SUPPLEMENTARY INFORMATION

to the article

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

Lorenza Cassano,[a] Lorenzo Pasotti,[b] Michela Casanova,[b] Debora Dallera,[b] Paolo Magni,[b] Andrés R. Alcántara,[c] Daniela Ubiali,[a]* and Marina Simona Robescu[a]*

[a.] *Department of Drug Sciences, University of Pavia, Viale Taramelli 12, I-27100, Pavia, Italy.*
[b.] *Department of Electrical, Computer and Biomedical Engineering, University of Pavia, via Ferrata 5, Pavia, Italy.*
[c.] *Department of Chemistry in Pharmaceutical Sciences, University Complutense di Madrid, Plaza de Ramon y Cajal s/n, Madrid, Spain.*

*Corresponding authors: daniela.ubiali@unipv.it

marinasimona.robescu@unipv.it

Table of contents

Supplementary Figures

q-^{1}H-NMR

Green metrics calculations

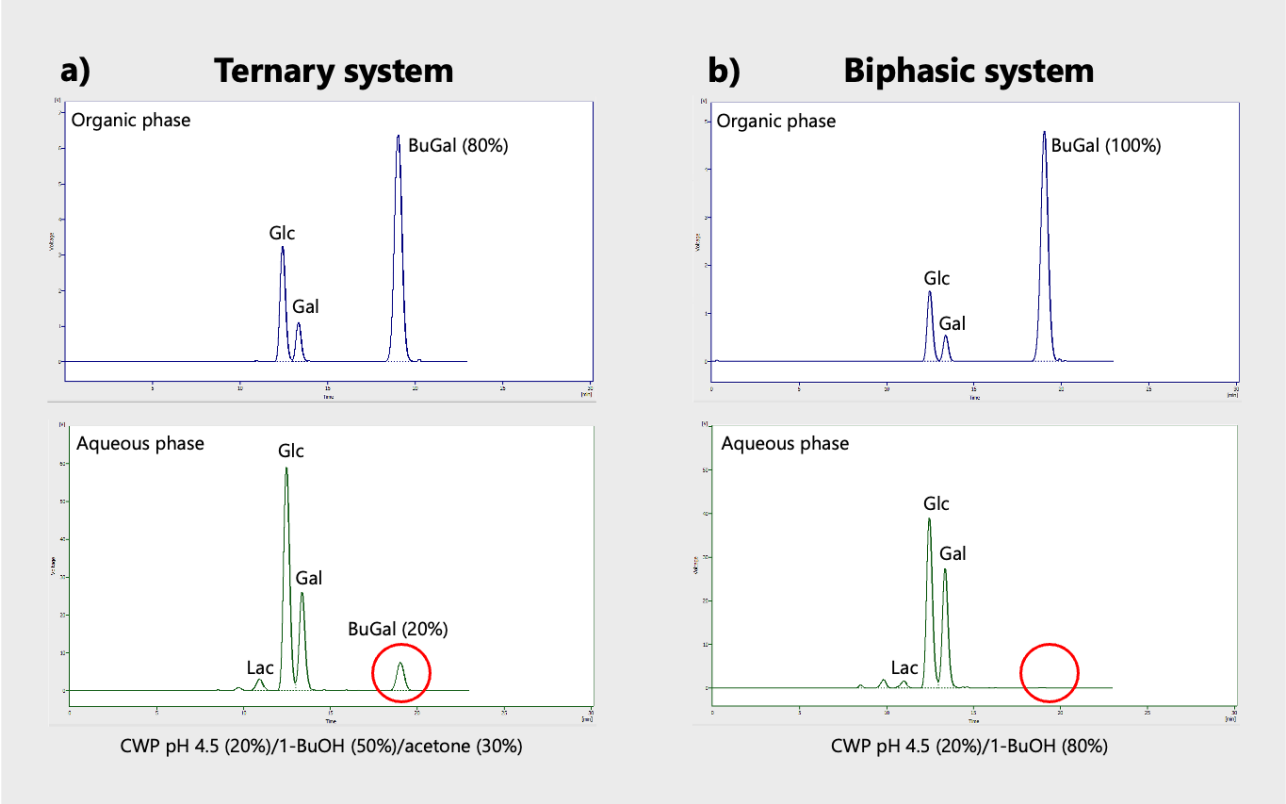

Figure S1. HPLC-ELSD monitoring of the aqueous and organic phases: (**a**) ternary system and (**b**) biphasic system.

