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Chemically vs Enzymatically Synthesized Polyglycerol-Based Esters: A Comparison between Their Surfactancy

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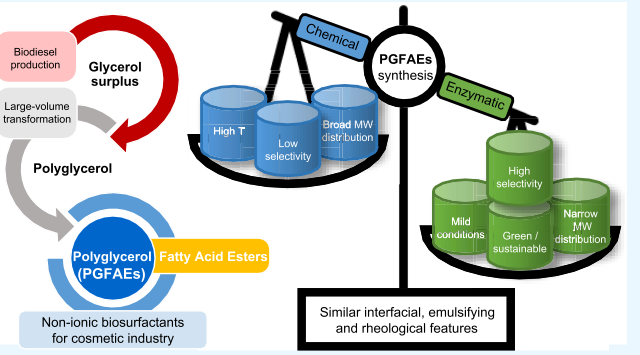
ABSTRACT: Polyglycerol fatty acid esters (PGFAEs) are gaining interest in several industrial sectors due to their excellent surfactant properties and their wide range of hydrophilic−lipophilic balance (HLB) values. Moreover, they can be prepared from renewable resources, i.e., fatty acids and glycerol. In this study, polyglycerol-2 stearic acid esters (PG2SAEs) were synthesized by the enzymatic esterification of polyglycerol-2 (PG2) and stearic acid (SA) using the immobilized lipase Novozym 435 as a biocatalyst in a solvent-free system. Reaction conditions, *i.e.*, temperature (80 °C), reactant ratio (1:1.8), and enzyme loading (2.7% w/w), were finely optimized; furthermore, biocatalyst recycling was studied by assessing the residual activity of the lipase after each reaction cycle, up to 20 times. The composition of the enzymatically synthesized products (**E**) was roughly evaluated by chromatographic methods and mass spectrometry and compared with that of the esters obtained by acid-catalyzed esterification (**C**). Then, the surfactant properties of the prepared polyglycerol-based surfactants were investigated by interfacial tension studies. Specifically, the emulsifying capacity and stability and the rheological behavior of O/W emulsions prepared in the presence of **E** were deeply investigated in comparison with those of the chemically synthesized and commercially available product **C**.



INTRODUCTION

By 2026, global glycerol production is expected to surpass 5 million tons due to the widespread adoption of biodiesel.[1] According to a report of Research & Markets, the global market demand for this chemical is estimated to reach just 4 million tons by 2027.[2] Thus, the large surplus accumulation of glycerol (about 10% by weight as an unavoidable byproduct of biodiesel production)[3] represents a major burden, both in environmental (since it cannot be simply discarded in the environment) and in economic (small biodiesel producers are forced to pay for incinerating it, since purification steps required for its selling are excessively expensive) terms.[4]

Much research effort has already been made in finding new large-volume applications for this compound, considering it a valuable cheap raw material within the context of a glycerol-based biorefinery. According to the literature, glycerol can be converted into valuable commodity chemicals through catalytic selective oxidation, dehydration, etherification, esterification, acetylation, halogenation, carboxylation, and other processes.[5] Among other transformations, etherification or polymerization is extremely promising because it yields polyglycerols (PGs), which are widely used as intermediates in the production of polyglycerol fatty acid esters (PGFAEs). These products are gaining prominence as non-ionic surfactants in cosmetic, pharmaceutical, and food industries because they display a broad range of polarity or hydrophilic−lipophilic balance (HLB) values. The polarity of each compound can be tuned by varying the fatty acid used for the esterification, the length of the PG chain, and the degree of esterification (ranging from monoesters to polyesters).[6] The possibility of controlling these parameters results in different products with multifunctional properties and a wide range of formulating options.[7] PGFAEs can be used as water-in-oil (W/O) or oil-in-water (O/W) emulsifiers in complex food systems such as margarines, cakes, creams, and toppings, as well as in the lubricant, polymer, and ink industries.[6,8,9] One particular class, specifically polyglycerol-2 fatty acid esters (PG2FAEs), brought a huge revolution in the cosmetic industry because its water absorption capacity helps reduce the fatty sensation produced by glycerol-based formulations.[10] Moreover, PG2FAEs can also increase the value of cosmetic formulations by forming a "mesh structure"

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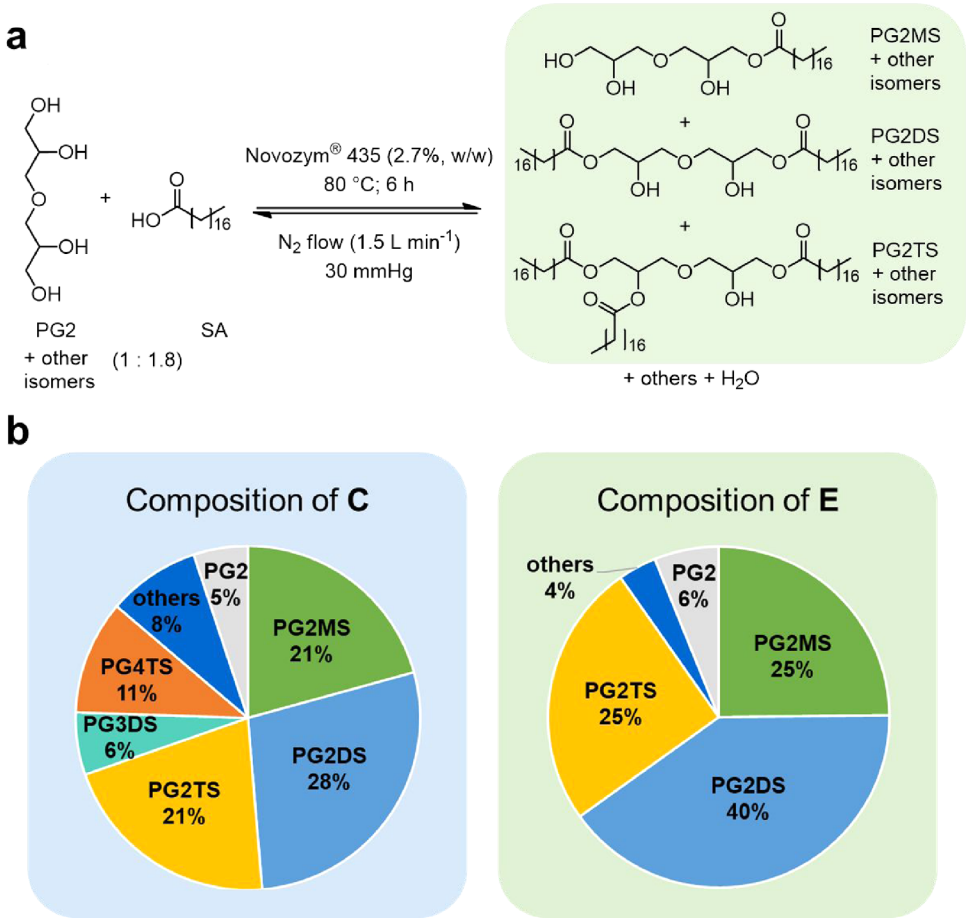

