RESEARCH ARTICLE

From Lactose to Alkyl Galactoside Fatty Acid Esters as Non-Ionic Biosurfactants: A Two-Step Enzymatic Approach to Cheese Whey Valorization

Riccardo Semproli,[+a] Marina Simona Robescu,[+a] Sara Sangiorgio,[b] Eleonora Pargoletti,[b] Teodora Bavaro,[a] Marco Rabuffetti,[b] Giuseppe Cappelletti,[b] Giovanna Speranza,*[b] and Daniela Ubiali*[a]

[a] R. Semproli, Dr. M.S. Robescu, Dr. T. Bavaro, Prof. D. Ubiali
Department of Drug Sciences
University of Pavia
Viale Taramelli 12
Pavia I-27100 (Italy)
E-mail: daniela.ubiali@unipv.it

[b] S. Sangiorgio, Dr. E. Pargoletti, Dr. M. Rabuffetti, Prof. G. Cappelletti, Prof. G. Speranza
Department of Chemistry
University of Milano
Via Golgi 19
Milano I-20133 (Italy)
E-mail: giovanna.speranza@unimi.it

[+] These authors have contributed equally to this work and share first authorship

Supporting information for this article is given via a link at the end of the document.

Abstract: A library of alkyl galactosides was synthesized to provide the "polar head" of sugar fatty acid esters to be tested as non-ionic surfactants. The enzymatic transglycosylation of lactose resulted in alkyl β-D-galactopyranosides, whereas the Fischer glycosylation of galactose afforded isomeric mixtures of α- and β-galactopyranosides and α- and β-galactofuranosides. *n*-Butyl galactosides from either routes were enzymatically esterified with palmitic acid, used as the fatty acid "tail" of the surfactant, giving the corresponding *n*-butyl 6-*O*-palmitoyl-galactosides. Measurements of interfacial tension and emulsifying properties of *n*-butyl 6-*O*-palmitoyl-galactosides revealed that the esters of galactopyranosides are superior to those of galactofuranosides, and that the enantiopure *n*-butyl 6-*O*-palmitoyl-β-D-galactosides, prepared by the fully enzymatic route, leads to the most stable emulsion. These results pave the way to the use of lactose-rich cheese whey as raw material for the obtainment of bio-based surfactants.

Introduction

Surfactants are amphiphilic compounds widely used in every industrial sector. Application of surfactants ranges from household detergents and personal care products to food processing, agricultural and oilfield chemicals, cleaners, paints and coatings, textile, plastics, adhesives, and others.[1] The growing interest of consumers and producers toward safer and more environmentally friendly products is driving the search for new bio-based and biodegradable surfactants.[2]

Sugar fatty acid esters (SFAE), usually called sugar esters, are non-ionic surfactants which, besides excellent emulsifying, stabilizing and detergency properties, overshadow petrochemical-derived surfactants in that they are tasteless, odorless, non-toxic, non-harmful to the environment, and fully biodegradable.[3] SFAE are constituted by a sugar moiety which acts as the "polar head" of the surfactant, and by a fatty acid "tail". The hydrophilic-lipophilic balances (HLB) of SFAE can be fine-tuned and thus customized for a specific application by controlling the degree of esterification and the nature of sugar and fatty acid residues. Moreover, the components of SFAE can be bio-derived from natural resources or from waste up-grading, thus producing bio-based surfactants.[4,5] Generally speaking, the chemical synthesis of SFAE requires harsh reaction conditions (hazardous solvents, high temperature, acid or base catalysts) resulting in high energy consumption, formation of undesirable by-products (*e.g.*, due to caramelization of sugars), and low regioselectivity. The regioselective chemical synthesis of SFAE can only be achieved through protection groups strategies, which are hardly acceptable for industrial processes.[6]

Enzyme-based synthesis can often circumvent the above-mentioned drawbacks: enzymatic reactions generally occur under milder conditions without the need for tedious protection/deprotection steps. Moreover, biotransformations of naturally occurring substrates provide more environmentally friendly synthetic routes allowing for the final product to be labelled as natural as well.[7] Sugar fatty acid esters can be obtained, indeed, through an esterification reaction between a sugar and a fatty acid catalyzed by a lipase.[6]

Tuning the reaction conditions of the enzymatic esterification between fatty acids and sugars for the SFAE preparation is a challenging task owing to the opposite solubility profiles of these reagents. A key issue is the quest for the solvent or co-solvent that can solubilize both the sugar and the fatty acid moieties without deactivating the enzyme.[8,9] To circumvent this constraint, the sugar can be indeed derivatized into a less polar precursor (*i.e.*, alkyl glycoside), followed by a solvent-free lipase-mediated esterification.[10–12] The SFAE that are currently available on the market include molecules derived from sucrose and sorbitan, as well as fatty acid glucamides and alkyl polyglycosides.[13] Amongst all sugar-based surfactants, sucrose and glucose esters are the most studied and applied derivatives. The use of lactose to produce sugar esters has been scarcely reported to date[14] despite recent studies have shown the high potential of lactose

esters.[15-18] Galactose-based fatty acid esters have been poorly investigated, too.[14]

Lactose is a naturally occurring disaccharide, found in milk, composed of D-galactose and D-glucose. The world milk production was estimated to be nearly 906 million tons in 2020.[19] From the production of 1 kg of cheese, around 9-10 L of cheese whey (CW) are generated. CW is very rich in lactose, proteins, lipids, and mineral salts, but has also high disposal costs and environmental burden.[20]

In the frame of applying a bio-based circular economy approach, this study aims at exploring the use of lactose, which can be easily accessed through cheese whey permeate (CWP), resulting from protein recovery by ultrafiltration, as a cheap and abundant substrate for the production of SFAE. Lactose was indeed used as starting material in the enzymatic transglycosylation mediated by the immobilized β-galactosidase from *Aspergillus oryzae* in the presence of naturally occurring alcohols to synthesize a library of alkyl galactosides. 1-Butyl β-D-galactopyranoside (**13b**, Scheme 1a) was enzymatically esterified with palmitic acid producing the corresponding SFAE (**17b**). The emulsifying properties of **17b** were evaluated and compared with those of the SFAE (**17ad**) obtained by a chemoenzymatic approach previously described for the obtainment of *n*-butyl glucoside fatty acid esters.[10] These latter compounds (*n*-butyl 6-*O*-palmitoyl-glucosides, labelled as **SER** herein) were used as reference standards.