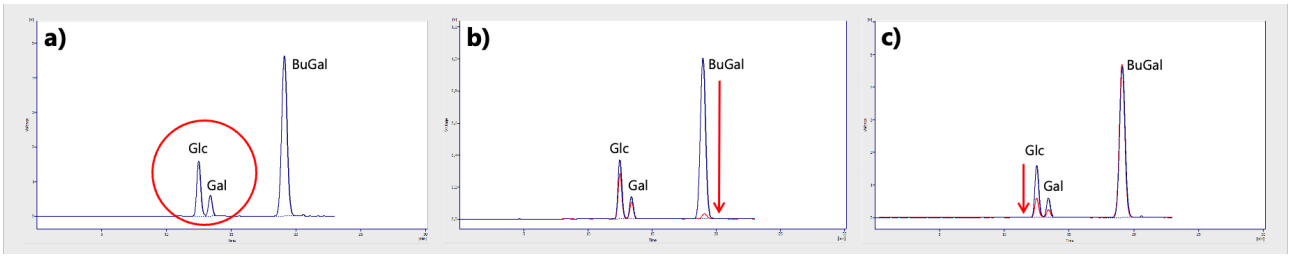

Figure S2. HPLC-ELSD monitoring of organic phase purification process by catch and release strategy using Amberlite® XAD4: (**a**) initial organic phase composition, (**b**) BuGal adsorption onto the resin (red chromatogram and red arrow), and (**c**) product desorption by EtOH (red chromatogram) overlapped with the starting solution (blue chromatogram).

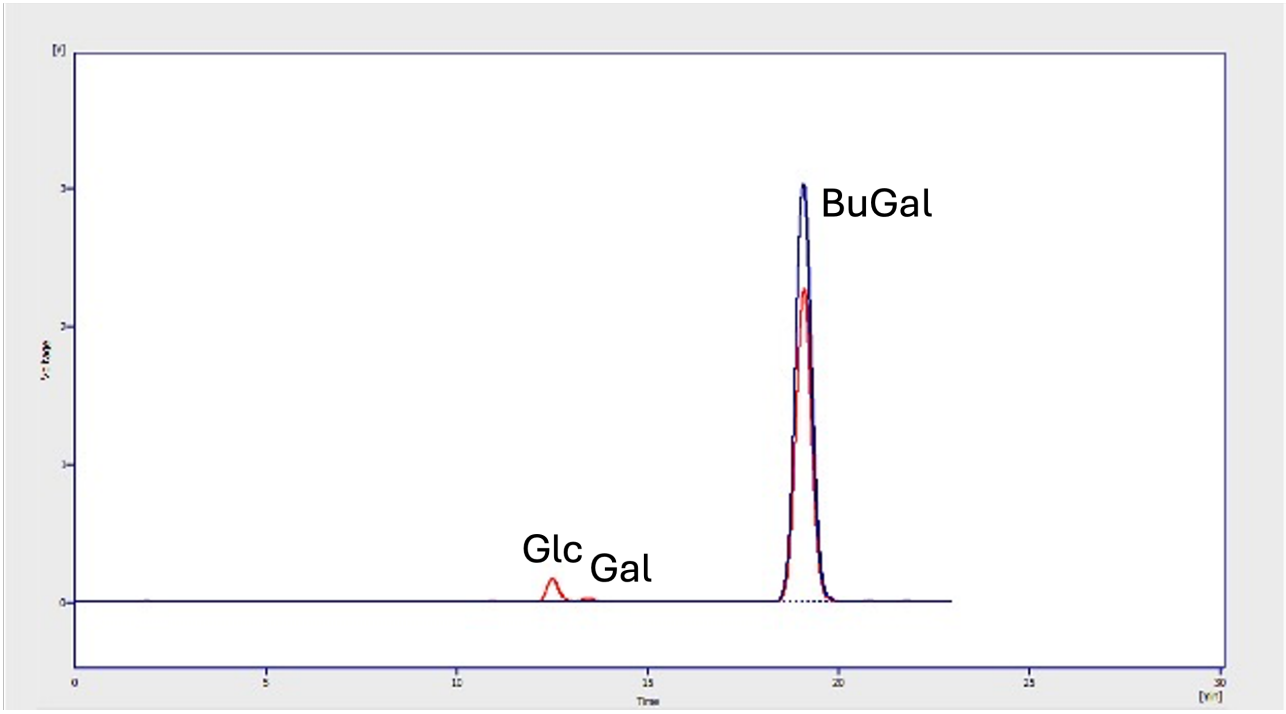

Figure S3. HPLC-ELSD analysis of a solution of BuGal purified via flash chromatography (10.4 mg/mL) (blue) and via L/L extraction + catch and release strategy + elution with EtOH (10.8 mg/mL) (red).