Figure 1. (a) Enzymatic synthesis of polyglycerol-2 stearic acid esters (**E**) by Novozym 435-catalyzed esterification of polyglycerol-2 (PG2) and stearic acid (SA). (b) Composition of polyglycerol-2 stearic acid esters obtained by acid-catalyzed (**C**) and enzymatic (**E**) esterification. Polyglycerol-2 mono-, di-, and stearates (PG2MS, PG2DS, PG2TS); polyglycerol-3 stearate (PG3DS); polyglycerol-4 stearates (PG4TS); other esters; and unreacted polyglycerol-2 (PG2) were detected.

that increases the viscosity of the system, generating emulsions with resistance to heat and stability over time.[6]

Nowadays, PGFAEs are industrially produced batchwise by direct esterification between PGs and a saturated fatty acid in solvent-free conditions. The process usually takes place in the presence of a homogeneous acid or alkaline catalyst (which has to be neutralized at the end of the reaction), at high temperatures (160−230 °C), and under reduced pressures in order to remove the water generated during the reaction.[6,11] However, the high temperatures and the use of a nonselective catalyst result in polycondensation reactions that tend to broaden the distribution of products.

On the other hand, PGFAEs can also be produced by enzymatic esterification catalyzed by a lipase. The reaction occurs under milder conditions (80 °C), does not require a neutralization step, and it is characterized by higher selectivity, which leads to fewer side reactions and a simplified product mixture. Moreover, if a supported enzyme is used as a biocatalyst, it can be easily separated from products and recycled.

Recently, the exploitation of lipases for industrial purposes is receiving increasing attention due to their huge potential as catalysts to produce a multitude of chemicals. Specifically, Novozym 435, a commercially available lipase from *Candida antarctica* type B and immobilized on an acrylic support, is widely used in many industrial processes thanks to its high regioselectivity and thermal stability, as well as a relaxed substrate specificity.[12] Regarding the cosmetic industry, lipases can be considered key enzymes: apart from water, esters are often the most used ingredients in a cosmetic product since they can be applied as emulsifiers, emollients, thickeners, detergents, *etc.* in different cosmetic formulations.[13]

Indeed, the use of Novozym 435, to yield PGFAEs and other industrially relevant esters in a more sustainable way, has been already reported.[14−16] However, to the best of our knowledge, none of the studies deeply investigated how the use of an immobilized enzyme can affect the production process. To date, the implementation of enzymes has not been affordable in many industrial contexts, specifically for cosmetic industries, which still rely on the cheaper homogeneous catalysis for the production of PGFAEs.[13,17−19]

Nevertheless, during the last few years, sustainability has been increasingly perceived as a key factor by the final consumer, especially in the cosmetic field. Therefore, it has become of paramount importance to conduct in-depth studies on the reusability of immobilized enzymes for the synthesis of PGFAEs in order to improve the sustainability of their production at the industrial scale.

In this paper, the optimum reaction conditions for the enzymatic esterification of polyglycerol-2 (PG2) with stearic acid (SA) in the presence of Novozym 435 were investigated along with the recovery and recycling of the immobilized

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a

Number of usage cycles	U / (μmol min^{-1} g^{-1})
Fresh	3700 ± 400
Washed	2800 ± 500
0	2600 ± 100
1	2800 ± 300
2	2490 ± 70
3	1920 ± 60
4	2400 ± 300
5	2000 ± 600
6	2400 ± 500
7	2600 ± 200
11	2300 ± 100
16	2230 ± 70
20	2200 ± 100

b

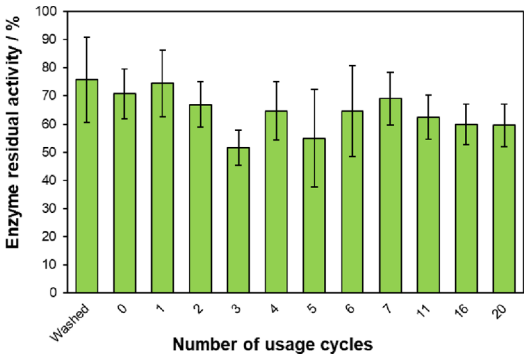

Figure 2. Reusability of Novozym 435. (a) Units of enzyme activity (U) and; (b) percentage of enzyme residual activity according to the number of usage cycles. All of the values are the means of three replicates. Error bars represent the standard deviation within the data set.

enzyme. Moreover, the product mixture obtained by the enzymatic approach (**E**) was characterized and compared to the one yielded through the classical approach based on homogeneous catalysis (**C**). Finally, in order to assess the physicochemical properties of both products, almond oil/water interfacial tension studies were performed, standard formulations were generated, and their stability was evaluated in detail.

RESULTS AND DISCUSSION

Optimization of Reaction Conditions. Reaction conditions for the synthesis of polyglycerol-2 stearic acid esters (PG2SAEs, **E**) were initially selected on the basis of literature data.[10,15,20] Specifically, a mixture of polyglycerol-2 (PG2)/stearic acid (SA) (molar ratio 1:1.5) and 3% (w/w) immobilized lipase (Novozym 435) was used at 80 °C, under a N_2 flow (1.5 L min^{-1}) and reduced pressure conditions (30 mmHg). However, in such reaction conditions, an accumulation of a significant amount of unreacted PG2 was observed at the bottom of the reaction flask, thus affecting the homogeneity of the product. To overcome this drawback, a series of experiments was planned, by keeping fixed the N_2 flow and the pressure, while varying the molar ratio of reagents, the temperature, and the biocatalyst amount, in order to find the optimal operative conditions.

Effect of the Molar Ratio of Reactants. It is worth noting that in the initially selected operating conditions, the reaction was considered completed when the mixture reached an acid value lower than 5 mg_{KOH} g^{-1}, which corresponds to an almost total consumption of SA. Thus, the observed unreacted PG2 could be attributed to a preference of the enzyme for the already esterified PG2 with respect to the underivatized one. When the molar ratio between the reagents was increased from 1:1.5 to 1:1.8, keeping the remaining parameters constant, unreacted PG2 did not accumulate at the bottom of the flask anymore. In this way, a homogeneous final product was obtained.