Results and Discussion

Immobilization of β-galactosidase from *A. oryzae*

β-Galactosidase used for the transglycosylation reactions was covalently immobilized on glyoxyl Sepabeads™ as a result of the screening of different carriers and conditions (see Supporting information, Table S1). Following previously reported protocols,[21,22] the enzyme was first immobilized by ionic interaction on an aminated carrier. Instead of using agarose activated with ethylendiamine (monoaminoethyl-*N*-aminoethyl-agarose or MANAE-agarose), the "ready-to-use" aminated Relizyme EA-112/S was used. The immobilization was fast (2 h) and high yielding (60%), but protein leakage (40%) detected in the reaction medium (McIlvaine buffer pH 4.3) after 2 h incubation discouraged the use of this immobilized biocatalyst in the transglycosylation reaction. Ionic immobilization is reversible and the enzyme can be desorbed depending on the conditions used. Post-immobilization cross-linking can assist in the stabilization of the enzyme-carrier interactions.[23] However, driven by a recent report[24] on the covalent immobilization of the β-galactosidase from *A. oryzae* on glyoxyl agarose and the use of the immobilized biocatalyst in the synthesis of **13b** by transglycosylation of lactose, we also moved to aldehyde-activated carriers by using glyoxyl Sepabeads™ in this case. These carriers display the same activation group, but differ about the nature of the matrix, being the poly-hydroxylated agarose sharply hydrophilic as compared to the polymethacrylate Sepabeads™. Sepabeads™ was subjected to epoxide ring opening in sulphuric acid followed by oxidation of the resulting diols into aldehyde groups with sodium periodate.[25] Upon incubation of the enzyme with an aldehyde-activated carrier, imine bonds are formed as a result of the reaction between ε-amino groups of surface Lys and aldehydes. Chemical reduction of the Schiff's bases is recommended and usually performed to get C-N stable bonds. However, the reduction step is generally a sore point for enzyme activity retention and the β-galactosidase from *A. oryzae* was not an exception (see Supporting information, Table S1). Therefore, the non-reduced biocatalyst was used in the transglycosylation reactions. Owing to the poor stability of the β-galactosidase at the high pH required for the immobilization (pH 10),[24,26] the immobilization was performed at 4 °C with satisfactory results (immobilization yield= 58%, activity recovery= 20%).

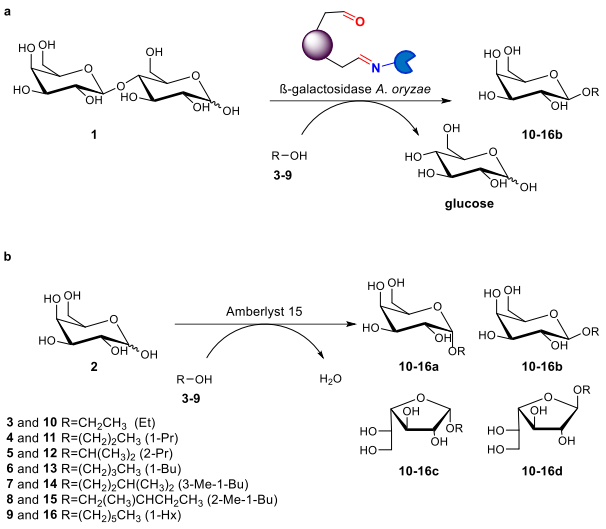
Synthesis of alkyl β-D-galactopyranosides (10b-16b) by enzymatic transglycosylation of lactose

Although galactosidases are hydrolytic enzymes, they can be used also in synthesis through either a "direct glycosylation" ("reverse hydrolysis") or a transglycosylation approach. In this latter route, a preformed activated glycoside is used and the nucleophile (water in the "normal" hydrolytic pathway) can be replaced by other nucleophiles, such as an acceptor alcohol. Transglycosylation results in higher yields than reverse hydrolysis which requires high concentrations of both the carbohydrate and the alcohol, as well as to finely tune the medium engineering.[27] Following this strategy, lactose can be converted into valuable chemicals, such as prebiotics and sweeteners (*i.e.* galactooligosaccharides, lactulose, lactosucrose, and galactosylpolyhydroxyalcohols).[28] The transglycosylation of lactose here reported was carried out according to the method described by Ahumada[24] which was extended, with some modifications, to the preparation of the library of alkyl β-D-galactopyranosides **10b**-**16b** (Scheme 1a and Table 1).

The highest yield (45%) was obtained with 1-BuOH (**6**) as shown in Table 1. It is worth mentioning that the reaction system is affected by the type of alcohol used: a ternary homogeneous system was formed with the C4 alcohol (**6**), whereas C5 and C6 alcohols (**7**-**9**) generated a biphasic system, regardless the addition of acetone (up to 40% v/v). Low yields obtained for short-chain alcohols (**3**-**5**) can be ascribed to some precipitation of lactose as well as a partial enzyme inactivation. On the other hand, the low yields achieved with long-chain alcohols (**7**-**9**) are consistent with previous reports.[29-30] This trend might be a constraint for using glycosidases in the preparation of alkyl glycosides endowed with surfactant properties, which would require longer chains (typically C8-C14). Nevertheless, even "shorter" alkyl glycosides result to be sufficiently less hydrophilic derivatives than their sugar counterpart, thus allowing for the straightforward preparation of SFAE by a direct enzymatic esterification with fatty acids in a solvent-free system.[10] This approach answers the need to balance the opposite solubility profiles of the sugar "polar head" of the surfactant and the fatty acid "tail". In addition, at least for 1-butyl glucosides, the alkyl chain was shown to positively affect the HLB (hydrophilic-lipophilic balance).[10]

Alkyl galactosides can be also synthesized by chemical glycosylation of galactose with alcohols (see next paragraph and Scheme 1b). Enzymatic transglycosylation has the advantage of a higher selectivity (pure β-D-galactopyranosides are obtained) and a lower reaction temperature. Moreover, in the frame of upcycling lactose into new bio-based products, this approach allows to use directly lactose from CWP as feedstock. On the other hand, the enzymatic approach is characterized by lower yields, although not optimized yet. 1-Butyl β-D-galactopyranoside

(**13b**) was selected out of the library of alkyl β-D-galactopyranosides (**10b**-**16b**) for the next esterification step, as a continuation of our previous work on the chemoenzymatic synthesis of 6-*O*-palmitoyl-*n*-butyl glucosides and the assessment of their surfactant properties.[10]



Scheme 1. Synthesis of alkyl galactosides (**10**-**16**). a) Enzymatic transglycosylation of lactose (**1**) with aliphatic alcohols (**3**-**9**) catalyzed by immobilized β-galactosidase from *Aspergillus oryzae* in McIlvaine buffer pH 4.3/alcohol/(acetone), r.t., 6 h. b) Fischer glycosylation of galactose (**2**) with aliphatic alcohols (**3**-**9**) catalyzed by Amberlyst® 15, reflux-120 °C, 1.5-6 h.