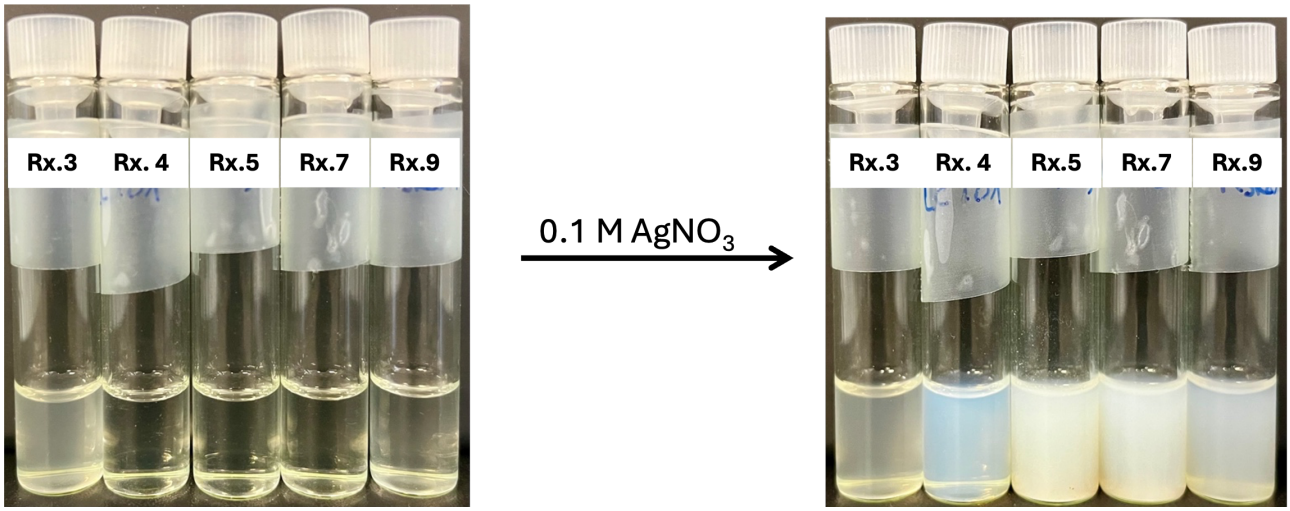

Figure S4. Silver nitrate precipitation test on BuGal from flash chromatography (Rx. 3), L/L extraction + catch and release strategy +elution with EtOH (Rx. 4, 5, 7) and L/L extraction + catch and release strategy+ elution with 1-BuOH (Rx. 9).

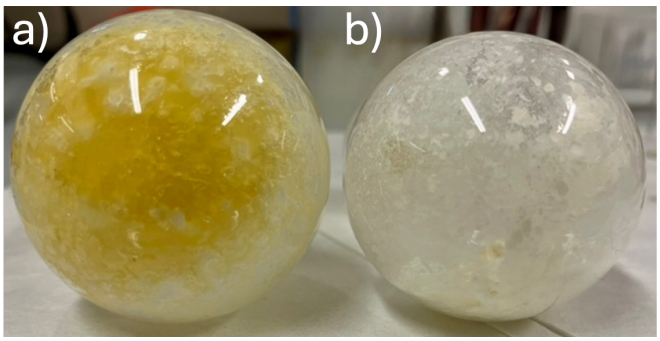

Figure S5. BuGal obtained via L/L extraction + catch and release strategy (**a**) after elution with EtOH and (**b**) after elution with 1-BuOH.

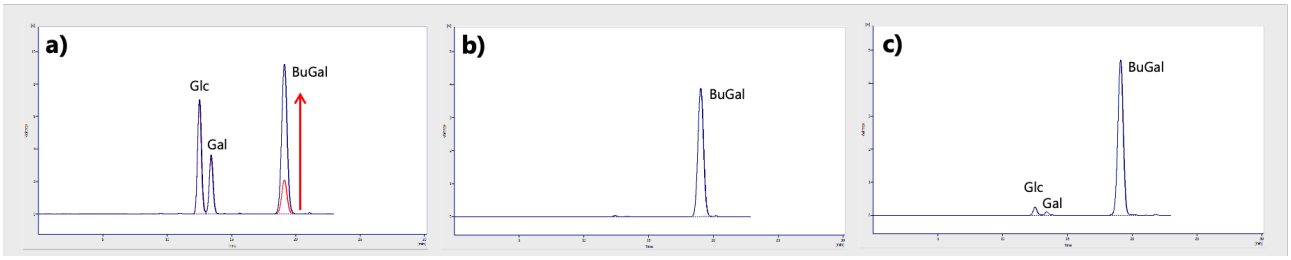

Figure S6. HPLC-ELSD monitoring of organic phase purification process by catch and release strategy and elution with 1-BuOH: (**a**) Initial water volumes (I (red) and II (blue)) from 1-BuOH elution (these volumes were discarded even if BuGal was present), (**b**) first 1-BuOH volume (III) and (**c**) final product obtained.

