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Green metrics-guided redesign of cheese whey permeate upcycling *via* biocatalysis

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Valorizing industrial side-streams through (bio)catalysis can contribute to the transition toward circular manufacturing by implementing sustainable and effective waste-to-value processes. In this study, cheese whey permeate, an abundant effluent of the dairy industry, was upcycled into the hydrophilic headgroup 1-butyl β-D-galactopyranoside of sugar fatty acid ester surfactants *via* enzymatic transglycosylation. This biotransformation was systematically redesigned by using a comprehensive green metrics-guided approach to enhance process sustainability, while ensuring operational simplicity and scalability. By moving from a homogeneous ternary reaction medium to a biphasic system and replacing flash chromatography with a streamlined downstream process involving liquid–liquid extraction and a recyclable hydrophobic resin, the process was successfully scaled-up to 1.5 L yielding the product on the gram-scale. Process redesign resulted in 8-fold reduction of (Environmental)-factor, about 60-fold improvement of solvent eco-impact, and 27-fold decrease in global warming potential (GWP). Moreover, the redesigned process enabled closed-loop material flows facilitating the direct recovery and recycling of materials (1-BuOH and the hydrophobic resin) within the same reaction unit. Finally, the recovered sugar-enriched aqueous stream from the enzymatic transglycosylation was reused as a nutrient-rich growth medium for microbial cultivation, paving the way for a future highly integrated biomanufacturing framework.

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Sustainability spotlight

While enzyme-catalyzed transformations of biomass are frequently perceived as inherently "green" or "sustainable" processes, rigorous quantitative assessment of reaction metrics often challenge this assumption. This study evaluates the greenness of the biocatalytic synthesis of the "polar head" of a sugar-based surfactant from whey permeate. By conducting a comprehensive assessment from biotransformation to downstream processing, we identified critical environmental hot spots. This evidence guided a strategic redesign that reduced the *E*-factor 8-fold, solvent eco-impact 60-fold, and GWP 27-fold, aligning the process with UN SDGs 12, 13 and 9. Our findings demonstrate that early-stage quantitative analysis is pivotal for steering experimental choices and process redesign.

1 Introduction

The integration of biocatalysis with circular economy principles is key to develop sustainable and effective waste-to-value processes.[1,2] Coupling the use of renewable feedstocks with enzymes as biocatalysts reduces the need for waste treatment while promoting material reuse. The use of enzymes further enhances these processes by offering advantages like high selectivity, activity under mild reaction conditions, biodegradability, and low toxicity of biocatalysts.[2,3] However, the inherent "greenness" of combining biocatalysis with the use of renewable feedstocks is not *a priori* guaranteed and therefore, quantitative environmental metrics must be implemented since the earliest research phases to assess and guide the process development.[4–6] Moreover, when developing any chemical process, a scalable and efficient method for product isolation and purification (downstream processing, DSP) must be envisaged and considered. The reaction system can significantly influence subsequent downstream preparative applications. The importance of DSP is often underestimated even if its contribution to the overall environmental footprint of a process is frequently substantial, often reaching the same order of magnitude as, or even exceeding, the environmental impact of the core biotransformation. Therefore, decisions regarding the reaction system should always be evaluated in light of their impact on subsequent isolation procedures.[7]

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The early identification of the factors in both the upstream (reaction) and downstream (purification) units which contribute most significantly to the overall environmental footprint in a developing biocatalytic process is crucial. This approach provides critical support for knowledge-based optimization and decision-making, enabling strategic adjustment and modification to the process design to achieve an optimal eco-efficiency.

A wide array of metrics is available to comprehensively assess the overall sustainability of a (bio)process.[8] These metrics can be broadly classified into mass-based metrics (*e.g.*, atom economy (AE), (Environmental)-factor, process mass intensity (PMI), reaction mass efficiency (RME)),[9,10] energy-related ones (*e.g.*, (Climate)-factor, E^+-factor),[11,12] and those focusing on environmental and human health (EcoScale, Eco-Impact, Benign index (BI), safety hazard index (SHI)).[10] The development of a sustainable process requires a comprehensive approach that takes into account the various aspects of greenness. Consequently, integrating a range of green metrics into a single assessment framework is essential for identifying the hot spots – what factors contribute mostly to the environmental impact – and guiding the decision-making process.

This paper reports the improvement of a biocatalytic process applied to the valorization of whey permeate (WP), an abundant waste stream of dairy industry, through a green metrics assessment. WP was previously employed as a renewable feedstock for a two-step bienzymatic synthesis of sugar fatty acid esters (SFAE), which display surfactant properties (Scheme 1).[13] In this setup, WP was employed in a transglycosylation step, providing both the substrate (lactose) and the reaction medium (water) for synthesizing the polar head 1-butyl β-D-galactopyranoside (BuGal). The resulting polar head was then used in a subsequent enzymatic transesterification with different fatty acid derivatives ("apolar tails") to produce a panel of SFAE, which exhibited promising interfacial properties.[13] The biocatalytic transglycosylation reaction was originally performed at laboratory scale and purified by standard flash chromatography technique.[13] In this paper, this reaction was scaled-up (10–15×) by developing a process with a reduced environmental impact. To this end, a comprehensive gate-to-gate approach was adopted, covering both the upstream (reaction) and downstream (purification) units, and addressing the valorization of the generated wastewater. This study explored different reaction conditions and purification strategies to determine their overall influence on the ultimate environmental impact of the process.