Effect of Temperature. Since the reaction is performed under vacuum in a solvent-free medium, the minimum reaction temperature to ensure complete SA melting (mp 69.3 °C) was found to be 80 °C. According to the producer, the immobilized lipase Novozym 435 works across a wide temperature range (20−110 °C).[21] However, in our hands, attempts to increase the temperature to 90 °C, in order to increase the reaction rate, resulted in partial inactivation of the enzyme, as indicated by the complete loss of activity after the enzyme recovery and recycling. Therefore, 80 °C was maintained as the optimal working temperature.

Effect of the Enzymatic Catalyst Concentration. In the enzymatic synthesis of PG2SAEs (**E**), the immobilized biocatalyst is the most expensive material. From an industrial point of view, it is mandatory to study the influence of its amount on the reaction outcome, *i.e.*, to find the minimum quantity needed, thus reducing the overall production costs. To this aim, several experiments were performed by varying the enzyme amount (from 2.5 to 3.0%, w/w) on the basis of the time required for the completion of the esterification reaction. The optimal amount of Novozym 435 to complete the reaction in 6 h was found to be 2.7% (w/w); see Figure 1a.

Composition of Ester Mixtures (E and C). With the aim to define if the enzymatic approach could represent a valid alternative to the classical acid-catalyzed approach, in terms of both production and final application, for the preparation of PG2SAEs, it is important to compare the composition of the product mixtures obtained through the two different routes. Thus, to establish the compositions of both the enzymatic (**E**) and the commercial (**C**) product, they were submitted to purification by flash chromatography. Due to the complexity of both of the product mixtures, multiple purification steps were carried out and each isolated fraction was submitted to ESI-MS analysis. Figure 1b depicts the noticeable differences between the two products: **E** is mainly constituted by PG2 monostearate (PG2MS), PG2 distearate (PG2DS), and PG2 tristearate (PG2TS) in a relative ratio of roughly 3:4:3, respectively; whereas **C** shows a more complex profile. In the commercial product (**C**), the relative ratio among PG2MS, PG2DS, and PG2TS is similar to that of **E**. However, other ester products with higher MWs were present; in particular, polyglycerol-3 distearate (PG3DS, 6% w/w) and polyglycerol-4 tristearate (PG4TS, 11% w/w) were identified. This different product distribution profile is mainly due to the nature of the catalyst used, as well as the temperature set during the reaction. Indeed, the immobilized lipase from *Candida antarctica* type B is extremely regioselective for primary hydroxyl functions. Since PG2 is a mixture of structural isomers (the ether bond between the two condensed glycerol molecules can occur between positions 1−1, 1−2, or 2−2), the esterification reaction can occur up to 4 times per PG2 molecule, depending on the isomer, and leading mainly to PG2DS for statistic reasons. On the contrary, the use of a nonselective homogeneous acid catalyst and the higher temperature set in the commercial approach can induce condensation or polymerization reactions of PG2, which lead to the formation of PG3- and PG4-based species. Moreover, unlike the enzymatic reaction, the acid catalysis could promote the

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formation of esters on secondary hydroxyl groups, as corroborated by the comparison of the GC chromatograms of **C** and **E** (see the Supporting Information, Figure S17). Indeed, from the qualitative point of view, the chromatographic analyses disclosed a higher number of species found in **C**, with respect to **E**, insofar as mono- and diesters are concerned.

Enzyme Recycling. A key advantage of using immobilized enzymes is their better overall stability (in terms of both storage and operational stability against denaturation by heat or organic solvents) in comparison to free enzymes. In addition, the efficient recovery of products enabled by their use and their easy recycling for several batches result in increased catalytic productivity.[22] To fully exploit these strengths and to lower the production costs, it is necessary to investigate the reusability of the immobilized enzyme. Thus, we planned a series of recycling experiments to find the best procedure to recover the biocatalyst, as well as a quick and easy assay to evaluate the residual enzymatic activity. This latter parameter, defined as the unit of enzyme activity (*U*, Figure 2a) and the percentage of residual activity (Figure 2b) of the recycled immobilized enzyme with respect to fresh Novozym 435, was determined according to the Aguieiras modified method.[23]

Optimization of the Recycling Procedure. As a first approach, recovery of the product mixture was performed by filtration. The filtrated enzyme beads were then submitted to a washing procedure before recycling, using a proper solvent to remove the traces of products and unreacted materials from the biocatalyst. According to the literature, EtOH easily dissolves unreacted PG2 trapped in enzyme beads,[23] as well as stearic acid and the esters produced. However, in our hands, the lipase esterification activity dropped after using this solvent in the washing procedure. Our findings agree with those reported by Chen and Wu, who observed that linear alcohols are toxic to the immobilized lipase, with the degree of deactivation inversely proportional to the number of carbon atoms.[24] Different studies suggest the use of solvents other than EtOH for the washing procedure, *i.e.*, *n*-hexane,[25] *n*-BuOH,[23] or *t*-BuOH.[26] However, according to the principles of green chemistry, organic solvents are undesired in green processes and, moreover, they tend to induce partial enzyme deactivation. Therefore, an alternative method of enzyme recycling was investigated: at the end the reaction, Novozym 435 was recovered by simple decantation and it was stored in the minimum amount of product mixture without any further washing procedure. This strategy appeared effective in maintaining the lipase activity, as determined by activity assays. Indeed, the successive reactions were carried out without increasing the reaction time (6 h).

Reusability of the Biocatalyst. Once the optimal reaction conditions, the recycling procedure, and the assay to evaluate the residual activity of the enzyme were established, the optimal number of usage cycles was studied. At the end of each cycle, a small amount of the used Novozym 435 was taken up from the reaction mixture and its esterification residual activity was evaluated. According to the modified Aguieiras method, the enzyme beads must be washed before performing the assay to remove traces of unreacted PG2 and SA. To this aim, a solvent mixture of *n*-BuOH/*n*-hexane 1:1 was selected, and a possible enzyme partial denaturation due to the treatment with solvents was investigated. Using fresh Novozym 435 as the reference, a 25% reduction in lipase residual activity just as a result of washing was observed; see Figure 2a. Therefore, Novozym 435 was submitted to 20 consecutive reaction cycles, and only 15% reduction (Figure 2b) in its residual activity was observed (40% considering the loss of activity intrinsically due to the chosen assay). Furthermore, the reaction times of each cycle remained constant (6 h), corroborating the previous results.