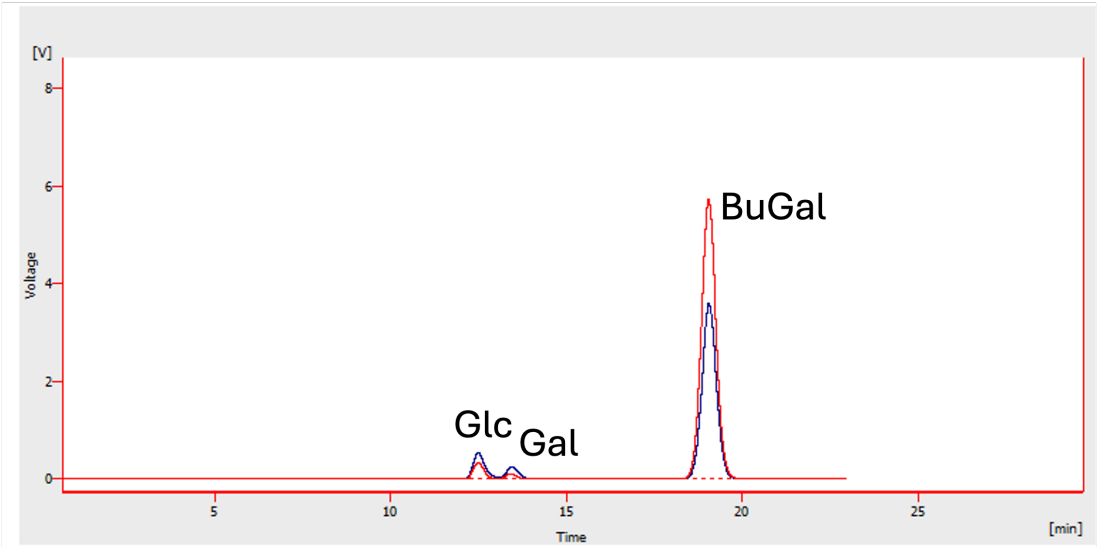

Figure S7. HPLC-ELSD analysis of a solution of BuGal purified via L/L extraction + catch and release strategy + elution with 1-BuOH (10.7 mg/mL) (red) and a solution of BuGal purified with L/L extraction + catch and release strategy + elution with EtOH (11.1 mg/mL) (blue).