2 Results and discussion

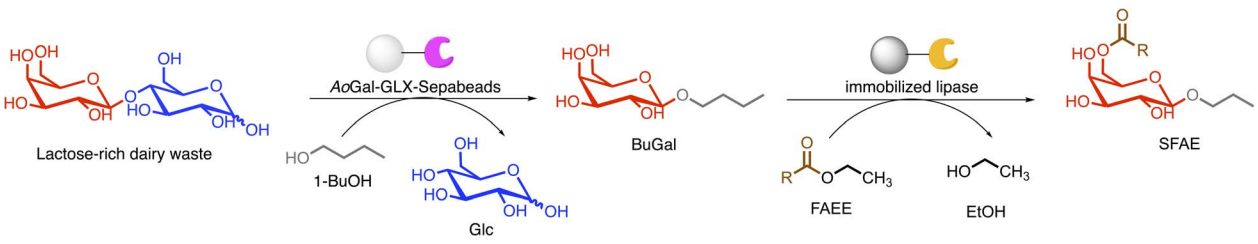
2.1 Enzymatic transglycosylation of WP for the synthesis of 1-butyl β-D-galactopyranoside: preliminary green assessment

As stated previously, WP containing lactose (Lac; 45 g per L) and traces of glucose (Glc; 0.09 g per L) and galactose (Gal; 0.10 g per L), was recently upcycled by transforming it in BuGal, the "polar head" of SFAE *via* enzymatic transformation.[13] After adjusting its pH at 4.5, WP was directly submitted to a transglycosylation reaction in a homogeneous ternary system (20/30/50 WP/acetone/1-BuOH) catalyzed by an in house immobilized β-galactosidase from *Aspergillus oryzae* (*Ao*Gal) to give BuGal in 40% yield (~250 mg) after 2.5 h at 30 °C (Table 1, Entry 1).[13] In this setup WP was used not only as a source of substrates, but also as a source of water reducing the overall water footprint of the biotransformation.

A preliminary green assessment was performed at this early stage to drive process optimization through an eco-conscious design approach. As reported in Table 2 (Entry 1) the *E*-factor of the reaction unit was equal to 347.60, mainly ascribed to the poor atom economy of the reaction. In the transglycosylation reaction just the galactose-unit of lactose is incorporated into the final product (BuGal) while the glucose-unit is generated as waste. Moreover, the fixed "non-adjustable" concentration of lactose within WP waste stream directly influences the titre of product that can be obtained. When considering the DSP unit, the *E*-factor raises up to 1797.40 and GWP increases by around two orders of magnitude (from 150 to 12 000 kg CO_2) due to the solvents employed in the purification. Moreover, while methanol is an acceptable solvent for virtually all ranking lists, the use of dichloromethane adds significant (hazardous) environmental burden. This is corroborated also by a huge composite Eco-impact value (12 044 760).

The preliminary green assessment highlighted four critical factors that mainly impacted the overall sustainability of the process: (i) unrecovered Glc co-product which contributes to the waste generation; (ii) high environmental impact due to the use of standard, lab-scale work-up and purification protocols (*i.e.*, flash chromatography), (iii) use of undesirable critical solvents in the purification protocol (*i.e.*, dichloromethane) and, (iv) scale penalty resulting from the mg scale of the biotransformation.

In this work, we focused on optimizing the reaction system to overcome all these issues.



Scheme 1 WP upcycling into sugar fatty acid esters through a two-step enzymatic biotransformation. *Ao*Gal = *Aspergillus oryzae* β-galactosidase, GLX-Sepabeads = glyoxyl-Sepabeads, Glc = glucose, BuGal = 1-butyl β-D-galactopyranoside, FAEE = fatty acid ethyl esters, SFAE = sugar fatty acid esters.

Table 1 Overview of the biotransformations described in the text[a]

Entry	Lactose source	Sugar content (g per L)[c]	Reaction system	Scale (L)	Purification method	Amount obtained (g)	Product & impurities (%) (ELSD + enzymatic assay[d])	Product & impurities (%)(RID)	Product & impurities (%) (q-^{1}H NMR)
1	WP	Lac 45 Glc 0.09 Gal 0.10	Ternary	0.1	Flash chromatography	0.250[b] (Y = 40%)	—	—	—
2	CWP	Lac 126 Glc n.d. Gal 1.82	Ternary	0.1	Flash chromatography	0.525 (Y = 30%)	—	—	—
3	CWP	Lac 126 Glc n.d. Gal 1.82	Biphasic	0.1	Flash chromatography	0.485 (Y = 28%)	96.3 BuGal	n.d.	98.0 BuGal
4	CWP	Lac 120 Glc n.d. Gal 1.82	Biphasic	0.1	L/L extraction + XAD4 resin + elution with EtOH	0.804 (Y = 35%)	75.0 BuGal 8.0 Glc 1.6 Gal 15.4 salts	74.9 BuGal 7.3 Glc 1.5 Gal 16.3 salts	68.8 BuGal 31.2 others
5	CWP	Lac 123 Glc n.d. Gal 1.77	Biphasic	1	L/L extraction + XAD4 resin + elution with EtOH	7.0 (Y = 26%)	56.0 BuGal 10.0 Glc 3.5 Gal 30.5 salts	67.0 BuGal 9.0 Glc 4.4 Gal 19.6 salts	64.9 BuGal 35.1 others
6	CWP	Lac 123 Glc n.d. Gal 1.77	Biphasic	1	L/L extraction + XAD4 resin + elution with EtOH	7.8 (Y = 26%)	52.0 BuGal 11.2 Glc 2.5 Gal 34.3 salts	62.0 BuGal 11.0 Glc 6.3 Gal 20.7 salts	55.2 BuGal 44.8 others
7	CWP	Lac 142 Glc n.d. Gal 2.14	Biphasic	1.5[e]	L/L extraction + XAD4 resin + elution with EtOH	7.20 (Y = 12%)	41.0 BuGal 15.2 Glc 4.6 Gal 39.2 salts	56.3 BuGal 14.4 Glc 9.0 Gal 20.3 salts	54.0 BuGal 46.0 others
8	CWP	Lac 119 Glc 0.65 Gal 2.24	Biphasic	1	L/L extraction + XAD4 resin + elution with 1-BuOH	3.14 (Y = 14%)	71.0 BuGal 5.3 Glc 3.0 Gal 20.7 salts	74.0 BuGal 4.8 Glc 2.0 Gal 19.2 salts	78.5 BuGal 21.5 others
9	CWP	Lac 113 Glc 1.98 Gal 5.49	Biphasic	1	L/L extraction + XAD4 resin + elution with 1-BuOH	3.30 (Y = 17%)	80.4 BuGal 6.7 Glc 3.0 Gal 9.9 salts	82.0 BuGal 6.3 Glc 2.1 Gal 9.7 salts	83.0 BuGal 17.0 others