Table 1. Reaction yields (enzymatic transglycosylation and chemical glycosylation) and isomeric ratio of mixtures **10ad-16ad**.

R-OH (**3**-**9**)	Enzymatic transglycosylation (yield %)	Chemical glycosylation (yield %)	Isomeric ratio (a/b/c/d)
3 (R=Et)	**10b**[31] (23)	**10ad**[32] (73)	13/20/27/40
4 (R=1-Pr)	**11b**[33] (33)	**11ad**[34,35] (78)	24/27/19/30
5 (R=2-Pr)	**12b**[36,37] (25)	**12ad**[37,38] (36)	18/20/28/34
6 (R=1-Bu)	**13b**[36,39] (45)	**13ad**[38,39] (81)	46/38/6/10
7 (R=3-Me-1-Bu)	**14b** (29)	**14ad** (95)	37/29/14/20
8 (R=2-Me-1-Bu)	**15b**[40] (19)	**15ad** (88)	43/34/10/13
9 (R=1-Hx)	**16b**[41] (23)	**16ad** (32)	37/32/14/17

Synthesis of alkyl galactoside isomeric mixtures (10ad-16ad) by Fischer glycosylation of galactose

Another way to upgrade CWP is the use of the lactose constituents, *i.e.* glucose and galactose that can be both obtained by β-galactosidase-mediated hydrolysis of lactose.[42] Recently, due to the lactose price fluctuation, the demand for manufacturing monosaccharides and other "lactose-derived bioactives" from inexpensive whey permeate is growing.[28] Specifically, galactose can be isolated directly from whey permeate after protein removal, lactose enzymatic hydrolysis and several purification steps, including activated carbon treatment, electrodialysis, ion purification and simulated moving bed chromatography.[43]

Following the already established strategy to synthesize sugar-based surfactants,[10] namely the preparation of sugar derivatives apolar enough to allow the successive solvent-free enzymatic esterification, D-(+)-galactose (**2**) was submitted to Fischer glycosylation reactions with the selected naturally occurring alcohols (**3**-**9**). Similar experimental conditions (0.1 M final concentration of the reaction mixture, 10% w/w Amberlyst® 15, 25% w/w 3 Å molecular sieves, 90-120 °C and 1.5-6 h) were successfully applied to all the reactions, obtaining alkyl galactoside isomeric mixtures (**10ad**-**16ad**) in a variable yield depending on the alcohol used (Table 1). Modest isolated yields were obtained from Fischer glycosylation of galactose with 2-PrOH (**5**) and 1-HexOH (**9**) (yield$_{\mathbf{12ad}}$ = 36%; yield$_{\mathbf{16ad}}$ = 32%), probably because of steric hindrance effects in the case of **5**, and lower sugar solubility in the case of **9**. On the contrary, high yields (73-95%) were achieved for all the other alcohols, both branched and linear.

It is important to underline that this industrially relevant reaction is usually performed in presence of a homogenous acid catalyst, *i.e. p*-toluenesulfonic acid or sulphamic acid, which requires a neutralization step.[44] We employed an alternative and more sustainable method based on the use of Amberlyst® 15, which is a strongly acidic cation exchange resin that can be eventually filtered and recovered.[45]

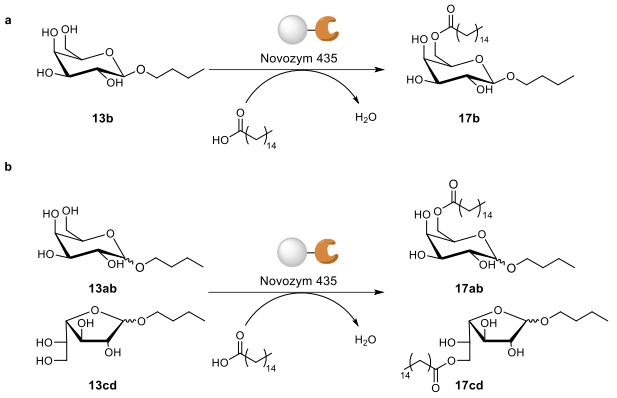
Unlike the enzymatic transglycosylation, which produces only the β-galactopyranoside isomer, the Fischer glycosylation leads to an isomeric mixture of α-/β-pyranosides and α-/β-furanosides (Table 1), whose relative ratio strictly depends on the sugar, the alcohol and the experimental conditions in which the reaction is carried out.[46] According to ^{1}H NMR analysis in D_2O, the shorter is the chain length of the alcohol (for EtOH, 1-PrOH and 2-PrOH), the higher is the amount of furanoside derivatives produced, till a plateau is reached of the relative ratio between the two structural isomeric forms of about 1:1 for longer chain alcohols (1-BuOH, 3-Me-1-BuOH, 2-Me-1-BuOH and 1-HexOH).

Synthesis of *n*-butyl 6-*O*-palmitoyl-galactosides (17ad) *via* solvent-free enzymatic esterification

Galactosides, obtained both through enzymatic and chemical approaches, were used as "polar heads" for the preparation of biosurfactants. Thus, focusing on the alkyl galactoside obtained with the highest yield from the enzymatic transglycosylation, namely 1-butyl β-D-galactopyranoside (**13b**), and in connection with our previous studies on alkyl glucosides,[10] this compound was submitted to the known protocol[10] of solvent-free Novozym® 435-catalyzed esterification with palmitic acid to give *n*-butyl 6-*O*-palmitoyl-*β*-D-galactopyranoside (**17b**, yield$_{\mathbf{17b}}$ = 15%) (Scheme 2 and Table 2).

As in the synthesis of alkyl glucoside fatty acid esters,[10] the reaction was carried out in a glass oven B-585 Kugelrohr at the optimal working temperature of the immobilized lipase (80 °C), under rotation (400 rpm) instead of magnetic stirring to avoid enzyme beads wrecking, and under reduced pressure conditions (30 mmHg) to favor water removal and to shift the equilibrium towards product formation. Even in the case of **17b**, Novozym® 435 catalyzes regioselectively the esterification of the primary hydroxyl group, as confirmed by the Heteronuclear Multiple Bond

Correlation (HMBC) ^{1}H-^{13}C NMR experiment, carried out in DMSO-d_6 (see Supporting information, Figure S39).