Interfacial Properties and Evaluation of Emulsion Stability. To unravel the interfacial features of the PG2SAE obtained through enzymatic synthesis and to assess its performances with respect to the commercial surfactant, IFT measurements were performed at different concentrations according to the Du Noüy ring tensiometric method.[27,28] As displayed in Figure 3a, the trend is quite similar, showing a constant IFT decrease (down to ≈2 mN m^{-1}) from 0.25 g L^{-1} for the **C**-based system and an abrupt reduction at around 0.37 g L^{-1} for the **E**-based emulsion. Literature data about IFT

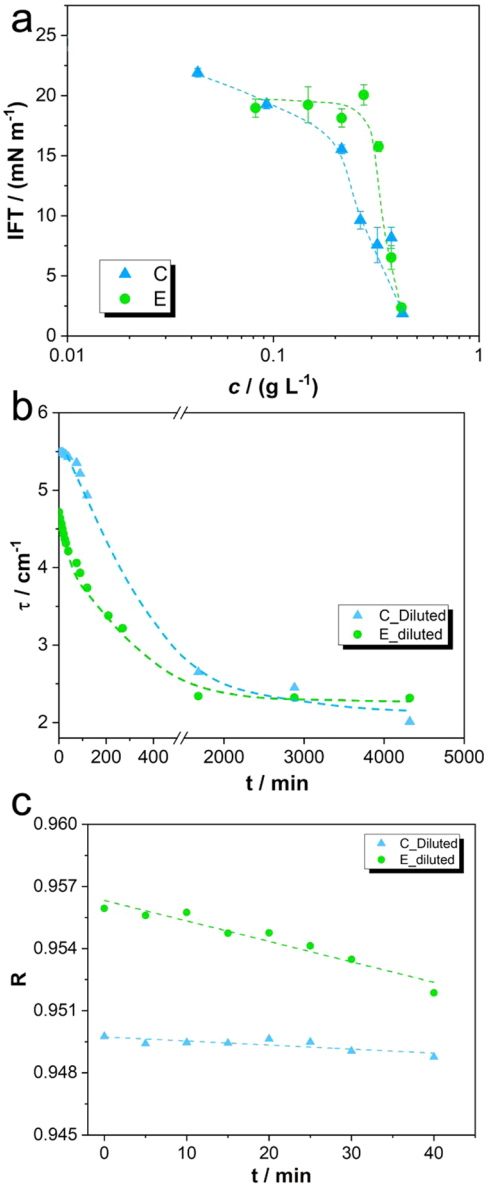

Figure 3. (a) Oil/water interfacial tension (IFT) trends in the presence of **C** and **E** surfactants. (b) Turbidity trend over time for both **C**- and **E**-based diluted emulsions. (c) Corresponding turbidity ratio (*R*) values within the first 40 min.

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measurements of PGFAEs are very scarce; indeed, most of them[29−31] are related to surface tension measurements of polyglycerol esters (ranging from diglycerol to octaglycerol) having diverse saturated and unsaturated fatty tails (derived from, e.g., lauric, palmitic, oleic, and linoleic acids). Specifically, a surface tension value of around 27 mN m^{-1} was obtained for both polyglycerol-2 monooleate[29] and polyglycerol-2 monolaurate.[30] Only in the case of polyglycerol-2 monooleate,[29] IFT was evaluated (5.5 mN m^{-1} at 0.05 g L^{-1}). In the present work, the minimum IFT value for both **C** and **E** resulted to be around 2 mN m^{-1} at a concentration 1 order of magnitude higher with respect to those of the above-mentioned papers,[29,30] probably due to the higher complexity of the surfactant mixture. This effect is consistent with the data of Charlemagne and Legoy,[31] that, by testing a mixture of polyglycerol-2 monooleate and dioleate, obtained a surface tension of 30 mN m^{-1} at 0.1 g L^{-1} (double than that of single polyglycerol-2 monooleate).[29] Indeed, our study does give new insights into the interfacial behavior of these compounds. Moreover, considering the similar interfacial properties obtained for **E** and **C**, it is possible to assume that polyglycerol-2 monostearates (PG2MS) and tristearates (PG2TS) might play a greater role in reducing the IFT, being present in similar ratios within the mixtures (see Figure 1b). Thus, the other species having higher molecular weights (corresponding to 4 and 23% of **E** and **C**, respectively) may be unlikely to affect the interfacial behavior.

To further corroborate the previous outcomes, emulsifying properties and the resulting O/W emulsion stability over time were finely evaluated by turbidity tests, optical microscopy images, and rheological measurements.[22,27,28,32] In particular, the turbidity change over time has been already reported to be strongly linked to emulsion-breaking phenomena since this parameter decreases at each wavelength with the occurrence of destabilization.[33,34] Since the turbidimetric method is based on UV−vis spectroscopy, dilution was necessary to make the measurements more reliable being the starting emulsions very milky, as already reported.[33] As shown in Figure 3b, the diluted emulsions exhibit a small destabilization degree probably due to the Ostwald ripening mechanism leading to the formation of larger oil droplets. Moreover, by computing the turbidity ratio (R) within the first 40 min, the quantitative assessment of the emulsion instability was made (see Figure 3c). Notably, R decreases faster in the presence of an unstable emulsion.[33] However, in the present study, the slopes of the turbidity ratios are minimal (10^{-5} min^{-1} for the **C**-based system vs 10^{-4} min^{-1} for the **E**-based one), highlighting a good stability even if a slightly worse behavior was observed for the enzymatic surfactant-based emulsion. By means of confocal microscopy, the droplet size was evaluated resulting in the observation of a quite homogeneous distribution of micrometric droplets for all of the investigated systems (Figure 4a,c), appearing slightly smaller in the case of commercial surfactant-based emulsions (<5 μm for the **C**-emulsion vs <10 μm for the **E**-based system; Figure 4a). Conversely, after an accelerated aging test made of three consecutive hot−cold cycles, oil droplets became bigger (around 20−30 μm), as clearly visible in Figure 4b,d. In addition, to further unveil the stability of the systems, the creaming index (CI) was determined after the aging tests, giving almost the same CI percentage of about 12% (Figure 4e) for both **C**- and **E**-based emulsions, comparable to the recent results in the case of similar systems.[35,36]