[a] Reaction conditions: 2–30 U immobilized AoGal, 30 °C, 2.5–17 h. Ternary system: (C)WP/acetone/1-BuOH 20/30/50; biphasic system: CWP/1-BuOH 20/80. [b] As reported in Semproli *et al.*[13] [c] Gal and Lac (g per L) were determined by the enzymatic kit K-LACGAR; Glc (g per L) was determined by the enzymatic kit K-GLU, n.d. not determined, the kit was not available in the lab at the time the reaction was performed. [d] Gal (%) was determined by the enzymatic kit K-LACGAR; Glc (%) was determined by the enzymatic kit K-GLU, n.d. not determined, the kit was not available in the lab at the time the reaction was performed. The percentage of others (%) was determined by HPLC-ELSD or HPLC-RID calibration curve by analyzing a solution of the purified product (10 g per L) or by q-^{1}HNMR of a solution 5–20 g per L of the product in DMSO-*d*6 in the presence of 1,3,5-trimethoxybenzene (1 mg) as internal standard. [e] Reaction performed with 1-BuOH recovered from entries 5–6 and reused.

Table 2 Green metrics

Rx. (entry Table 1)	*E*-factor[a] (w/o DSP)	*E*-factor[a] (w DSP)	*E*-factor[a] (w DSP but considering recovered materials)	E^+-factor[b] (w/o DSP)	Solvent Eco-Impact[c]	GWP (gate to gate, w/o DSP)[d]	GWP (gate to gate, w DSP)[d]
#1	347.60	1797.40	—	755.40	12 044 760	150	12 000
#2	168.11	1579.35	—	1489.25	12 014 743	80	6000
#3	183.30	1597.32	—	1613.40	12 016 887	80	6500
#4	151.07	313.27	—	1334.94	88 910	80	280
#5–#6	202.01	323.58	116.32	359.65	113 718	100	240
#7	373.78	539.02	131.20	566.47	161 003	80	250
#8–#9	355.75	579.91	220.62	633.20	208 056	190	450

[a] kg waste per kg product.[9] [b] kg waste (including energy) per kg product.[11] [c] Impact units per kg of product.[23] [d] kg of CO_2 per kg product.[12]

2.2 Enzymatic transglycosylation of concentrated whey permeate for the synthesis of 1-butyl β-D-galactopyranoside: new setup

Starting from the conditions described in the literature, we replaced the starting biomass with concentrated whey permeate (CWP) to increase lactose concentration and hopefully product titre. It is worth mentioning that CWP is a heterogeneous starting material, generated by cheese whey processing plants. Following whey concentration *via* reverse osmosis and the separation of high value whey proteins *via* ultrafiltration, the resulting permeate stream contains mostly lactose but also mineral salts, fats, and B vitamins.[14,15] Different batches of CWP were employed during the project which exhibited minor differences in their sugar concentrations: Lac (110–142 g per L), Glc (<2%) and Gal (<6%) (Table 1).

As shown in Table 1, the isolated yield of BuGal obtained starting from CWP (31%) (Entry 2) was comparable to that obtained starting from WP (40%) (Entry 1). However, the titre of product recovered, as expected, was higher in the CWP reaction (525 mg) compared to WP (250 mg), due to the 3× higher initial lactose concentration, that addressed its use in this study. As reported in Table 2 (Entry 2) the green assessment of the reaction unit confirmed that using CWP as starting material improved the environmental efficiency compared to WP, proven by lower *E*-factor (168.11 *vs.* 347.60) and GWP (80 *vs.* 150). Conversely, the DSP unit remained the primarily source of environmental burden. While a higher reaction yield slightly lowered the *E*-factor (from 1797.40 to 1579.35), this high value was still attributable to the significant use of solvents for chromatographic purification, reflected also by a comparable composite Eco-impact value (12 014 743). Additionally, the required extension of the incubation time (17 h instead of 2.5 h) raised the E^+-factor from 755.40 to 1489.25.

To develop a new process that allows for easier purification and large-scale setup, efforts were directed toward replacing flash chromatography with liquid/liquid (L/L) extraction. The use of acetone in the reaction mixture promotes the formation of a homogeneous system between CWP (aqueous phase) and 1-BuOH, which are otherwise almost immiscible. Our first attempt focused on selective distillation of acetone (which has a boiling point lower than water and 1-BuOH) to enhance phase separation. Based on literature data,[16] BuGal was expected to partition selectively into the 1-BuOH phase, enabling simple L/L extraction. However, as shown in Fig. S1a, HPLC-ELSD analysis of the separated phases, after acetone removal, revealed that the product was not partitioned exclusively into the organic phase: approximately 20% BuGal remained in the aqueous phase, thus resulting in product loss. Furthermore, this setup made it quite challenging to determine the time needed to complete acetone distillation; moreover, the complex miscibility profile of the ternary reaction mixture prevented from its complete removal, as it was confirmed by the co-extraction of the product in the aqueous phase.

We addressed the poor partitioning of BuGal by switching to a heterogeneous biphasic system, fully replacing the volume of acetone (30%) with 1-BuOH. This new setup proved to be successful yielding quantitative L/L extraction of BuGal into the organic phase, as shown in Fig. S1b, while natural sugars (Glc, Gal) were recovered mainly into the aqueous phase. However, due to the partial miscibility between water and 1-BuOH,[17] the volume of aqueous phase was significantly lower (5–7 mL) than the theoretical expected volume (20 mL). To enhance the separation, 10 mL of aq. 1% w/v NaCl was added to the separatory funnel allowing to recover 15 mL final volume of aqueous stream. The recovered aqueous stream, once the reaction was scaled-up, was successfully used as a growth medium for bacterial cells (see Sections 2.4 and 2.5). Moreover, the partial miscibility between water and 1-BuOH resulted in detectable levels of Glc and Gal co-extracted into the organic phase (Fig. S1b). Therefore, an additional purification step had to be implemented to eliminate the residual sugars (see Section 2.3).