Scheme 2. Synthesis of *n*-butyl 6-*O*-palmitoyl-galactosides **17ad** by enzymatic esterification of **13ad** with palmitic acid catalyzed by Novozym® 435 in a solvent-free system, 80 °C, 8 h, *s.v.*

Table 2. Reaction yields (enzymatic esterification) and isomeric ratio of mixtures **17ad**.

Substrate	Product	Yield (%)	Isomeric ratio (α/β)
13b	**17b**	15	0/100
13ad	**17ab**	21	94/6
	17cd	14	20/80

In parallel, the same experimental conditions were applied to the isomeric mixture of 1-butyl galactosides (**13ad**), obtained through the chemical route, to achieve an isomeric mixture of *n*-butyl 6-*O*-palmitoyl-galactosides. The remarkable difference in polarity between pyranoside and furanoside ester isomers allowed the separation of the two species, which was performed by flash chromatography (*n*-hexane/EtOAc, 2:8), affording *n*-butyl 6-*O*-palmitoyl-galactopyranosides (**17ab**, $yield_{17ab}$ = 21%) and *n*-butyl 6-*O*-palmitoyl-galactofuranosides (**17cd**, $yield_{17cd}$ = 14%). Regarding the former, the Heteronuclear Single Quantum Coherence (HSQC) ^{1}H-^{13}C NMR correlation spectrum (see Supporting information, Figure S85) showed the presence of the β-anomer only in traces (about 6%), as further confirmed by the purity assessment through quantitative TLC and image analysis (see Supporting information, Figure S94). On the contrary, in the case of furanosides, the β-anomer seemed to be prevailing (α/β furanosides; 20:80). Beside the anomeric effect, the low yield of the *n*-butyl β-D-galactopyranoside ester, which ranges from 33% in the mixture of 1-butyl galactopyranosides to 6% in the corresponding *n*-butyl 6-*O*-palmitoyl-galactopyranosides (**17ab**), might depend on the different interaction of these substrates in the catalytic site.

The two-step chemoenzymatic process for the synthesis of *n*-butyl 6-*O*-palmitoyl-galactosides (**17ad**) was carried out also by cutting off intermediate and final chromatographic steps. The crude from the Fischer glycosylation (containing 1-butyl galactoside isomeric mixture **13ad** and traces of unreacted **2**) was directly reacted with palmitic acid according to the solvent-free enzymatic esterification protocol. The reaction mixture was taken up in EtOAc, filtered to remove the biocatalyst and the esters were extracted in EtOAc from 1 M NaOH solution, thus affording the isomeric mixture of *n*-butyl 6-*O*-palmitoyl-galactosides (**17ad**, $yield_{17ad}$ = 35%) after EtOAc evaporation. The mixture **17ad** was obtained in comparable yield with respect to the reported above methods involving chromatographic separations, resulting in 90 ± 2% purity (about 10% of unreacted 1-butyl galactoside isomeric mixture, **13ad**) and a 52/4/11/33 relative ratio between α-/β-pyranosides and α-/β-furanosides, which were assessed by quantitative TLC and image analysis (Figure S94).

Interfacial features study and emulsifying properties evaluation

For a deeper insight in the surfactant properties of the isomeric mixture of α-/β-pyranosides and α-/β-furanosides of *n*-butyl 6-*O*-palmitoyl-galactosides (**17ad**), it is mandatory to study the contribute of each isomer.

The stabilization of a disperse system, *i.e.* emulsion, can be achieved by reducing the interfacial tension (IFT) between two immiscible phases. Surfactants, due to their amphiphilic nature, are adsorbed at interphases, thus modifying the interfacial tension.[47] Therefore, in the present case, the IFT reduction (Figure 1a) between water (milli-Q) and sunflower oil was evaluated by adding the isomeric mixture of *n*-butyl 6-*O*-palmitoyl-galactosides (**17ad**), as well as the single isomeric components (*n*-butyl 6-*O*-palmitoyl-galactopyranoside **17ab** containing 6% of **17b**, *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside **17b**, *n*-butyl 6-*O*-palmitoyl-galactofuranoside **17cd** containing about 6% of *n*-butyl 6-*O*-palmitoyl-galactopyranoside isomers **17a** and **17b**), at increasing concentration (0.1, 1.5 and 3.0 mM in sunflower oil), up to the solubility limit of some of them.

At 0.1 mM, all the tensides are comparable, showing a negligible IFT reduction with respect to the starting IFT value between sunflower oil and water in absence of a surfactant (26 mN m^{-1}; grey histogram in Figure 1a). On the contrary, at 3.0 mM, except for the furanoside derivatives (**17cd**), all the prepared tensides (**17ab**, **17b**, **17ad**) resulted to be able to reduce the IFT to a value lower than 2 mM m^{-1}, thus indicating extremely promising interfacial features. However, at an intermediate surfactant concentration (1.5 mM), some differences can be easily appreciated: particularly, only *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside (**17b**) seems to reach an IFT value close to 2 mN m^{-1}. This result may be due to a better surfactant chain orientation, which favors the packing capacity of the molecules adsorbed at the interface, thus reducing the IFT significantly. Concomitantly, it is worth noting that the *n*-butyl 6-*O*-palmitoyl-galactoside isomeric mixture (**17ad**) showed intermediate interfacial features with respect to pure **17ab** and **17b**, due to the presence of furanosides (**17cd**) that hinder the IFT reduction at this concentration. These results are fully in agreement with those reported in the case of other sugar-based esters (**SER** family in the Table of Figure 1)[10,48] bearing *n*-butyl glucoside as the "polar head" and palmitic acid as the fatty acid "tail".