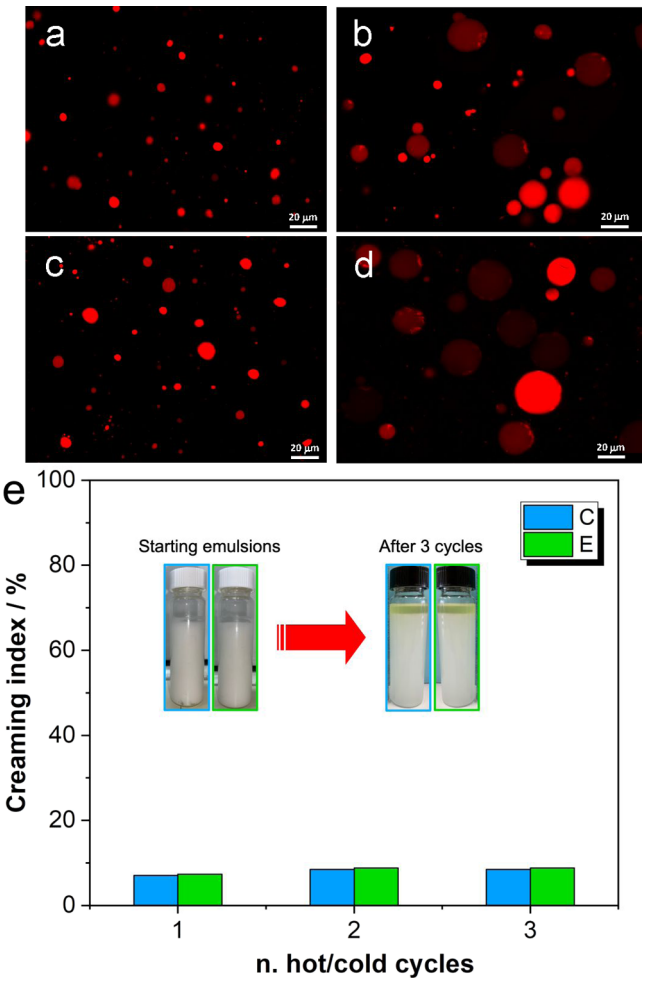

Figure 4. Confocal microscopy images relative to fresh (a) **C**-based, (c) **E**-based and aged (b) **C**-based and (d) **E**-based emulsions. The aging process constituted by three hot−cold aging cycles (bar: 20 μm). (e) Creaming index relative to **C**- and **E**-aged emulsions (inset: photos of fresh and aged systems).

Finally, with respect to the rheological features, shear stress vs shear rate curves (Figure 5a) display a nonlinear viscoplastic behavior for all of the studied emulsions. Figure 5b illustrates the viscosity as a function of the shear rate resulting in a significant increase of η at low D values. Thus, the studied systems are characterized by a shear-thinning behavior, which is reported to be specific of emulsions, gels, and polymer solutions, and it is generally considered to be the result of microscale structural rearrangements within the fluid itself.[32] Then, by using Casson's equation (eq 1), yield stress (τ_c) values[37] were computed: this parameter has a fundamental role in structured fluids because a higher τ_c value helps prevent phase separation, sedimentation, or emulsion aggregation.

To our best knowledge, no rheological data concerning similar emulsions stabilized by PGFAE surfactants are reported in the recent literature. However, the calculated τ_c values (around 2 Pa) are comparable to those of other emulsion systems. Specifically, Zadymova et al.[37] reported a τ_c value of 0.87 Pa for heavy oil emulsions characterized by a phase volume (ϕ_V) of 0.21 in the presence of 5% Tween 80 surfactant. Moreover, a similar value (τ_c = 0.90 Pa) was obtained in the case of a sunflower oil/water emulsion (ϕ_V = 0.30) stabilized by gelatinized groundnut flour (5%).[38]

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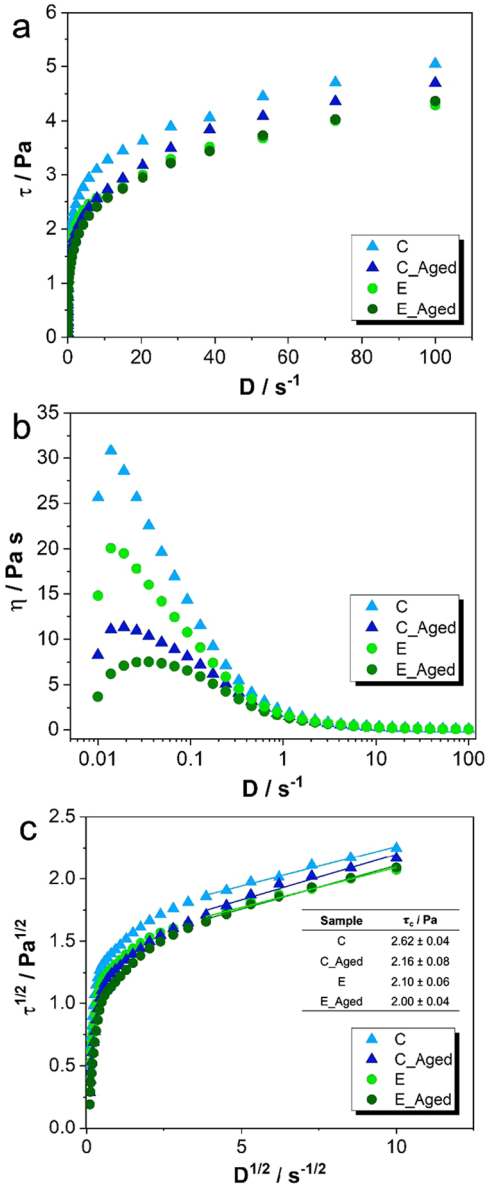

Figure 5. (a) Shear stress vs shear rate trend relative to both fresh and aged **C**- and **E**-based emulsions. (b) Shear viscosity vs shear rate for all of the investigated systems. (c) Casson's plot and computed yield stress (τ_c) values in the table in the inset.

Furthermore, in the present case, a slightly higher τ_c value (2.6 Pa) was obtained for the **C**-based system with respect to the **E**-based one (2.1 Pa; see the table in the inset of Figure 5c), and this might be due to the greater complexity of the **C** sample, composed of several polyglycerol-2 stearic acid esters having an overall lower apolar character with respect to the **E** one. For this reason, the final O/W emulsion, whose stability should theoretically be greater using tensides with high HLB, may turn out to be more stable. On the contrary, after the hot−cold cycles, we observed a higher τ_c reduction for the **C**-based formulation witnessing a possible emulsion destabilization, and this is probably a consequence of the partial solubility loss of some of its components (hypothetically those with a higher molecular weight, as PG3DS and PG4TS). Instead, since the **E**-based sample is less complex in structure, it seems to be slightly more stable after the aging test.

CONCLUSIONS

The results obtained within this study indicate that the enzymatic approach, based on the use of Novozym 435, can be a valid alternative to the commonly used acid catalysis for the esterification reactions of PG2 with SA, allowing one to work under milder reaction conditions and improving the sustainability of the process. Moreover, the reusability of the biocatalyst (up to 20 times), by storing it in the minimum amount of reaction mixture, permits a significant reduction of the production costs, thus confirming the potential advantage of this enzymatic route for industrial applications. Finally, the physicochemical study highlights the comparability of the interfacial features, the emulsifying capacity, and the rheological behavior of the disperse systems prepared in the presence of the two polyglycerol-based tensides (**E** and **C**). These findings suggest that the enzymatically obtained product (**E**) represents a potential candidate to substitute the currently used one (**C**), in terms of both sustainable production and final application.