2.3 BuGal purification by using a "catch-and-release" hydrophobic resin

As stated before, the presence of residual natural sugars in the organic phase required the implementation of an additional purification step. We investigated the use of a hydrophobic resin to selectively adsorb BuGal, thus exploiting the lipophilicity of its alkyl chain. Amberlite® XAD4, a hydrophobic resin commonly employed in the industrial recovery of 1-BuOH from acetone/ethanol/butanol fermentation broths, was selected for this purpose.[18,19] The organic phase was first dried under vacuum to remove 1-BuOH, which could otherwise saturate the resin, and then the crude was resolubilized in water (Fig. S2a). The wet resin was added in multiple batches, monitoring product adsorption by HPLC-ELSD. As shown in Fig. S2b, the addition of the last batch of resin resulted in more than 95% of product adsorption, while sugar adsorption remained negligible. These results confirmed that the alkyl chain of BuGal significantly enhances its lipophilicity, enabling its selective separation. Following adsorption, desorption was carried out to isolate the product. The resin was washed several times with EtOH (as suggested by the resin supplier), and the eluates were monitored by HPLC-ELSD until BuGal was no longer detected (Fig. S2c). The collected fractions were dried under vacuum to yield the solid product. Product purity was comprehensibly assessed using a multi-analytical approach by analyzing a ∼10–20 g per L aqueous solution. Enzymatic assays were used to quantify residual sugars while HPLC-ELSD was employed for BuGal quantification. Furthermore, HPLC-RID and q-^{1}HNMR were also employed to generate a complete purity profile of the final product. Avoiding flash chromatography purification led to a reduced product purity due to the retention of residual sugars (Glc and Gal) and to the co-elution of inorganic salts contained in the starting CWP raw material, which were significantly present in the purified product (Fig. S3). While Glc and Gal were quantitatively determined using both enzymatic assays and HPLC-RID, the presence of inorganic salts could not be directly assessed by any of the techniques employed. To address this issue, a qualitative test using silver nitrate precipitation was performed to detect the presence of chlorides (as marker for inorganic chlorides). Chlorides were detected in all

samples resulting from the simplified L/L extraction protocol. In contrast, no chloride detection was observed in the product isolated *via* flash chromatography (Fig. S4). It is worth mentioning that the BuGal quantification results obtained by the different analytical methods were largely in agreement, particularly for the higher-purity samples. Inconsistencies were minimal, with a maximum variation of 10% observed for BuGal values in only a small number of cases (see Table 1). Also, the quantification of Glc and Gal by HPLC-RID and enzymatic assays were consistent, although minor variations were occasionally noted in the Gal values (see Table 1).

The new setup based on L/L extraction on small scale resulted in a yellow gummy product, which after repeated washings with diethyl ether and drying under vacuum, gave 804 mg of a yellow solid (Entry 4, Table 1). The analyses revealed that BuGal represented around 75% of the sample mass based on the calibration curve both in HPLC-ELSD and HPLC-RID (587 mg; 35% isolated yield, Table 1, Entry 4), with minor residues of Glc (8%) and Gal (1.6%). The missing 15.4% of the sample mass was likely attributable to salts derived from the complex CWP matrix, undetectable by HPLC.[15] For comparison, a biphasic reaction mixture was also purified by standard flash chromatography achieving a comparable isolated yield (29% *vs.* 35%, Table 1, Entry 3). However, the resulting 10 g per L solution of the product obtained *via* flash chromatography exhibited a higher BuGal concentration (Fig. S3), directly attributed to the absence of natural sugars and, most importantly, of salts contained in the starting biomass. As BuGal is an intermediate building block for SFAE synthesis and must undergo subsequent reaction and purification steps, we deemed its purity level (around 75%) acceptable at this stage, also taking into consideration the advantages of the new purification setup. The new setup of the reaction (heterogeneous biphasic system instead of homogeneous ternary system) and the simplified downstream processing allowed to reduce by 5-fold the *E*-factor (with purification) from 1597.32 (Entry 3, Table 2) to 313.27 (Entry 4, Table 2) as well as to almost halve the kg of CO_2/kg product produced thanks to the reduction of the amount of solvent used for the downstream. Moreover, a significant reduction (135-fold) was achieved in Eco-impact value (from 12 016 887 to 88 910) thanks to the elimination of dichloromethane, whereas a slight reduction of E^+-factor (from 1613.40 to 1334.94) was observed. In all the calculations, the immobilized biocatalyst had to be counted as a waste due to its low stability as a result of a non-optimal immobilization protocol. Specifically, the binding chemistry of immobilized β-galactosidase from *Aspergillus oryzae* relies on the formation of imine-bonds between the carrier and the enzyme.[20] Stabilization of imines into C–N stable covalent bonds could not be performed owing to enzyme inactivation upon chemical reduction. A screening of different immobilization methods and different immobilization carriers was previously performed, but this immobilization proved to be the most effective.[20] However, the use of an immobilized biocatalyst was essential for the new DSP setup: it guaranteed simple biocatalyst removal from the reaction mixture by filtration and enabled efficient liquid/liquid separation without needing complex steps such as ultrafiltration to prevent from the formation of emulsions which are common with free (crude) enzymes.[21]

Even if green metrics were improved by the new L/L extraction setup, the yet small lab-scale of the biotransformation still hindered the green metrics of the process. Moreover, the small volume of aqueous phase recovered did not allow its use as bacterial cell growth medium. Thus, we further scaled-up the process.