The ability of reducing the IFT was also observed in the cases of *n*-butyl 6-*O*-palmitoyl-glucopyranoside (**SERab**), *n*-butyl 6-*O*-palmitoyl-glucofuranoside (**SERcd**), and *n*-butyl 6-*O*-palmitoyl-glucoside isomeric mixture (**SERad**). Generally, the IFT reduction is strongly driven by the degree of disorder of the interfacial film, which is related to the interfacial entropic contributions of water/oil/surfactant molecules. When no surfactant is present, oil

and water molecules at the interface lose their degrees of freedom, since parallel alignments occur, due to strong short-range van der Waals interactions between the two phases. Conversely, the addition of a surfactant pushes away the oily molecules from the water surface, making them more disordered, by increasing their rotational and translational modes.[49] As reported in the Table of Figure 1, all the galactoside-based esters (**17ab**, **17cd** and **17ad**) led to a strong IFT reduction, much more than the corresponding glucoside-based isomers (**SERab**, **SERcd** and **SERad**). These results could be explained by looking into the chemical structures: **17** and **SER** compounds are epimers in the C-4 position, being the orientation of the OH(4) group the only difference. According to the literature,[50] this behavior is strictly connected to a different ability of matching the tetrahedral hydrogen bond network of water. Sugars with an axial OH(4) group, such as galactose, match the three-dimensional water structure much worse than sugars with an equatorially-oriented OH(4), as in the case of glucose. The mismatch causes a distortion of the water structure in the primary hydration shell, which is also transmitted to the more distant layers, inducing a more disordered structure in the water configuration and, consequently, the increase in the total entropy of the system.[49]

Finally, water in sunflower oil (W/O) emulsions (Φ_V = 0.13) were prepared by ultrasounds using a micro-tip sonicator in the presence of the biosurfactant, and their stability was evaluated within 72 h. Figure 1c shows the decrease of turbidity over time, strictly related to the emulsion destabilization phenomena:[10] compound **17b** leads to the most stable emulsion after 72 h ageing (see also the pictures in the inset of Figure 1c), whereas the other tensides were not able to stabilize the disperse systems over time, undergoing water droplets sedimentation processes and irreversible coalescence mechanisms. Hence, these results confirm the IFT data previously obtained.

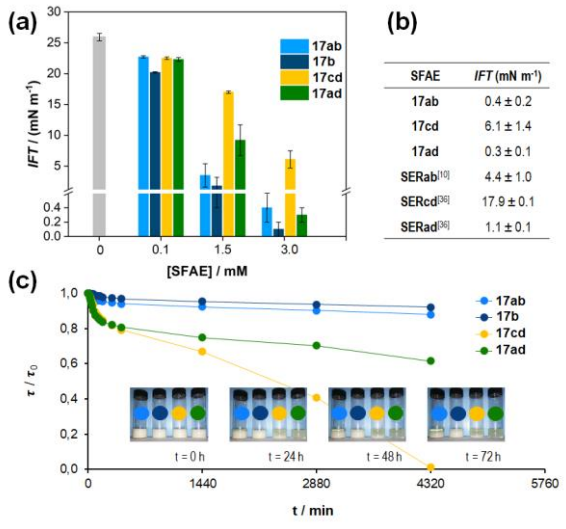


Figure 1. a) Sunflower oil/water interfacial tension (IFT) data for the synthesized surfactants (**17ab**, **17b**, **17cd**, **17ad**). b) Table: comparison of IFT values at 3 mM with other sugar-based esters used as references (**SERab**,[10] **SERcd**,[48] and **SERad**[48]) c) Normalized turbidity values (τ) of the prepared emulsions over time at fixed wavelength (550 nm). Insets: photos of the four samples at 0, 24, 48 and 72 h.

Conclusion

Sugar fatty acid esters find application as emulsifiers in the cosmetic and food industry.[51-53]

In this work, a general procedure for the preparation of some SFAE starting from lactose through a two-step enzymatic approach catalyzed by the immobilized β-galactosidase from *Aspergillus oryzae* and the immobilized CalB (Novozym® 435), or from galactose through a two-step chemoenzymatic strategy was set-up. The exploitation of lactose as a raw material, either as such or as a source of galactose, for the production of surfactants answers the need for tackling the issue of cheese whey disposal in dairy industry, while upcycling this abundant and cheap feedstock, which is independent from season and climate and is not in competition with food. Starting from the results achieved herein, we aim indeed at using lactose-rich CWP as raw material both for the synthesis of the "polar head" of the surfactant, and as fermentation medium for the obtainment of microbial lipids to be used as fatty acid "tail".[54]

According to the preliminary physicochemical study here reported, *n*-butyl galactoside fatty acid esters were shown to generally possess promising interfacial features, although to a different extent; in particular, the enantiopure *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside (**17b**), which was prepared enzymatically from lactose, leads to the most stable emulsion.

Experimental Section

General

All chemicals were from Sigma-Aldrich (Milano, Italy), if not stated otherwise. Anhydrous lactose was purchased from Honeywell Fluka (Rodano, Italy). All solvents were from Merck Life Science (Milano, Italy) and were used without further purification. β-Galactosidase from *Aspergillus oryzae* was purchased from Sigma-Aldrich (Milano, Italy). Lipase B from *Candida antarctica* immobilized on an acrylic carrier (Novozym® 435) was kindly supplied by Novozymes (Denmark). Sepabeads™ EC-EP/S and Relizyme™ EA-112/S were a gift of Resindion s.r.l. (Binasco, Italy).

Electrospray ionization mass spectra (ESI-MS) were recorded on a Thermo Finnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, UK). ^{1}H and ^{13}C NMR spectra were recorded at 400.13 and 100.61 MHz, respectively, on a Bruker AVANCE 400 spectrometer (Bruker, Karlsruhe, Germany) interfaced with a workstation running a Windows operating system TOPSPIN software package, at 300 K. Chemical shifts (δ) are given in parts per million (ppm) and were referenced to the solvent signal (DMSO-d_6, δ_H 2.50 ppm and δ_C 39.52 ppm, and D_2O, δ_H 4.79 ppm, from TMS, respectively). The ^{13}C NMR signal multiplicities were based on APT (attached proton test) spectra. The ^{13}C NMR signals were assigned with the aid of ^{1}H-^{13}C correlation experiments (heteronuclear single quantum correlation spectroscopy, HSQC, and heteronuclear multiple bond correlation spectroscopy, HMBC). The fatty acid profile of commercial sunflower oil was determined by gas chromatography/mass spectrometry (GC/MS) analysis, after base-catalyzed transmethylation, using the protocol FIL-IDF 182:1999, reported elsewhere.[10]

Immobilization of β-galactosidase from *A. oryzae* on glyoxyl Sepabeads™

Glyoxyl Sepabeads™ was prepared as previously reported.[25] Briefly, Sepabeads™ EC-EP/S (20 g) was suspended in 0.5 M H_2SO_4 (260 mL) under mechanical shaking for 2 h at r.t., then filtered under vacuum and washed thoroughly with distilled H_2O until neutral pH. The resin was then oxidized by incubation with 0.1 M $NaIO_4$ (80 mL) for 2 h at r.t., filtered, washed thoroughly with distilled H_2O and stored at 4 °C till use. β-

Galactosidase from *A. oryzae* (17 mg; 5 IU) was dissolved in 50 mM $NaHCO_3$ buffer pH 10 (10 mL) containing 1% D-(+)-galactose (w/v) under stirring at 4 °C till a clear solution was obtained. Glyoxyl Sepabeads™ (1 g) was added to the mixture to start the immobilization. At the endpoint (24 h), the immobilized enzyme was filtered, washed thoroughly with distilled water, and stored at 4°C till use.