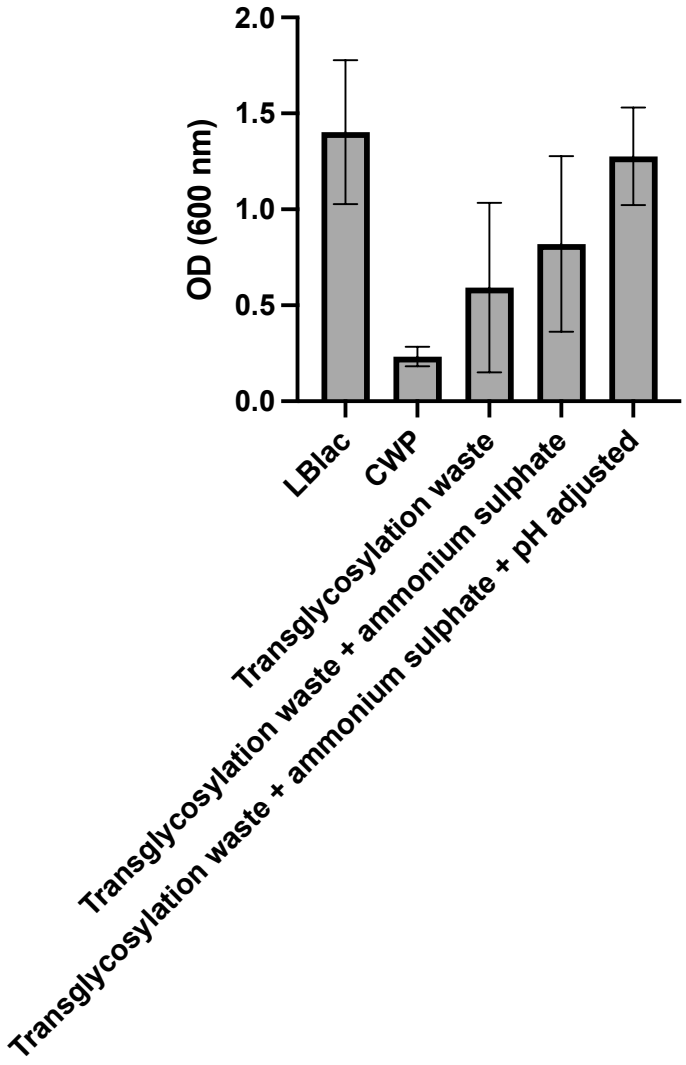

Figure S8. Cell growth performance in the transglycosylation waste stream. Bacterial densities (in terms of optical density at 600 nm; OD600) reached after 24-h cultivation in the indicated media. Statistical comparisons via t-test showed that the cell density reached in CWP is significantly lower than in all the other conditions, and that the cell density reached in LBlac is significantly higher than in transglycosylation waste (untreated or with ammonium sulfate added) with a p-value cutoff of 0.05. Bars correspond to the average value of at least two independent replicates, and error bars indicate standard deviations.

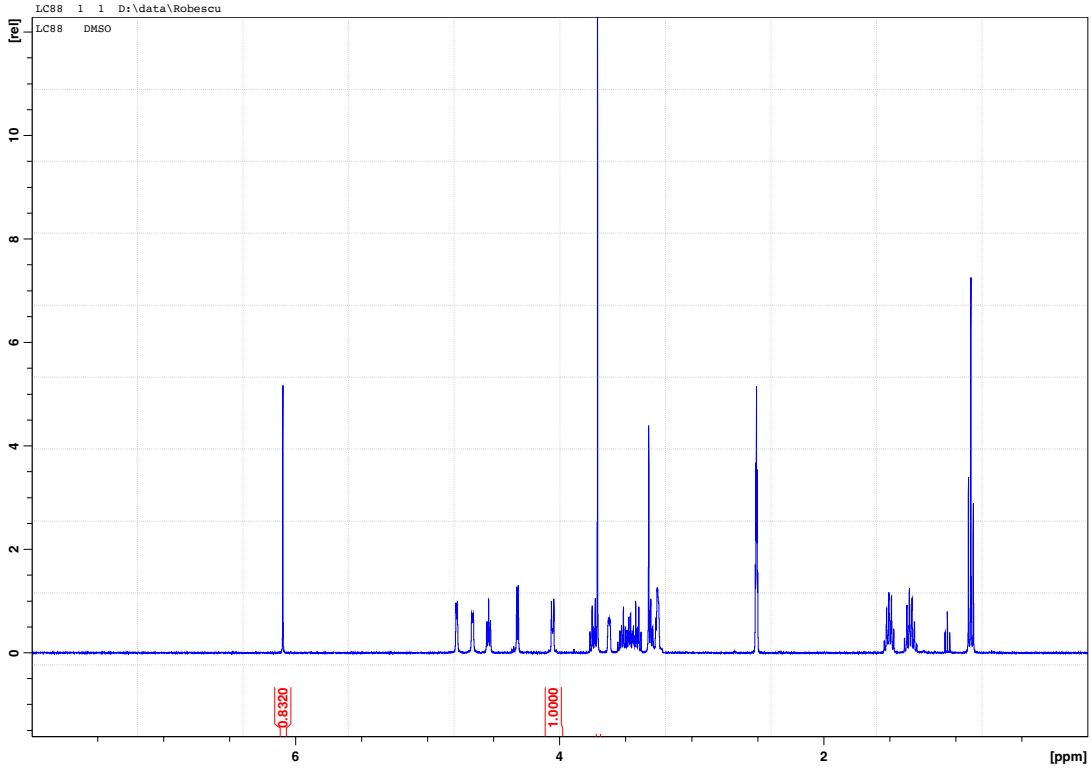

Figure S9. ^{1}H-NMR of BuGal isolated by flash chromatography (Entry 3, Table 1).