2.4 Scale-up

Following process downstream redesign, the reaction was successfully scaled-up to 1 L (10-fold). After enzyme filtration and phase separation, the aqueous phase enriched in natural sugars (73.8 g per L Glc and 48.2 g per L Gal, Table 3) was recovered (∼168 mL) and the organic phase was dried under vacuum yielding a crude solid, which was purified following the procedures previously described. At this stage, after drying the organic phase, 1-BuOH (∼800 mL) could be recovered for further reuse in a subsequent transglycosylation reaction (see below). The 1 L scale reaction was performed in duplicate confirming the reproducibility of the isolated yield (Table 1, Entries 5 and 6). The isolated yield of the 1 L scale reaction was slightly lower (26%) compared to the reaction performed on a 100 mL-scale (35%). We hypothesized that this difference was a consequence of the scale-factor, as the larger volume (10× increase in reaction volume) may have impaired the efficiency of system mixing and consequently the mass transfer.[22] Upon scale-up, also the purity profile of the final product exhibited some variations. Specifically, the amount of salt residues increased slightly ranging from 20% to 35%, while the natural sugar content remained consistent with the small-scale results: Glc content remained constant (10%), whereas Gal showed a slight increase (ranging from 2.5 to 6.0%). The product was obtained as a yellow sticky solid (Fig. S5a). The *E*-factor whether considering (323.58) or not (202.01) the purification, were similar to the small-scale reaction values, while the E^+-factor was reduced by a 4-fold factor (from 1334.94 to 359.65) (Table 2, Entries 4 and 5). Furthermore, in this case, the implementation of a resource circularity framework into process design significantly improved the overall greenness of the process. Specifically, the straightforward recovery and recycling of 1-BuOH, the reuse of the sugar-enriched aqueous phase for subsequent cell growth, and the recovery and regeneration of the Amberlite® XAD4 adsorbent (which replaces non-regenerable silica) allow these materials to be excluded from the *E*-factor waste

Table 3 Transglycosylation aqueous waste stream composition

Aqueous phase (Table 1)	Volume (mL)	Glc (g per L)[a]	Gal (g per L)[a]
Entry 5	168	73.8	48.2
Entry 6	168	63.3	27.1
Entry 7	300	69.0	56.0
Entry 8	155	56.6	36.2
Entry 9	158	67.9	38.8

[a] Based on calibration curve in HPLC-RID.

calculation, further improving this metric (from 323.58 to 116.32) (Table 2).

To further improve the sustainability of the process and reduce the environmental metrics, a further reaction was performed on a 1.5 L scale (15-fold scale-up). Interestingly, 1-BuOH was recovered from the previous two 1 L scale reactions and recycled for the 1.5 L reaction. The isolated yield was lower than those obtained using fresh 1-BuOH (12%) probably due to water residues from previous reactions that saturated 1-BuOH thereby altering the ratio between the reactants in the biphasic system (Table 1, Entry 7). This was also confirmed by the efficient recovery of the totality of the aqueous phase (300 mL) from the reaction, achieved without the need for supplementary aq. NaCl solution addition. Due to the lower yield obtained in this reaction, the green metrics were slightly higher (Table 2, Entry 7) compared to the reactions performed with fresh 1-BuOH (Table 2, Entries 5 and 6), but aligned with the optimized conditions. Before reusing 1-BuOH the removal of water should be taken into consideration.

Despite achieving favourable green metrics after scaling-up the transglycosylation reaction and implementing an alternative purification setup, further issues emerged during the synthesis of SFAE when using BuGal eluted from XAD4 resin with EtOH (which contained <20% Glc/Gal and 20–40% inorganic salts) (see Section 2.6). We also attempted to optimize the transesterification reaction in order to be able to use the crude BuGal obtained. Temperature was lowered and the reaction setup was switched from a solvent-free system[13] to a solvent-based system. Residual sugars and the inorganic content prevented from obtaining satisfactory yields of SFAE. It became clear that residual sugars and inorganic salts had to be reduced as much as possible. Rather than introducing a costly extra purification step, we hypothesized that the issue stemmed from the water miscibility and polarity of EtOH used in the elution step which could interact with the wet resin and the sugars/inorganic salts allowing their elution. We therefore replaced EtOH (suggested by the resin supplier) with 1-BuOH during the elution step, aiming to sharply concentrate the water eluate containing sugars and salts into the initial fractions. We tested our hypothesis by performing two additional transglycosylation reactions on a 1 L scale and using 1-BuOH to elute the XAD4 resin. As expected, the first two elution volumes contained water (from the wet resin) and a high concentration of Glc and Gal, although BuGal was partly eluted as well (Fig. S6a). These eluates were discarded. The third volume eluted was a mixture of water and 1-BuOH, which immediately exhibited phase separation upon standing for few minutes. This eluate contained few sugars and a high amount of product (Fig. S6b). From the fourth to the seventh fraction only 1-BuOH was eluted containing solely the product. The fractions from 3 to 7 were pooled and dried under reduced pressure affording the product as a white solid (Fig. S5b). HPLC-ELSD analysis confirmed the higher purity of BuGal upon elution with 1-BuOH (Fig. S7). Although discarding the first mixed fractions resulted in a lower yield (14–17%, Table 1), the highly purified BuGal could be successfully used in the subsequent transesterification reaction for SFAE synthesis, achieving yields comparable to the BuGal purified by flash chromatography (see Section 2.6). Due to the lower yield (<20%) the *E*-factor and the GWP of the reaction unit and DSP unit slightly increased (Entry 8–9, Table 2) compared to the same reactions where the product was eluted with EtOH (Entry 5–6, Table 2). The composite Eco-impact is also higher since 1-BuOH has a higher composite score than EtOH (181 *vs.* 90). However, the product obtained by this new setup could be successfully employed as substrate in the subsequent transesterification reaction for the synthesis of SFAE (see Section 2.6).