Synthesis of alkyl β-D-galactopyranosides (10b-16b) by enzymatic transglycosylation of lactose (general protocol)

Enzymatic transglycosylation reactions (Scheme 1a) were performed as reported by Ahumada et al.[24] with slight modifications. Anhydrous lactose (**1**, 0.342 g, 1 mmol) was dissolved in McIlvaine buffer pH 4.3 (20 mL) and the desired alcohol (**3**-**9**, 50 mL) was added under magnetic stirring. Acetone (30 mL or 40 mL) was added to the mixture when 1-BuOH (**6**) or C5/C6 alcohols (**7**-**9**) were used, respectively, thus generating a ternary homogeneous system or a biphasic system. For water-miscible alcohols (**3**-**5**), no acetone was used. The immobilized β-galactosidase (2 g, 1.6 IU) was added to the reaction and the mixture was stirred at r.t. After 6 h (TLC monitoring, DCM/MeOH, 85:15, detection by H_2SO_4 5% v/v in EtOH), the suspension was filtered under vacuum and the immobilized enzyme was washed with the same reaction mixture (20 mL) by replacing the buffer with distilled water. The filtrate was evaporated under reduced pressure and purified by flash chromatography (DCM/MeOH, 85:15). The crude was added with silica and MeOH, dried under reduced pressure and loaded on the silica column. Purified alkyl β-D-galactopyranosides (**10b-16b**) were characterized by TLC, ESI-MS, and NMR analysis (see Supporting information). Yields are listed in Table 1.

Synthesis of alkyl galactoside isomeric mixtures (10ad-16ad) by Fischer glycosylation of galactose (general protocol)

D-(+)-Galactose (**2**, 0.900 g, 5 mmol) was suspended in dry naturally occurring alcohols (**3**-**9**, 50 mL) in the presence of the strongly acidic cation exchange resin Amberlyst® 15 (10 %, w/w) and 3 Å molecular sieves (25 %, w/w), under reflux or at 120 °C, according to the alcohol boiling points (Scheme 1b). After 1.5-6 h, depending on the alcohol, the reactions were stopped by filtration of the solid catalyst, the alcohols were removed under reduced pressure, and the reaction mixtures were submitted to flash chromatography (DCM/MeOH, 9:1) to give alkyl galactosides **10ad-16ad** as viscous syrups (yields are listed in Table 1). The isomeric mixtures of galactosides (**10ad-16ad**) were characterized by TLC, ESI-MS and NMR analysis (see Supporting information). NMR signals were identified by comparison with data reported in literature.[55] D-(+)-Galactose (**2**), Amberlyst® 15 and 3 Å molecular sieves were dried at 90 °C overnight prior to use.

Enzymatic synthesis of *n*-butyl 6-*O*-palmitoyl-galactosides (17b, 17ab, 17cd) (general protocol)

1-Butyl *β*-D-galactopyranoside (**13b**, 0.708 g, 3 mmol), palmitic acid (0.768 g, 3 mmol) and Novozym® 435 (10 %, w/w) were mixed and poured into a round-bottom flask. The mixture was heated to 80 °C while rotating the flask by means of a glass oven B-585 Kugelrohr (Büchi, Cornaredo, Italy). After fatty acid melting, the reaction was performed under reduced pressure (30 mmHg) (Scheme 2a and Table 2). After 8 h, the reaction mixture was taken up in EtOAc and the immobilized enzyme was removed by filtration. Then, the ester was extracted in EtOAc (2x) from 1 M NaOH, the organic phases were collected, dried over Na_2SO_4 and the solvent was removed under reduced pressure. The product was isolated by flash chromatography (*n*-hexane/EtOAc, 2:8), thus affording *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside (**17b**) (yield=15 %). The same experimental conditions were used for reacting the isomeric mixture of 1-butyl galactosides (**13ad**). *n*-Butyl 6-*O*-palmitoyl-galactopyranoside (**17ab**) (yield=21 %) and *n*-butyl 6-*O*-palmitoyl-galactofuranosides (**17cd**) (yield=14 %) were isolated by flash chromatography (Scheme 2b and Table 2). The products were characterized by TLC, ESI-MS and NMR analysis (see Supporting information).

Chemoenzymatic synthesis of *n*-butyl 6-*O*-palmitoyl-galactosides (17ad)

D-(+)-Galactose (**2**, 0.900 g, 5 mmol) was suspended in dry 1-BuOH (**6**, 50 mL, 546 mmol) in the presence of the strongly acidic cation exchange resin Amberlyst® 15 (10 %, w/w) and 3 Å molecular sieves (25 % w/w) at 120 °C. After 3 h, the solid catalyst was filtered off and the alcohol was removed under reduced pressure. Then, the resulting isomeric mixture of 1-butyl galactosides (**13ad**), palmitic acid (0.768 g, 3 mmol) and Novozym® 435 (10 % w/w) were mixed together and poured into a round-bottom flask. The mixture was heated to 80 °C while rotating the flask by means of a glass oven B-585 Kugelrohr (Büchi, Cornaredo, Italy). After fatty acid melting, the reaction was performed under reduced pressure (30 mmHg). After 8 h, the reaction mixture was taken up in EtOAc and the immobilized enzyme was removed by filtration. The organic phase was further diluted with EtOAc, washed with 0.1 M NaOH (3x), dried over Na_2SO_4, and the solvent was removed under reduced pressure, thus affording *n*-butyl 6-*O*-palmitoyl-galactosides (**17ad**) (yield=35 %).

Interfacial tension (IFT) measurements and emulsifying properties

The sunflower oil/water interfacial tension (IFT) values at 0.1, 1.5 and 3.0 mM of *n*-butyl 6-*O*-palmitoyl-α-D-galactopyranoside (**17ab**), *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside (**17b**), *n*-butyl 6-*O*-palmitoyl-galactofuranoside isomeric mixture (**17cd**), and *n*-butyl 6-*O*-palmitoyl-galactoside isomeric mixture (**17ad**) were measured at (25 ± 1) °C by means of a Gibertini tensiometer (Du Noüy ring method, Harkins-Jordan corrections).