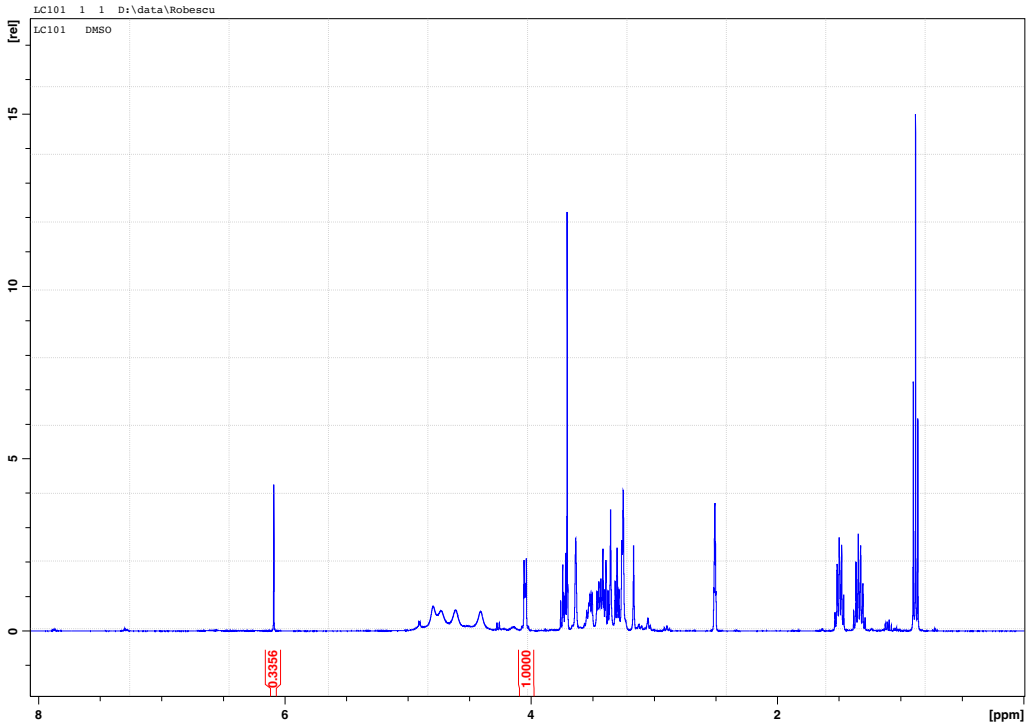

Figure S10. ^{1}H-NMR of BuGal isolated by L/L extraction and elution from XAD with EtOH (Entry 4, Table 1).

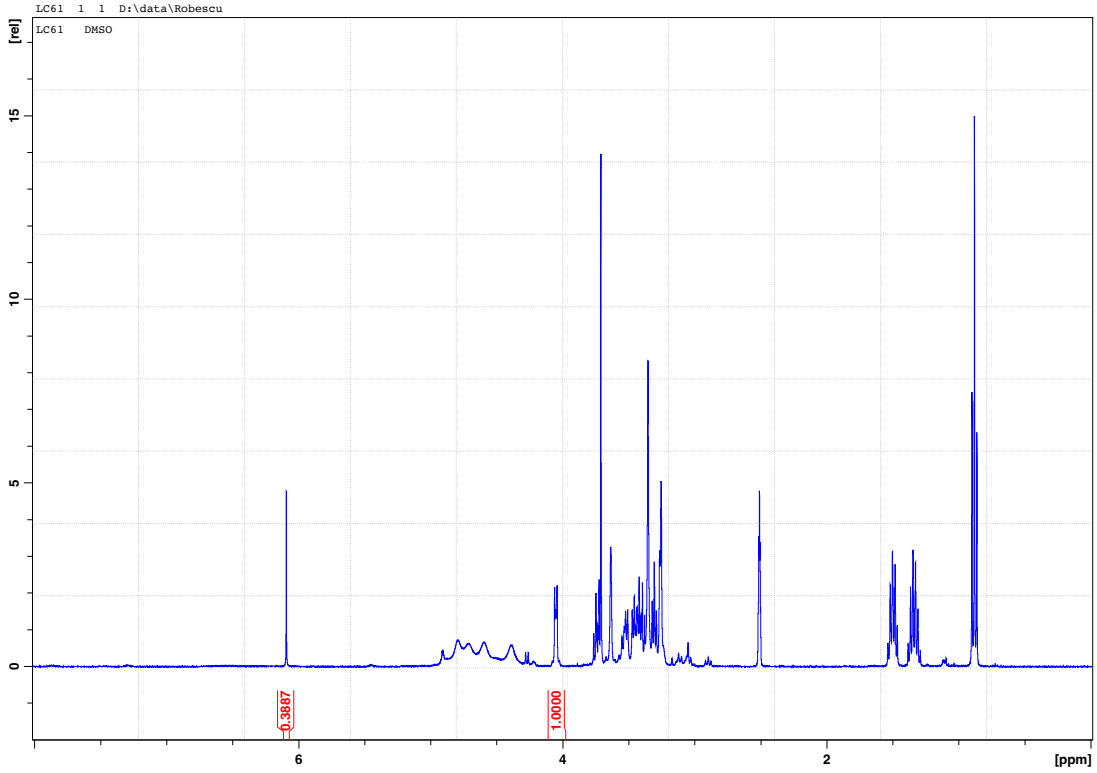

Figure S11. ^{1}H-NMR of BuGal isolated by L/L extraction and elution from XAD with EtOH (Entry 5, Table 1).

