose or fructose fine powder and 100 g of a crude-grade sugar ester, serving as the initial reaction medium, and placed on the magnetic stirrer plate, operated at 400 rpm in a beaker of 100 mL and room temperature (23.5 ± 1.2 °C) for 5 min. Afterwards, the

mixture was processed by a high speed homogenizer (VWR PowerMax AHS from Henry Troemner, Thorofare, NJ, USA) for 1 min at 12,000 rpm (805 g). Then, the homogenized slurry was centrifuged at 800 rpm (71 g) for 0.5 min, to remove larger particles via sedimentation. The supernatant was collected. The temperature of the resultant suspension after removal from the centrifuge was 24 °C. The samples were then treated by high pressure homogenation according to previously published procedures [22]. In brief, the sample was subjected to HPH at 300 MPa using a high pressure valve homogenizer (Model FPG 12500, Stansted Fluid Power, Essex, UK). The homogenization chamber was cooled to 1 °C using a controlled temperature water bath (Isotemp 3016D, Fisher). In addition, a tubular heat exchanger was applied immediately after homogenization for maintaining the temperature of the samples at 24 °C.

3.3. Lipase-Catalyzed Sugar Oleate Synthesis Using Solvent-Free Suspensions

The reactions were carried out according to the previous method developed by our group [23]. In brief, a 100 mL of stirred tank bioreactor (STBR) containing the reaction mixture in the absence of CALB was placed in a sealed desiccator that contained $CaSO_4$ for 3 days at 350 rpm and 65 °C. Reactions were conducted in a 100 mL beaker on a 20 g scale in a sealed desiccator operated on a hot plate/stirrer at 350 rpm and 65 °C for 4 days. All reactions were performed in duplicate, with data reported in the figures representing average values ± standard deviation.

3.4. Composition of Reaction Medium

The water concentration for the reaction mixture was determined using a coulometric Karl-Fischer titrator (Denver Instrument Company, Aurora, CO, USA) in the presence of methanol, according to our previous paper [24]. The concentrations of oleic acid and sugar esters were measured by a HPLC system consisting of a dual-pump system from Agilent (Walnut Grove, CA, USA) and a model Mark III evaporative light scattering detector from WR Grace (Deerfield, IL, USA) with a reversed phase C18 column (4.6 mm × 250 mm, pore diameter 5 μm) from Grace at 25 °C. An isocratic solvent system comprising of acetone/acetonitrile/acetic acid (45/45/10 *v*/*v*/*v*) was delivered at flow rate of 1.0 mL· min^{-1} [24].

Prior to measure the saccharide content using HPLC, 40 mg-sized aliquots of reaction mixture were mixed with n-hexane and water (500 μL of each), based on our pervious method [15] at 35 °C for 2 h using a thermomixer (model 022670158, Eppendorf AG, Hamburg, Germany). An analytical Prevail Carbohydrate ES column (4.6 mm × 250 mm, pore diameter 5 μm) from Grace was used to measure the saccharide content at 25 °C with an isocratic solvent system containing acetonitrile/deionized water (80/20 *v*/*v*) at flow rate of 1 mL· min^{-1}. Standard curves for saccharide concentration in a fatty acid/saccharide-fatty acid esters liquid phase *versus* peak area were obtained and it is independent of the reaction mixture's composition.

3.5. Specific Gravity and Surface Tension

The specific gravity of sugar esters at 22 °C was measured by using a calibrated metal pycnometer. The surface tension (γ) of aqueous solutions of sucrose laurate, sucrose oleate and fructose oleate at several different concentrations (C) and 22 °C was measured by a dynamic contact angle analyzer (model DCA 312 from Cahn, Newington, NH, USA). All measurements were repeated until the difference between two values was less than 0.2 mN· m^{-1} at room temperature. Deionized water was used as a reference standard. Plot of C (log scale) *vs.* γ exhibit a linear decrease of γ *vs.* log C at low C, and a constant, minimal γ at high C. The intersection of lines for the two regions was used to determine the critical micellar concentration (CMC). The slope of the low-C region was used to calculate the surface excess (Γ) according to Gibbs Equation [34]:

$$\Gamma = -\frac{1}{RT}\frac{d\gamma}{d\ln C} \quad (1)$$

where R is the ideal gas law constant and T is the temperature. The specific surface area (A_s) is inversely related to Γ:

$$A_s = (\Gamma N_A)^{-1} \quad (2)$$

where N_A is Avogadro's number.

3.6. Emulsification Capacity and Stability

The emulsification capacity and stability were determined based on a previously published procedure with minor modifications [26]. Oil (hexadecane or dodecane, 1 mL) was vigorously mixed with 4 mL of 2 g/L aqueous surfactant solution (sucrose oleate, fructose oleate, sucrose laurate and Tween® 80), then allowed to settle in the absence of stirring for 20 min. The turbidity of the sample, reflecting the amount of oil emulsified, was measured as the absorbance at 600 nm *versus* time (1.0 cm path length) using a model UV-1700 spectrophotometer from Shimadzu (Columbia, MD, USA).