METHODS

Materials and Methods. Diglycerol (or polyglycerol-2, PG2, purity >90%, with traces of glycerol, triglycerol, and tetraglycerol) was supplied by Inovyn (United Kingdom), and stearic acid (SA, purity >98%) was supplied by Giomavaro (Italy). All other chemicals and solvents were purchased from Sigma Aldrich, Fluka, Titolchimica (Italy). Polyglycerol-2 stearic acid esters (PG2SAEs) produced through acid-catalyzed esterification (**C**) by Res Novare (Italy) were used as reference samples. The immobilized lipase from *Candida antarctica* type B, Novozym 435 (declared enzymatic activity: 5000 LU g^{-1}), was kindly provided by Novozymes (Denmark). Silica gel 60, 40−63 μm (Merck), was used for flash column chromatography. Analytical thin-layer chromatography (TLC) was performed on silica gel F_{254} precoated aluminum sheets (0.2 mm layer, Merck, Germany); components were detected by spraying with a $Ce(SO_4)_2/(NH_4)_6Mo_7O_{24} \times 4H_2O$ solution, followed by heating at 150 °C. Electrospray ionization mass spectroscopy (ESI-MS) analysis was recorded on a Thermo Finnigan LCQ Advantage spectrometer (United Kingdom).

Enzymatic Synthesis of Polyglycerol-2 Stearic Acid Esters: PG2SAEs (E). Polyglycerol-2 (PG2, 1 mol), stearic acid (SA, 1.8 mol), and Novozym 435 (2.7%, w/w) were added to a three-necked 250 mL flask. The system was heated to 80 °C, stirred by a N_2 flow (1.5 L min^{-1}) passing through a capillary and maintained under reduced pressure conditions (30 mmHg) using a vacuum pump (KNF laboport N840.3FT.18, Switzerland) (Figure 1a). The reaction was monitored at 30 min intervals by 0.1 M KOH titration: a sample of the product mixture (0.5 g) was dissolved in EtOH/*i*-PrOH at the ratio of 85:15, and phenolphthalein was used as a colorimetric indicator. The acid value (mg_{KOH} g^{-1}), defined as the quantity in milligrams of KOH required in order to neutralize the free acids present in 1 g of the substance,[39] was determined. The reaction was stopped at an acid value lower than 5 mg_{KOH} g^{-1}. The immobilized enzyme was recovered by decantation and stored in a minimum amount of product mixture for recycling.

Chromatographic Analysis. The product mixture obtained by the enzymatic approach (**E**), as well as the commercial one produced by chemical methods (**C**), was

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submitted to flash chromatography using *n*-hexane containing an increasing amount of EtOAc and MeOH as eluents (*n*-hexane/EtOAc, 9:1 (2 column volumes (CVs)); *n*-hexane/EtOAc, 7:3 (2 CV); *n*-hexane/EtOAc, 1:1 (2 CV); *n*-hexane/EtOAc, 3:7 (2 CV); *n*-hexane/EtOAc, 1:9 (2 CV); MeOH/EtOAc, 2:8 (2 CV)). Each isolated fraction was submitted to ESI-MS analysis to roughly define the percentage composition of the mixtures (Figure 1b). Further flash chromatographic purifications were carried out on isolated fractions considered not sufficiently pure; see the Supporting information. Moreover, the GC analysis of products **E** and **C** was carried out on an Agilent 7890N (Germany) equipped with an on-column injector and a Zebron ZB-1HT Inferno column, 30 m, 0.25 mm, 0.25 mm (Phenomenex). Before injection, the mixtures were derivatized by using *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)[40] and analyzed with the following temperature program: 15 min at 250 °C, heating at 5° min^{-1} up to 340 °C and 40 min at 340 °C.

Residual Activity of Novozym 435. The lipase esterification residual activity was evaluated according to the Aguieiras method,[23] with slight modifications based on the reaction proposed by Shankar and co-workers.[41] Briefly, lauric acid (1 mmol) and *n*-BuOH (1.4 mmol) were stirred at 50 °C under anhydrous conditions; then, recycled Novozym 435 (3%, w/w), previously washed with *n*-BuOH/*n*-hexane at a 1:1 ratio (50 mL), was added under magnetic stirring, in the presence of molecular sieves (10%, w/w). After 15 min, the amount of unreacted lauric acid was determined by titration with 0.04 M NaOH; specifically, a sample of the reaction mixture (0.1 mL) was dissolved in acetone/EtOH at a 1:1 ratio (40 mL), and phenolphthalein was used as a colorimetric indicator. The unit of enzyme activity (U) is defined as the amount of enzyme needed to consume 1 μmol of lauric acid per minute.[23] Fresh Novozym 435 was used as a standard, showing the maximum lipase esterification activity. Both titrations and reactions were performed in triplicates.

Interfacial Tension (IFT) Measurements. The almond oil/water interfacial tension (IFT) values were measured at (25 ± 1) °C by means of a Gibertini tensiometer following the Du Noüy ring method and by varying the amount (in the range between 0.04 and 0.40 g L^{-1} in oil) of PG2SAE obtained from chemical (**C**) and enzymatic (**E**) syntheses.[27,28] Data were reported as average values on three different replicates. Prior to tensiometric measurements, several parameters were introduced in the relative software to set up the method, such as liquid density, platinum ring, and wire radii, required by the Harkins−Jordan correction.

Emulsion Formation. Almond oil-in-water (Milli-Q) (O/W) emulsions were prepared using a Silverson Rotor-stator L5M-A, equipped with a square hole head. To preliminarily assess the emulsifying properties of both commercial and enzymatically obtained surfactants, a basic emulsion recipe was adopted. In particular, the surfactant (**C** or **E**) was dissolved at 80 °C in almond oil (15% w/w), whereas a mixture of glycerol (4.3% w/w) and xanthan gum (0.4% w/w; as the rheological modifier) was added in water (Milli-Q). Glycerol was used to help the xanthan gum solubilize in water. Then, the oil phase was added to the water phase and homogenized at 3000 rpm for 5 min to obtain a phase volume (Φ_V) of 0.08.

Turbidimetry Measurements. The emulsion stability within 72 h was assessed by the turbidimetric method,[27,28] following the absorbance value at 550 nm (by means of a Shimadzu UV−Vis spectrophotometer UV-2600) of both the as-prepared samples (**C** and **E**) and the 10-fold Milli-Q water-diluted ones (**C** diluted and **E** diluted). Moreover, the turbidity ratio (R), defined as the ratio of turbidity at high and low wavelengths (800 and 450 nm), was calculated. Indeed, R is a useful, rapid, and simple method to evaluate the stability of an emulsion against sedimentation. Subsequently, the slope of the turbidity ratio over time was calculated within 50 min.