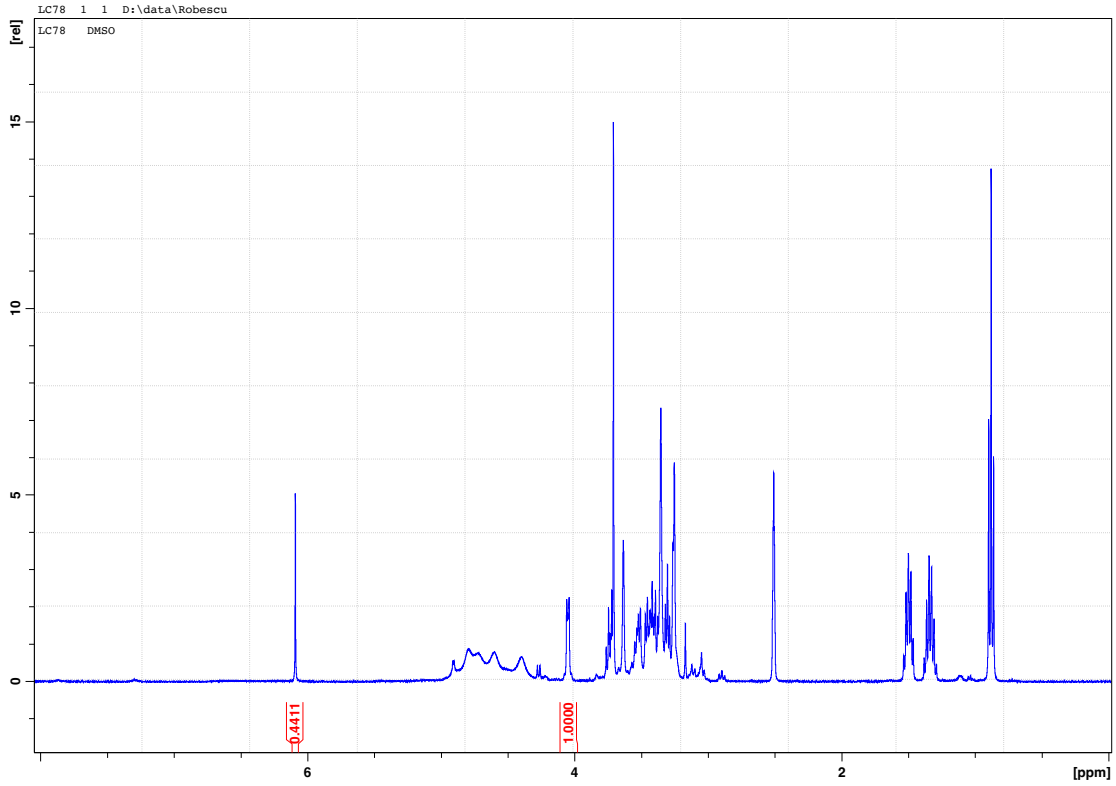

Figure S12. ^{1}H-NMR of BuGal isolated by L/L extraction and elution from XAD with EtOH (Entry 6, Table 1).