3.7. Antimicrobial Activity

Antimicrobial activities of commercial and enzymatically synthesized sugar fatty acid esters against test microorganisms were estimated by a published method [27]. Four bacteria commonly causing foodborne illness were selected in this study. For *Bacillus subtilis* and *Staphylococcus aureus*, a broth containing meat extract (1 g/L), yeast extract (2 g/L), peptone (5 g/L), and sodium chloride (5 g/L) was used. For *Lactobacillus plantarum* and *Pediococcus pentosaceus*, a broth containing meat peptone (15 g/L), sodium chloride (1 g/L), glucose (19 g/L), and glycerol (5 mL/L) was employed. Liquid medium was inoculated with microorganism and incubated for 24 h at 30 °C for *B. subtilis* and 37 °C for *L. plantarum*, *S. aureus* and *P. pentosaceus*, based on different optimum growth temperatures of microorganisms. Stock solutions of sugar esters were diluted to the desired concentration in the liquid media. For better solubility and dispersion of sugar esters, 0.1% Tween® 80 was added to the growth media. It was observed that the addition of 0.1% Tween® 80 to cultures had no marked effect on cell growth when compared to controls (data not shown). The assays were prepared in test tubes for overnight culture microorganism suspension in the presence or absence of the sugar esters solutions.

Antimicrobial activity was accessed by measuring the turbidity suspension (optical density; *OD*) of microorganisms through UV–vis spectrophotometry at 600 nm. A bacteria control without the addition of sugar ester was measured. An alcoholic control was prepared by addition of 1 mL of 96% ethanol instead of test sample solution and then inoculated with 1 mL bacterial suspension. The inhibition was calculated as the *OD* of the sample containing microorganism and sugar fatty acid ester at the defined time of measurement (OD_X) compared to the *OD* of the alcoholic control at defined time of measurement (OD_{AC}):

$$\text{Inhibition } (\%) = \left(\frac{OD_{AC} - OD_X}{OD_{AC}}\right) \times 100 \quad (3)$$

3.8. Antitumor Activity

Ascitic tumor cells of Ehrlich carcinoma were cultured in Roswell Park Memorial Institute (RPMI, Buffalo, NY, US) 1640 supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 25 mM), L-glutamine (2 mM), sodium bicarbonate (25 mM), 10% fetal bovine serum (FBS), 2-mercaptoethanol (50 μm) and antibiotics (100 International Units (IU)/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a 5% CO_2 incubator. Viability and cell density were determined by the trypan blue dye exclusion test [28].

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted based on a well-established method in literature [29]. Cells were transferred to 96 well plates with a cell density of ~2×10^5 mL^{-1} per well in the presence of 100 μL of RPMI 1640 in a CO_2 incubator for 24 h (37 °C, 5% CO_2). The medium was then removed and refilled with fresh medium including the different concentrations of sugar esters for 48 h. The cells were incubated for 24–48 h, followed

by the addition of 20 μL MTT, and stock solution (5 mg/mL in phosphate-buffered saline) to each well and incubation for 5 h. After the medium was removed, 200 μL of dimethyl sulfoxide was introduced into each well to dissolve the MTT metabolic product and the plate was shaken at 150 rpm for 5 min. The *OD* of the sample was measured at 600 nm. Untreated cells (basal) were used as a control (viability = 100%). Results are expressed as % viability related to the control:

$$Cell\ \ viability\,(\%) = (A\ \ treated/A\ \ untreated) \times 100 \quad (4)$$

where *A treated* and *A untreated* are the *OD* of the treated and untreated cells, respectively.

4. Conclusions

In this study, the ester content achieved previously for solvent-free immobilized *Rhizomucor miehei* lipase-catalyzed synthesis of sucrose oleate and fructose oleate (81%–83%) was increased to 90.0% and 96.6% ester in four days at 65 °C, respectively, by applying several innovative approaches. First, HPH was used to decrease the particle size of polysaccharides in the reaction medium to values <4 μm, leading to higher concentrations of suspended saccharide. Second, the water activity of the lipases' microenvironment was lowered by employing $CaSO_4$ to control the bioreactor's air headspace and using an immobilized lipase (from *Candida antarctica* B) possessing a hydrophobic matrix. The latter lipase altered the ester distribution in favor of diesters rather than monoesters, due to its regioselectivity. The resultant sugar esters lowered the surface tension to <30 mN/m, produced stable oil-in-water emulsions, and inhibited gram-positive foodborne pathogens and tumor cell growth to a similar extent as a commercially available sucrose ester. In conclusion, it was demonstrated that lipases can produce technical-grade biobased surfactants with utility in foods, cosmetics, personal care products, and pharmaceuticals under solvent-free conditions and in the absence of downstream purification.

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Abbreviations

The following abbreviations are used in this manuscript:

HIV	Anti-human immunodeficiency virus
FAO	Food and Agriculture Organization
HPH	High pressure homogenization
RML	*Rhizomucor miehei* lipase
CALB	*Candida antarctica* lipase B
PBBR	A packed-bed bioreactor
STBR	A stirred tank bioreactor
HLB	Hydrophilic–lipophilic balance
CMC	Critical micellar concentration
OD	Optical density
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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