Hot−Cold Cycles. Emulsion stability was further evaluated by aging tests, *i.e.*, five consecutive hot−cold cycles (24 h each) at 50/4 °C. Averaged droplet sizes of fresh (**E** fresh and **C** fresh) and aged (**E** aged and **C** aged) emulsions were acquired by using the Nikon A1 laser scanning confocal microscope (LSCM) working in an oil immersion (NA1.4) equipped with a 60× objective. Before each analysis, emulsions were stained with a dye, and images were acquired with an excitation wavelength of 561 nm, and the emitted signal was detected between 770 and 620 nm.

The creaming destabilization kinetics were evaluated by measuring the creaming index (CI) as the percentage of destabilized layer height (upper part) from the total emulsion height.[36]

Rheological Features. Rheological studies of emulsions (the two fresh emulsions, **E** and **C**, and the corresponding aged ones) were performed on a Modular Compact rheometer by Anton Paar, equipped with a cell P-PTD200/AIR SN82424770 and a cone plate CP50-1 (diameter: 49.981 mm; cone angle: 1.004°; cone truncation: 103 μm). All of the experiments were performed with a constant strain of 5%, in the range of frequency sweep 0.1−100.0 Hz, at 25 °C. Then, the shear rate (τ) and viscosity (η) vs shear stress (D) were obtained. Since the τ vs D curves showed a nonlinear behavior, the yield stress (τ_c) was computed according to Casson's equation[37] (eq 1)

$$\tau^{1/2} = \tau_c^{1/2} + \eta^{1/2}\cdot(\mathrm{d}\gamma/\mathrm{d}t)^{1/2} \quad (1)$$

where η is the apparent viscosity dependent on the shear rate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02922.

Mass spectrometry analysis of fractions isolated from flash chromatography of the product mixture obtained by enzymatic and chemical approaches; and gas chromatographic analysis comparison (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

η, shear viscosity; τ, shear stress/turbidity; D, shear rate; ESI-MS, electrospray ionization mass spectrometry; EtOAc, ethyl acetate; EtOH, ethanol; GC, gas chromatography; HLB, hydrophilic−lipophilic balance; *i*-PrOH, 2-propanol; IFT, interfacial tension; KOH, potassium hydroxide; MeOH, methanol; *n*-BuOH, 1-butanol; NaOH, sodium hydroxide; O/W, oil-in-water; PG, polyglycerol; PG2, polyglycerol-2; PG2DS, polyglycerol-2 distearate; PG2MS, polyglycerol-2 monostearate; PG2TS, polyglycerol-2 tristearate; PG2SAE, polyglycerol-2 stearic acid ester; PG3DS, polyglycerol-3 distearate; PG4TS, polyglycerol-4 tristearate; PGFAE, polyglycerol fatty acid ester; SA, stearic acid; TLC, thin-layer chromatography; W/O, water-in-oil

■ REFERENCES

(1) Ladero, M. New Glycerol Upgrading Processes. *Catalysts* **2021**, *11*, No. 103.
(2) BW Businesswire. Global Glycerol Industry (2020 to 2027) - Market Trends and Drivers. https://www.businesswire.com/news/home/20210115005399/en/Global-Glycerol-Industry-2020-to-2027---Market-Trends-and-Drivers---ResearchAndMarkets.com (accessed March, 2023).
(3) Quispe, C. A. G.; Coronado, C. J. R.; Carvalho, J. A., Jr. Glycerol: Production, consumption, prices, characterization and new trends in combustion. *Renewable Sustainable Energy Rev.* **2013**, *27*, 475−493.
(4) Gholami, Z.; Abdullah, A. Z.; Lee, K.-T. Dealing with the surplus of glycerol production from biodiesel industry through catalytic upgrading to polyglycerols and other value-added products. *Renewable Sustainable Energy Rev.* **2014**, *39*, 327−341.
(5) Zheng, Y.; Chen, X.; Shen, Y. Commodity Chemicals Derived from Glycerol, an Important Biorefinery Feedstock. *Chem. Rev.* **2010**, *110*, No. 1807.
(6) Norn, V. Polyglycerol Esters. In *Emulsifiers in Food Technology*, 2nd ed.; John Wiley & Sons, Ltd., 2015; pp 181−207.
(7) Clacens, J.-M.; Pouilloux, Y.; Barrault, J. Selective etherification of glycerol to polyglycerols over impregnated basic MCM-41 type mesoporous catalysts. *Appl. Catal., A* **2002**, *227*, 181−190.
(8) D'Avico, L.; Beltrami, R.; Pargoletti, E.; Trasatti, S. P. M.; Cappelletti, G. Insight into the release agents/PVD coatings interaction for plastic mold technology. *Coatings* **2020**, *10*, No. 281.
(9) Lotierzo, A.; Pifferi, V.; Ardizzone, S.; Pasqualin, P.; Cappelletti, G. Insight into the role of amines in Metal Working Fluids. *Corros. Sci.* **2016**, *110*, 192−199.
(10) Martínez, M.; Oliveros, R.; Aracil, J. Synthesis of Biosurfactants: Enzymatic Esterification of Diglycerol and Oleic Acid. 1. Kinetic Modeling. *Ind. Eng. Chem. Res.* **2011**, *50*, 6609−6614.
(11) Garti, N.; Aserin, A.; Zaidman, B. Polyglycerol esters: Optimization and techno-economic evaluation. *J. Am. Oil Chem. Soc.* **1981**, *58*, 878−883.
(12) Stauch, B.; Fisherc, S. J.; Cianci, M. Open and closed states of *Candida antarctica* Lipase B: protonation and the mechanism of interfacial activation. *J. Lipid Res.* **2015**, *56*, 2348−2358.
(13) Ansorge-Schumacher, M. B.; Thum, O. Immobilised lipases in the cosmetics industry. *Chem. Soc. Rev.* **2013**, *42*, 6475−6490.
(14) Peng, B.; Xiong, C.-Y.; Huang, Y.; Hu, J.-N.; Zhu, X.-M.; Deng, Z.-Y. Enzymatic Synthesis of Polyglycerol Fatty Acid Esters and Their Application as Emulsion Stabilizers. *J. Agric. Food Chem.* **2018**, *66*, 8104−8113.
(15) Wan, F. L.; Teng, Y. L.; Wang, Y.; Li, A. J.; Zhang, N. Optimization of oligoglycerol fatty acid esters preparation catalyzed by Lipozyme 435. *Grasas y Aceites* **2015**, *66*, No. e088.
(16) Todea, A.; Aparaschivei, D.; Badea, V.; Boeriu, C. G.; Peter, F. Biocatalytic Route for the Synthesis of Oligoesters of Hydroxy-Fatty acids and ϵ-Caprolactone. *Biotechnol. J.* **2018**, *13*, No. 1700629.
(17) Chandra, P.; Enespa; Singh, R.; Arora, P. K. Microbial lipases and their industrial applications: a comprehensive review. *Microb. Cell. Fact.* **2020**, *19*, No. 169.
(18) Houde, A.; Kademi, A.; Leblanc, D. Lipases and their industrial applications. *Appl. Biochem. Biotechnol.* **2004**, *118*, 155−170.
(19) DiCosimo, R.; McAuliffe, J.; Poulose, A. J.; Bohlmann, G. Industrial use of immobilized enzymes. *Chem. Soc. Rev.* **2013**, *42*, 6437−6474.
(20) García, E.; Ferrari, F.; García, T.; Martínez, M.; Aracil, J. Optimization of the enzymatic esterification of diglycerol and lauric acid. *J. Surfactants Deterg.* **2001**, *4*, 257−262.
(21) Ortiz, C.; Ferreira, M. L.; Barbosa, O.; dos Santos, J. C. S.; Rodrigues, R. C.; Berenguer-Murcia, A.; Briand, L. E.; Fernandez-Lafuente, R. Novozym 435: the "perfect" lipase immobilized biocatalyst? *Catal. Sci. Technol.* **2019**, *9*, 2380−2420.
(22) Jørgensen, H.; Pinelo, M.; et al. Enzyme recycling in lignocellulosic biorefineries. *Biofuels, Bioprod. Biorefin.* **2016**, *11*, 150−167.
(23) Aguieiras, E. C. G.; Ribeiro, D. S.; Couteiro, P. P.; Bastos, C. M. B.; de Queiroz, D. S.; Parreira, J. M.; Langone, M. A. P. Investigation of the Reuse of Immobilized Lipases in Biodiesel Synthesis: Influence of Different Solvents in Lipase Activity. *Appl. Biochem. Biotechnol.* **2016**, *179*, 485−496.
(24) Chen, J.-W.; Wu, W.-T. Regeneration of immobilized *Candida antarctica* lipase for transesterification. *J. Biosci. Bioeng.* **2003**, *95*, 466−469.
(25) Marín-Suárez, M.; Méndez-Mateos, D.; Guadix, A.; Guadix, E. M. Reuse of immobilized lipases in the transesterification of waste fish oil for the production of biodiesel. *Renewable Energy* **2019**, *140*, 1−8.
(26) Duan, Z. Q.; Du, W.; Liu, D. H. Novozym 435-catalyzed 1,3-diacylglycerol preparation via esterification in *t*-butanol system. *Process Biochem.* **2010**, *45*, 1923−1927.
(27) Sangiorgio, S.; Pargoletti, E.; Rabuffetti, M.; Robescu, M. S.; Semproli, R.; Ubiali, D.; Cappelletti, G.; Speranza, G. Emulsifying