2.5 Use of aqueous phase enriched in natural sugars for bacterial growth

To further expand the scope of CWP valorization, the transglycosylation aqueous streams rich of natural sugars (Table 3) were recovered from the redesigned biocatalytic process, and directly used as medium for the growth of *E. coli* W105Fe, an engineered strain able to produce bioethanol from different sugars, including the lactose contained in dairy waste.[24] Successful growth of W105Fe in transglycosylation aqueous streams would decrease the cost of inoculum preparation, for which a nutrient-rich medium (LBlac) is currently adopted. The first attempts of cell growth were not successful due to the presence of traces of 1-BuOH in the aqueous phase, considering their miscibility limit of around 10%.[17] To eliminate the traces of 1-BuOH the aqueous phase was concentrated under reduced pressure. A mixture of water and 1-BuOH (20–30 mL) was recovered and let to settle for phase separation. This volume was discarded and substituted with fresh distilled water. The resulting aqueous phase was further used for cell growth. After overnight growth, the strain reached cell density values, in terms of OD_{600}, that were 2.4-fold lower than a control culture grown in LBlac, used as a rich medium (Fig. S8). As shown in Fig. S8, the addition of ammonium sulphate to this aqueous waste stream and the adjustment of pH from 4.5 to about 6.5 (optimal for bacterial growth) resulted in the increase of cell density. In the best condition (0.5 g per L ammonium sulphate with pH adjustment), the strain reached cell density values that were only 1.1-fold lower than LBlac. These data demonstrate that the aqueous phase, generated as waste stream of the transglycosylation reaction, could be adapted for bacterial growth after minimal processing. All the cell density values obtained in aqueous phase samples in the different conditions tested were significantly higher (2.5- to 5.5-fold) than the OD_{600} of cultures grown in CWP (Fig. S8), probably due to the carbon source preference in the aqueous phase (glucose and, to a minor extent, galactose instead of lactose in CWP). To demonstrate that this level of growth was suitable for the preparation of bacterial inoculants for CWP fermentation, the cells grown in the aqueous phase were used to start lactose fermentation in test tubes. Data showed that the obtained bacterial biomass was able to support lactose fermentation at slightly lower values than LBlac, used as a control medium for preculture preparation. In particular, EtOH titre was 21.5 g per L in the samples prepared with the aqueous phase, while a 22.2 g per L concentration was reached in the control. Considering the

consumed sugars, the average conversion yields were 46% and 50% of the maximum theoretical yield, respectively. As a further control, a fermentation was started from a preculture in which pH was not adjusted, and the resulting fermentation performances were lower (EtOH titre 15.6 g per L, and 37% conversion) as expected, confirming the impact of aqueous phase processing to improve bacterial growth.

2.6 Transesterification of BuGal with OAEE for the synthesis of SFAE

BuGal obtained from L/L extraction and elution with EtOH (containing 15–20% Glc/Gal, 20–40% salts) was first subjected to a solvent-free transesterification reaction at 80 °C, catalyzed by Sustine 110 (previously Novozym® 435), using oleic acid ethyl ester (OAEE) as a model acyl donor (BuGal/OAEE ratio 1 : 3) following previous literature.[13] However, in these standard conditions the presence of Glu and Gal and other residues (salts) led to a highly viscous mixture that prevented from efficient mixing and, therefore, mass transfer. At this point, since OAEE is liquid at room temperature and can thus act both as reactant and solvent, we lowered the temperature from 80 °C to 50 °C. Nonetheless, without solvent and even at a lower temperature, sugars become sticky hampering the magnetic stirring and the immobilized lipase dispersion into the reaction mixture. To address this shortcoming, the reaction was performed in the presence of solvent (BuGal/solvent ratio 1 : 3 w/v), using *tert*-amyl alcohol, a lipase-compatible solvent, as reported in the literature.[25,26] The use of solvent resulted in a homogeneous mixture, enabling product formation with 17% SFAE yield (Table 4, Entry 2). The yield was lower compared to the same reaction performed with BuGal isolated by flash chromatography (36%) (Table 4, Entry 1). Thus, the Glc and Gal residual sugars and salts were eliminated from the BuGal intermediate by switching the elution solvent from EtOH to 1-BuOH (see Section 2.4). In this case, the isolated yield (41%) (Table 4, Entry 3) was comparable to that achieved by using BuGal isolated by flash chromatography.

3 Experimental

3.1 General

Ethyl oleate (OAEE) was purchased from Sigma-Aldrich (Italy). Lipase B from *Candida antarctica* immobilized on an acrylic carrier (Sustine 110, previously Novozym® 435) was kindly supplied by Novozymes (Denmark). Amberlite® XAD4 polymeric adsorbent resin was kindly supplied by DuPont (France). Concentrated cheese whey permeate (CWP) was kindly supplied by Serum Italia S.p.A. (Cazzago San Martino, Italy).

β-Galactosidase from *Aspergillus oryzae* was immobilized on glyoxyl-Sepabeads as previously reported.[20] Lactose/D-Galactose Rapid Assay Kit (K-LACGAR) and D-Glucose Assay Kit (GOPOD Format) were purchased from Megazyme NEOGEN (Ireland) and were used to determine lactose, galactose and glucose concentration in CWP and in the solution of BuGal following supplier indications.

The W105Fe bacterial strain was adopted for growth and fermentation assays. This strain is a derivative of *Escherichia coli* W (DSM 1116), engineered for enhanced EtOH production and tolerance, previously described and used for dairy waste fermentation.[24,27] L-broth (LB; 10 g per L NaCl, 10 g per L tryptone, 5 g per L yeast extract) was used as a rich medium for bacterial growth. LB agar plates were made by adding 15 g per L agar.

HPLC analyses for the transglycosylation reaction were run with an HPLC (VWR Hitachi Chromaster, Japan) equipped with a 5310 column oven, a 5260 autosampler, a 5160 pump, and an evaporative light scattering detector (ELSD) (SEDEX 100LT).

HPLC analyses for quantifying sugars and fermentation products in bacterial growth assays were run with an LC-2000 system (Jasco Corp., Tokyo, Japan), autosampler and an RI 10A refractive index detector (RID) (Shimadzu Corp., Kyoto, Japan).

q-^{1}HNMR spectra were recorded at 400 MHz on a Bruker AVANCE 400 spectrometer equipped with a TOPSPIN software package (Bruker, Karlsruhe, Germany) at 298 K. DMSO-*d*6 (Merck) was used as solvent and 1,3,5-trimethoxybenzene was used as internal standard. ^{1}H chemical shifts (δ) are given in parts per million (ppm) and referenced to the solvent signals (δH 2.51 ppm). Relaxation delay was set to 60 s and acquisition time at 8 s, 20 scans, automatic phase and baseline correction were used.