IFT values at 3 mM relative to other already reported sugar-based esters [10,48] *n*-butyl 6-*O*-palmitoyl-glucopyranoside labelled as **SERab**, *n*-butyl 6-*O*-palmitoyl-glucofuranoside (**SERcd**), and *n*-butyl 6-*O*-palmitoyl-glucoside isomeric mixture (**SERad**) were compared to the data of the herein investigated surfactants.

Water (milli-Q) in sunflower oil (W/O) emulsions (phase volume of Φ_V = 0.13) were prepared by ultrasound homogenizer, using a Thermo Fisher Q700 sonicator equipped with a 3 mm-titanium alloy microtip, following the operative conditions previously reported.[10] The synthesized surfactants were solubilized at 80 °C in oil (3.0 mM) and added in a fixed amount (0.06 % wt) to form the final emulsions. Their stability was evaluated within 72 h by means of turbidimetric measurements as reported elsewhere.[10,56]

Acknowledgements

This work was financially supported by the Cariplo Foundation (Italy) (call: “Circular Economy for a sustainable future 2020”, project BioSurf, ID 2020-1094).

Author Contributions

Conceptualization: GS, DU; data curation: RS, MSR, SS, EP, TB, MR; funding acquisition and project administration: GS, DU; investigation: RS, MSR, SS, EP; supervision: GS, DU, GC; visualization: RS, MSR, SS; writing-original draft: RS, MSR, SS; writing-review & editing: all authors

Keywords: biocatalysis • biosurfactants • emulsifiers • lactose • sugar fatty acid esters

[1] F. Begum, L. Xu, S. Amin, *Surfactants,* in *Kirk - Othmer Encyclopedia of Chemical Technology*, (Eds: John Wiley & Sons, Inc.), USA, **2020**.

[2] C. B. B. Farias, F. C. G. Almeida, I. A. Silva, T. C. Souza, H. M. Meira, R. de C. F. Soares da Silva, J. M. Luna, V. A. Santos, A. Converti, I. M. Banat, L. A. Sarubbo, *Electron. J. Biotechnol.*, **2021**, *51*, 28–39.

[3] A. Ducret, A. Giroux, M. Trani, R. Lortie, *Biotechnol. Bioeng.*, **1995**, *48*, 214–221.

[4] S. Siebenhaller, J. Kirchhoff, F. Kirschhöfer, G. Brenner-Weiß, C. Muhle-Goll, B. Luy, F. Haitz, T. Hahn, S. Zibek, C. Syldatk, K. Ochsenreither, *Front. Chem.*, **2018**, *6*, 421.

[5] A. Bhadani, A. Kafle, T. Ogura, M. Akamatsu, K. Sakai, H. Sakai, M. Abe, *Curr. Opin. Colloid Interface Sci.*, **2020**, *45*, 124–135.

[6] B. Pérez, S. Anankanbil, Z. Guo, in *Fatty Acids: Chemistry, Synthesis, and Applications*, (Eds: M. U. Ahmad, Academic Press and AOCS Press), Jina Pharmaceuticals, Inc., Libertyville, IL, USA, **2017**, Ch. 10.

[7] D. G. Hayes, G. A. Smith, in *Biobased Surfactants*. *Synthesis, Properties, and Applications*, (Eds: D. G. Hayes, D. K.Y. Solaiman, R. D. Ashby, Academic Press and AOCS Press), **2019**, Ch. 1.

[8] A. M. Gumel, M. S. M. Annuar, T. Heidelberg, Y. Chisti, *Process Biochem.*, **2011**, *46*, 2079–2090.

[9] Z. Yang, Z.-L. Huang, *Catal. Sci. Technol.*, **2012**, *2*, 1767–1775.

[10] S. Sangiorgio, E. Pargoletti, M. Rabuffetti, M. S. Robescu, R. Semproli, D. Ubiali, G. Cappelletti, G. Speranza, *Colloid Interface Sci. Commun.*, **2022**, *48*, 100630.

[11] F. Björkling, S. E. Godtfredsen, O. Kirk, *J. Chem. Soc., Chem. Commun.*, **1989**, *14*, 934–935.

[12] P. F. Monsan, F. Paul, V. Pelenc, E. Boures, *Ann. N. Y. Acad. Sci.*, **1996**, *799*, 633–641.

[13] M. S. Ortiz, J. G. Alvarado, F. Zambrano, R. Marquez, *J. Surfactants Deterg.*, **2022**, *25*, 147–183.

[14] M. C. Pereira Gonçalves, J. P. Romanelli, J. R. Guimarães, A. C. Vieira, B. Pereira de Azevedo, P. W. Tardioli, *Crit. Rev. Biotechnol.*, **2021**, *41*, 865-878.

[15] M. K. Walsh, R. A. Bombyk, A. Wagh, A. Bingham, L. M. Berreau, *J. Mol. Catal. B: Enzym.*, **2009**, *60*, 171–177.

[16] M.-Y. Liang, M. G. Banwell, Y. Wang, P. Lan, *J. Agric. Food Chem.*, **2018**, *66*, 12594–12603.

[17] J. Staroń, J. M. Dąbrowski, E. Cichoń, M. Guzik, *Crit. Rev. Biotechnol.*, **2018**, *38*, 245–258.

[18] M. Verboni, S. Lucarini, A. Duranti, *Pharmaceuticals*, **2021**, *14*, 1306.

[19] FAO, *Dairy Market Review: Overview of global dairy market developments in 2020*, **2021**.

[20] A. F. Pires, N. G. Marnotes, O. D. Rubio, A. C. Garcia, C. D. Pereira, *Foods*, **2021**, *10*, 1067.

[21] H. Zaak, S. Peirce, T. de Albuquerque, M. Sassi, R. Fernandez-Lafuente, *Catalysts*, **2017**, *7*, 250.

[22] E. A. Barraza-González, R. Fernández-Lafuente, D. E. Zazueta-Alvarez, J. A. Rojas-Contreras, J. Lòpez Miranda, N. O. Soto-Cruz, P. G. Vàzquez-Ortega, *Rev. Mex. Ing. Quim.*, **2022**, *21*, 1–11.

[23] I. Serra, C. D. Serra, S. Rocchietti, D. Ubiali, M. Terreni, *Enzyme Microb. Technol.*, **2011**, *49*, 52–58.

[24] D. Ahumada, F. Arenas, F. Martìnez-Gòmez, C. Guerrero, A. Illanes, C. Vera, *Front. Bioeng. Biotechnol.*, **2020**, *8*, 859.