properties of sugar-based surfactants prepared by chemoenzymatic synthesis. *Colloid Interface Sci. Commun.* **2022**, *48*, No. 100630.
(28) Semproli, R.; Robescu, M. S.; Sangiorgio, S.; Pargoletti, E.; Bavaro, T.; Rabuffetti, M.; Cappelletti, G.; Speranza, G.; Ubiali, D. From lactose to alkyl galactoside fatty acid esters as non-ionic biosurfactants: a two-step enzymatic approach to cheese whey valorization. *ChemPlusChem* **2023**, *88*, No. e202200331.
(29) Martínez, M.; Oliveros, R.; Aracil, J. Synthesis of Biosurfactants: Enzymatic Esterification of Diglycerol and Oleic Acid. 1. Kinetic Modeling. *Ind. Eng. Chem. Res.* **2011**, *50*, 6609−6614.
(30) Matsumura, S.; Maki, M.; Toshima, K.; Kawada, K. Enzymatic Synthesis, Surface Activity, Antimicrobial Properties and Biodegradability of Di-and Triglycerol Fatty Acid Esters. *J. Jpn. Oil Chem. Soc.* **1999**, *48*, 681−892.
(31) Charlemagne, D.; Legoy, M. D. Enzymatic Synthesis of PolyglyceroI-Fatty Acid Esters in a Solvent-Free System. *J. Am. Oil Chem. Soc.* **1995**, *72*, 61−65.
(32) Hong, I. K.; Kim, S. I.; Lee, S. B. Effects of HLB value on oil-in-water emulsions: Droplet size, rheological behavior, zeta-potential, and creaming index. *J. Ind. Eng. Chem.* **2018**, *67*, 123−131.
(33) Song, M. G.; Jho, S. H.; Kim, J. Y.; Kim, J. D. Rapid Evaluation of Water-in-Oil (w/o) Emulsion Stability by Turbidity Ratio Measurements. *J. Colloid Interface Sci* **2000**, *230*, 213−215.
(34) Olietti, A.; Pargoletti, E.; Diona, A.; Cappelletti, G. A novel optimized mold release oil-in-water emulsion for polyurethane foams production. *J. Mol. Liq.* **2018**, *261*, 199−207.
(35) Gu, Y. S.; Decker, E. A.; McClements, D. J. Influence of pH and carrageenan type on properties of *β*-lactoglobulin stabilized oil-in-water emulsions. *Food Hydrocolloids* **2005**, *19*, 83−91.
(36) Castel, V.; Rubiolo, A. C.; Carrara, C. R. Droplet size distribution, rheological behavior and stability of corn oil emulsions stabilized by a novel hydrocolloid (*Brea gum*) compared with gum arabic. *Food Hydrocolloids* **2017**, *63*, 170−177.
(37) Zadymova, N. M.; Skvortsova, Z. N.; Traskine, V. Y.; Kulikov-Kostyushko, F. A.; Kulichikhin, V. G.; Malkin, A. Y. Rheological properties of heavy oil emulsions with different morphologies. *J. Pet. Sci. Eng.* **2017**, *149*, 522−530.
(38) Adeyi, O.; Ikhu-Omoregbe, D.; Jideani, V. Emulsion Stability and Steady Shear Characteristics of Concentrated Oil-in-Water Emulsion Stabilized by Gelatinized Bambara Groundnut Flour. *Asian J. Chem.* **2014**, *26*, 4995−5002.
(39) *European Pharmacopoeia 7.0. Vol. 1. Council of Europe: European Directorate for the Quality of Medicines and Healthcare, Assay 01/2008:20501, Acid value, Strasbourg*, 2010, p 137.
(40) Pappalardo, V. M.; Boeriu, C. G.; Zaccheria, F.; Ravasio, N. Synthesis and characterization of arabinose-palmitic acid esters by enzymatic esterification. *Mol. Catal.* **2017**, *433*, 383−390.
(41) Shankar, S.; Agarwal, M.; Chaurasia, S. P. Study of reaction parameters and kinetics of esterification of lauric acid with butanol by immobilized *Candida antarctica* lipase. *Indian J. Biochem. Biophys.* **2013**, *50*, 570−576.

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