3.2 Enzymatic transglycosylation of CWP for the synthesis of 1-butyl β-D-galactopyranoside

Before setting up the transglycosylation reactions, CWP was filtered by vacuum filtration using a sintered glass filter to remove a white precipitate that was observed after thawing. The pH of CWP (pH 6.7), appearing as a clear yellow solution, was adjusted to 4.5 by adding 85% H_3PO_4. Two reaction systems were evaluated on a 100 mL scale: homogeneous ternary system (CWP/1-BuOH/acetone 20 : 50 : 30) and biphasic system (CWP/1-BuOH 20 : 80). The biphasic system was also scaled-up to 1 L

Table 4 Transesterification of BuGal with OAEE catalyzed by Sustine 110[a]

Entry	Source of BuGal	Isolated yield (%)
Entry 1	BuGal from flash chromatography	34–36
Entry 2	BuGal from L/L extraction + XAD4 + elution with EtOH	17.5
Entry 3	BuGal from L/L extraction + XAD4 + elution with 1-BuOH	41

[a] Reaction conditions: BuGal (0.720 mmol), OAEE (670 mg, 2.160 mmol), *tert*-amyl alcohol (500 μL), Sustine 110 (10% w/w), 50 °C, 72 h.

using "fresh" 1-BuOH (2 reactions) as well as to 1.5 L using 1-BuOH recovered and recycled from previous reactions. Transglycosylation reactions were set up in a 250 mL–3 L round-bottom flask. The reaction was started by the addition of immobilized *Ao*Gal-GLX-Sepabeads (2–30 g, 2–30 U) and the mixture was maintained under stirring at 30 °C for 17 hours. The reaction was stopped by filtration under vacuum of the immobilized enzyme. The immobilized enzyme was washed with 1-BuOH (10–100 mL). The downstream was performed by standard flash chromatography purification (see Section 3.3) or by L/L extraction and subsequent "catch and release" strategy by using Amberlite® XAD4 resin (see Section 3.4).

3.3 BuGal purification by flash chromatography

For standard flash chromatography purification, the filtrate was evaporated under reduced pressure. The obtained crude was solubilized in MeOH and added with silica (*ca.* 2.5 g), dried under reduced pressure, loaded on the silica column and purified (DCM/MeOH, 90 : 10). The purified product was obtained as a light-yellow solid: 525 mg of BuGal were obtained from the ternary system and 486 mg from the biphasic system performed on a 100 mL scale. The detailed reaction procedure is reported in the SI file.

3.4 BuGal purification by liquid/liquid extraction and "catch and release" strategy by using Amberlite® XAD4

The transglycosylation reaction was performed as described in the previous section. At the endpoint, after filtration and enzyme washing (with 10–250 mL 1-BuOH), the filtrate was transferred into a separatory funnel, washed with a 1% w/v NaCl solution (10–100 mL) and then it was left to settle overnight to achieve phase separation. The two phases were then recovered separately and analyzed by HPLC-ELSD to determine their composition. The organic phase was dried under reduced pressure, 1-BuOH was recovered to be reused, and the crude was resolubilized in H_2O (20–50 mL). Subsequently, Amberlite® XAD4 polymeric adsorbent resin was washed with water to eliminate NaCl and Na_2CO_3 used as preservatives. The wet resin (2–10 g × 7 batches) was added to the solution under mechanical stirring. Product adsorption onto the resin was monitored by HPLC-ELSD until its removal from the solution was almost complete. The resin was then filtered and subjected to five consecutive washings with EtOH (following supplier indications) (50 mL per batch) under mechanical stirring at room temperature until product detection in the filtrate (HPLC-ELSD) was almost negligible. The EtOH fractions containing the product were then collected and dried under reduced pressure. After drying a light-yellow oily solid was obtained which required some washings with diethyl ether (5–50 mL) and drying under vacuum. The product was obtained as a yellow solid (7.0–7.8 g) (Fig. S5a).

An alternative elution was setup by using 1-BuOH instead of EtOH. In this case the elution was performed by adding 5 mL of solvent in the first elution step, then 20 mL and finally 50 mL 1-BuOH for 5 times. In this case the first two volumes eluted were composed of water (and thus discarded), the third was a mixture of water and 1-BuOH and from the fourth volume onwards just 1-BuOH was recovered. The product was obtained as a white solid (3.1–3.3 g) (Fig. S5b).

The detailed reaction procedure is reported in the SI file.

3.5 BuGal characterization

To determine the purity of BuGal obtained both by flash chromatography purification and "catch and release" strategy, a solution (~10 g per L) was prepared by dissolving in water the solid obtained and analyzed by HPLC-ELSD and HPLC-RID. HPLC analyses were carried out by using a Supelco C-610H column (30 cm × 7.8 mm) (59320-U, Sigma-Aldrich) and 0.1% v/v formic acid (for ELSD detection) or 0.1% v/v phosphoric acid (for RID detection) in water as the mobile phase. The column was thermostated at 30 °C, flow rate was set at 0.5 mL min^{-1}. The detection by using the ELSD was carried out applying the following parameters: temperature: 60 °C; gain: dynamic; filter: 10 s; P: 3.5 bar. Retention times were: lactose (Lac) = 10.9 min; glucose (Glc) = 12.5 min, galactose (Gal) = 13.4 min, BuGal = 19.0 min.

The same solution was used for the quantification of the residual Glc and Gal present in the final product by two spectrophotometric assays using respectively the commercially available kit K-GLUC (GOPOD format) and the Lactose/D-Galactose Rapid Assay Kit (K-LACGAR), following the supplier's instructions.

^{1}H qNMR analyses were also performed to determine the titre of BuGal in the samples. The solid obtained from each reaction (5–20 mg) was added with the internal standard 1,3,5-trimethoxybenzene (1.0 mg) and solubilized in DMSO-*d*6 (700 μL). For quantification the area of the anomeric signal of BuGal (4.05 ppm) and the area of the aromatic protons of the internal standard (6.09 ppm) were considered (Fig. S9–S15).

3.6 Green metrics calculations

The detailed calculations are reported in the SI file.