[25] C. Temporini, P. Bonomi, I. Serra, A. Tagliani, T. Bavaro, D. Ubiali, G. Massolini, M. Terreni, *Biomacromolecules*, **2010**, *11*, 1623–1632.

[26] L. M. Huerta, C. Vera, C. Guerrero, L. Wilson, A. Illanes, *Process Biochem.*, **2011**, *8*, 245–252.

[27] K. Faber, in *Biotransformations in organic chemistry: a Textbook*, (Eds: Springer), Springer-Verlag Berlin Heidelberg, **2011**, Ch. 2.

[28] C. Vera, C. Guerrero, A. Illanes, *Syst. Microbiol. Biomanuf.*, **2022**, *2*, 393–412.

[29] Mohd. Younis Rather, S. Mishra, V. Verma, S. Chand, *Bioresour. Technol.*, **2012**, *107*, 287–294.

[30] E. Benesova, P. Lipovova, H. Dvorakova, B. Kralova, *Glycobiology*, **2010**, *20*, 442–451.

[31] Y. Shimizu, T. Maeda, Y. Hidaki, H. Tani, N. Morita, *Food Res. Int*., **2003**, *36*, 373–379.

[32] K. Yoshida, N. Iino, T. Kamata, K. Kato, *Chem. Pharm. Bull*., **1969**, *17*, 1123–1127.

[33] C. Vera, C. Guerrero, L. Wilson, A. Illanes, *Process Biochem*., **2017**, *53*, 162–171.

[34] E. Bourquelot, A. Aubry, *Compt. Rend*., **1916**, *163*, 312-315.

[35] J.W. Green, E. Pacsu, *J. Am. Chem. Soc*., **1938**, *60*, 2056–2057.

[36] Y. Ooi, T. Hashimoto, N. Mitsuo, T. Satoh, *Chem. Pharm. Bull*., **1985**, *33*, 1808–1814.

[37] Z. Rapi, T. Nemcsok, P. Bagi, G. Keglevich, P. Bakó, *Tetrahedron*, **2019**, *75*, 3993–4004.

[38] A.A. Chiocconi, O. Varela, R.M. de Lederkremer, *Carbohydr. Lett*., **1996**, *2*, 115–122.

[39] F. Cinget, R.R. Schmidt, *Synlett*., **1993**, *2*, 168–170.

[40] A. Trincone, *J. Chem. Soc., Perkin Trans*., **1991**, *11*, 2841–2844.

[41] G. Chen, Z. Li, L. Chen, S. Ji, W. Shen, *J. Surfactants Deterg*., **2016**, *19*, 1095–1105.

[42] T. Shintani, *Fermentation*, **2019**, *5*, 47.

[43] S. J. Yan, M. H. Kim, T. B. Kim, Y. M. Lee, S. B. Kim, S. W. Park, US 2016/0152652A1 **2016**.

[44] F. P. A. Kinanti, D. U. C. Rahayu, A. P. Gustianthy, Y. K. Krisnandi, *AIP Conf. Proc.*, **2021**, *2349*, 020018.

[45] R. L. Whistler, M. L. Wolfrom, in *Methods in carbohydrate chemistry. Volume 2: Reactions of carbohydrates*, Academic Press, New York, **1963**.

[46] P. M. Collins, R. J. Ferrier, in *Monosaccharides: their chemistry and their roles in natural products*, (Eds: Wiley & Sons), Chichester; New York, **1995**.

[47] K. S. Birdi, in *Surface and colloid chemistry: principles and applications*, (Eds: CRC Press), Boca Raton (FL), USA, **2010**, Ch. 3.

[48] S. Sangiorgio, E. Pargoletti, M. Rabuffetti, S. Nasserian, M.S. Robescu, R. Semproli, D. Ubiali, G. Cappelletti, G. Speranza, Chemoenzymatic Synthesis of Alkyl Glycoside Fatty Acid Esters and Investigation of their Emulsifying Properties, XLVI "A. Corbella" International Summer School on Organic Synthesis (ISOS 2022), Gargnano – Italy, 12 - 16 June **2022**.

[49] T. Bui, H. Frampton, S. Huang, I. R. Collins, A. Striolo, A. Michaelides, *Phys. Chem. Chem. Phys*., **2021**, *23*, 25075.

[50] N. V. Penkov, *Int. J. Mol. Sci*., **2021**, 22, 11969.

[51] N. R. Khan, V. K. Rathod, *Process Biochem.*, **2015**, *50*, 1793–1806.

[52] N. S. Neta, J. A. Teixeira, L. R. Rodrigues, *Crit. Rev. Food Sci. Nutr.*, **2015**, *55*, 595–610.

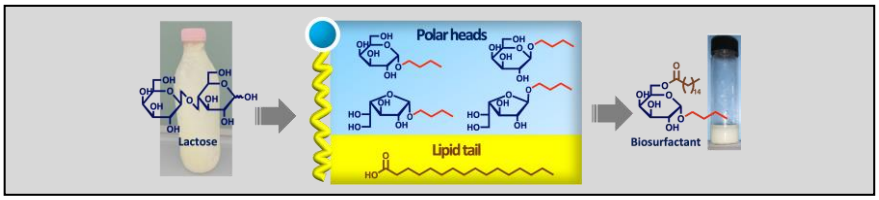
[53] A. Pellis, S. Cantone, C. Ebert, L. Gardossi, *New Biotechnol.*, **2018**, *40*, 154–169.

[54] S. Donzella, A. Fumagalli, S. Arioli, L. Pellegrino, P. D'Incecco, F. Molinari, G. Speranza, D. Ubiali, M. S. Robescu, C. Compagno, *Fermentation*, **2022**, *8*, 341.

[55] A. J. J. Straathof, J. Romein, F. van Rantwijk, A. P. G. Kieboom, H. van Bekkum, *Starch/Stärke*, **1987**, *39*, 362–368.

[56] C. Cionti, G. Vavassori, E. Pargoletti, D. Meroni, G. Cappelletti, *J. Colloid Interface Sci.*, **2022**, *628*, 82–89.

Entry for the Table of Contents



Sugar fatty acid esters were prepared starting either from lactose through a two-step enzymatic approach catalyzed by immobilized β-galactosidase from *Aspergillus oryzae* and immobilized CalB (Novozym® 435), or from galactose through a two-step chemoenzymatic strategy. *n*-Butyl galactoside fatty acid esters were shown to possess good interfacial features, being the pure β-anomer prepared by the fully enzymatic route the best emulsifier.

Project Twitter username: @BioSurf_project