3.7 Bacterial growth assays

A single bacterial colony from a streaked LB agar plate was used to inoculate 2 mL of LB supplemented with 40 g per L lactose (LBlac) that was incubated at 37 °C, 220 rpm overnight. The grown culture was 100-fold diluted in 2 mL of different media based on the glucose-rich waste of transglycosylation and grown in the same conditions as above for 24 hours in 15 mL tubes. After a 24-hour growth, bacterial density was measured in 200-μL samples in terms of optical density at 600 nm (OD_{600}) by using the Infinite F200 microplate reader (Tecan). Raw absorbance data were processed by subtracting the raw OD_{600} value of sterile media, used as a background. Different processing methods were evaluated for the transglycosylation aqueous stream to improve growth conditions: removal of the residual 1-BuOH under vacuum; adjustment of pH to about 6.5 by NaOH; addition of ammonium sulphate (0.5 g per L) as a nitrogen source. Growth in CWP and LBlac were also tested as controls.

3.8 Fermentation

Fermentation assays were carried out in test tubes to produce EtOH from lactose, present in CWP at high levels, using bacterial inoculants grown in the transglycosylation aqueous stream. Briefly, overnight cultures of W105Fe grown in 1.16 mL of transglycosylation waste (with or without pH adjustment to about 6.5, as indicated) or LBlac were centrifuged (4000 rpm, 15 min), the supernatant was discarded, and the bacterial pellet resuspended in 5 mL of fermentation medium (CWP supplemented with 150 mM PIPES and 0.5 g per L ammonium sulphate) in a 50 mL tube. The suspended culture was incubated at 37 °C and 220 rpm, for 72 h. The used growth conditions guaranteed high-oxygen fermentation by the engineered strain in a buffered medium to limit the pH drop due to CO_2 and production of organic acids from endogenous genes that were not deleted, as previously described.[27] Samples were withdrawn at the end of fermentation, filtered (0.2 μm) and stored at −20 °C until HPLC analysis for determining residual substrates (lactose, glucose, and galactose) and the main fermentation product (EtOH). Fermentation performance was expressed as EtOH titre, *i.e.*, its final concentration, and yield, *i.e.*, the percentage of the theoretical maximum conversion yield, considering that one molecule of substrate can be converted into four (for lactose) or two (for glucose and galactose) molecules of EtOH.

3.9 Synthesis of *n*-butyl 6-*O*-oleoyl-β-D-galactopyranoside

BuGal (0.720 mmol) (variable amount based on % purity) and oleic acid ethyl ester (OAEE; 670 mg, 2.160 mmol) were suspended in *tert*-amyl alcohol (500 μL) in a 4 mL glass vial. The reaction was started through the addition of Sustine 110 (10% w/w) and the mixture was maintained under magnetic stirring at 50 °C for 72 hours. At the endpoint, the mixture was diluted with ethyl acetate (5 mL), the enzyme was eliminated by decantation and the solvent was removed under reduced pressure. The crude reaction mixture was purified by dry column chromatography (gradient elution: from 100% *n*-hexane to 3 : 7 *n*-hexane/ethyl acetate). The purified product was obtained as a white solid.

4 Conclusions

Circular chemistry, driven by the integration of green metrics and circular design principles, is a key technology for the transition from traditional linear production models towards closed-loop manufacturing schemes.

The integration of biocatalysis with the upcycling of industrial waste-streams enables, in principle, the design of clean processes that recover and reintroduce waste into production cycles in accordance also with UN SDG 12. This synergy fosters the creation of value chains where side-streams are valorized, bridging the gap between waste management and sustainable chemistry. Biocatalysis is a key player of this circular transition as enzymes are exquisitely selective, renewable, biodegradable, and active under environmentally friendly reaction conditions.

However, a qualitative adherence to green chemistry and circular economy principles can be deceptive since enzymatic transformations and the use of renewable biomass are not sustainable by definition. To be truly sustainable, modern manufacturing processes must be designed from the laboratory stage to mitigate environmental "hot spots" within both the reaction and, especially, the downstream unit. This design-led approach is essential to demonstrate that greening reaction conditions is technically feasible. Furthermore, a "circularity mindset" is required already at the bench scale to ensure the recovery and reuse of reaction media and solvents which represent a key prerequisite for industrial adoption.

While the biocatalytic upcycling of industrial side-streams is increasingly documented in literature, practical examples of scalable, green metrics-optimized processes remain still scarce.

We applied a set of green metrics to the study and development of the biocatalyzed synthesis of 1-butyl β-D-galactopyranoside (or BuGal), a well-established bioprocess in our lab, to guide process optimization towards a reduced environmental footprint. The analysis of the bioprocess, from concentrated whey permeate (CWP), an abundant waste stream of dairy industry, to product downstream processing, assisted us in identifying and redesigning "hot spots" with a beneficial outcome on the greenness of the biotransformation. The results show a generalized reduction in the *E*-factor(s), particularly relevant when these metrics comprehensively consider purification step, by-product recycling (to feed a fermentation as a secondary process), and solvent reuse. *E*-factor decreased by about 10-fold, solvent eco-impact by about 60-fold, and Global Warming Potential (GWP) by about 30-fold with respect to the starting protocol, while the quality of the final product was maintained and the process streamlined.

The combination of biocatalysis (BuGal synthesis) and fermentation (bio-EtOH production) can be integrated into a biorefinery for the obtainment of fully bio-based sugar fatty acid esters (SFAE). Process synergies between biocatalytic and fermentation steps are beyond the use of a common feedstock. Bacterial biomass can be prepared from the aqueous waste of transglycosylation, effectively replacing other growth media, as demonstrated in this work. In addition, the microbially produced EtOH could be used as an energy source to fuel process operations as well as the synthesis of fatty acid ethyl esters (FAEE) as precursors of SFAE.

Author contributions

LC: investigation; validation; visualization. LP: investigation; formal analysis, writing – original draft. MC: investigation, validation; visualization. DD: investigation; validation; visualization. PM: conceptualization; formal analysis; resources; data curation; supervision; project administration; funding acquisition. ARA: investigation; methodology. DU: conceptualization; methodology; resources; data curation; supervision; project administration; writing – original draft. MSR: conceptualization; methodology; validation; data curation; visualization; supervision; writing – original draft. All authors: writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d6su00180g.

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