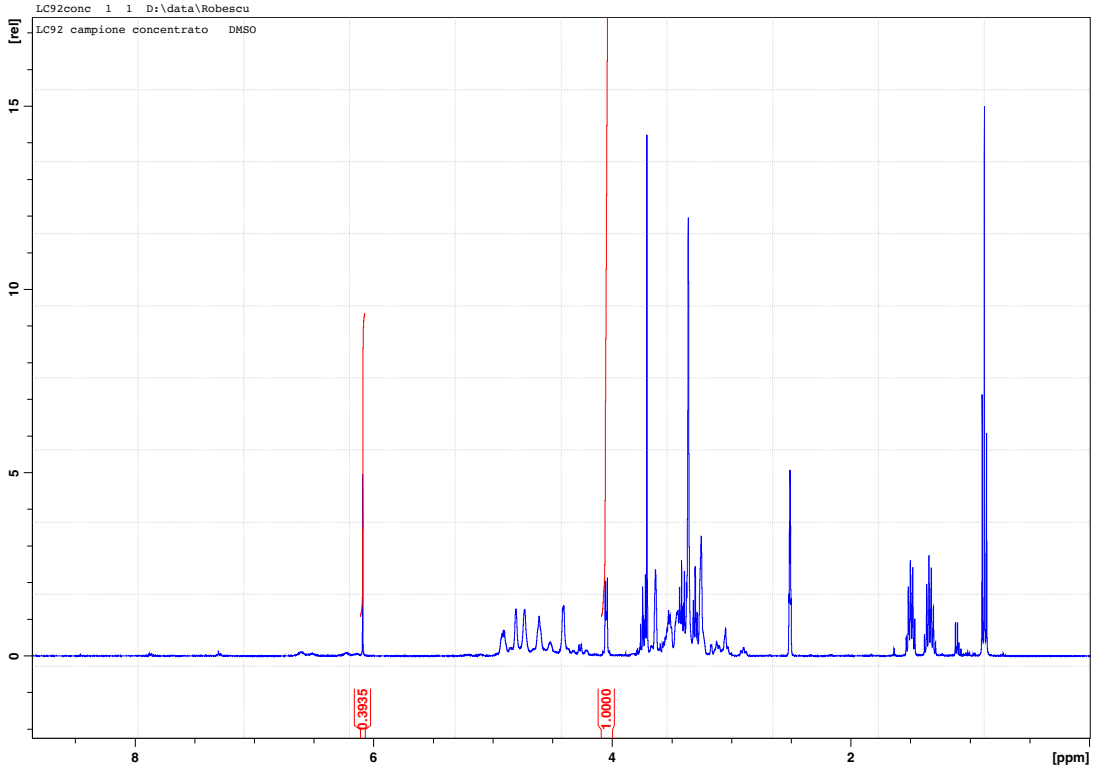

Figure S13. ^{1}H-NMR of BuGal isolated by L/L extraction and elution from XAD with EtOH (Entry 7, Table 1).

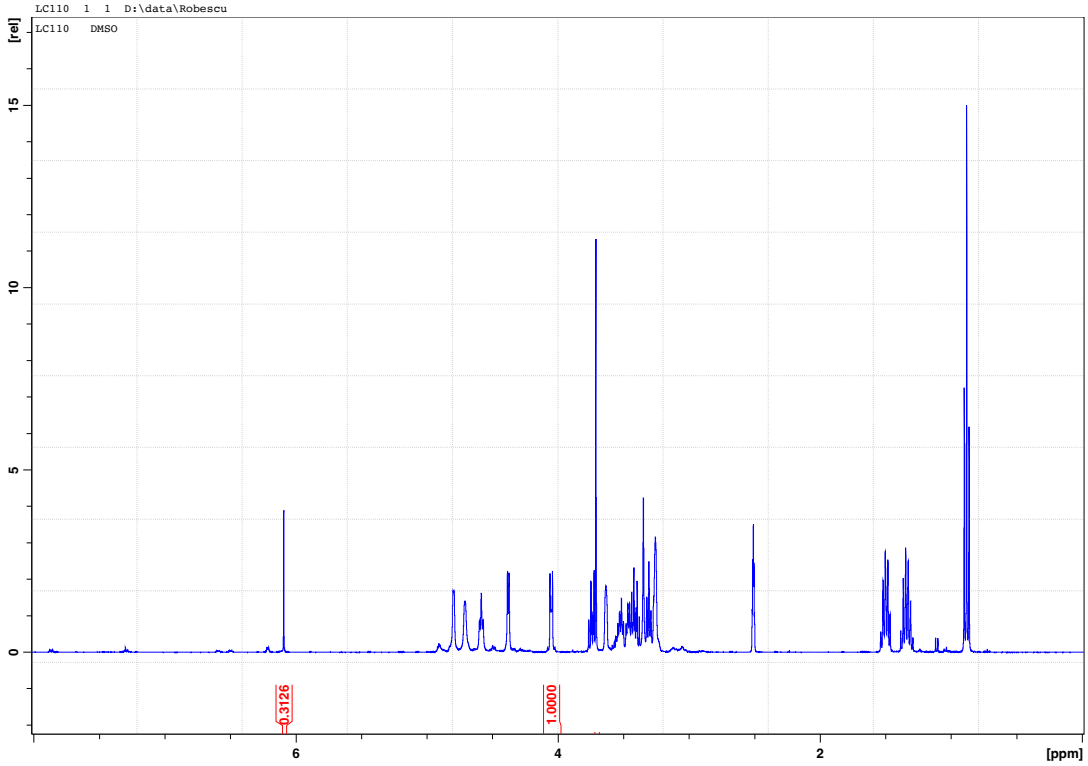

Figure S14. ^{1}H-NMR of BuGal isolated by L/L extraction and elution from XAD with 1-BuOH (Entry 8, Table 1).

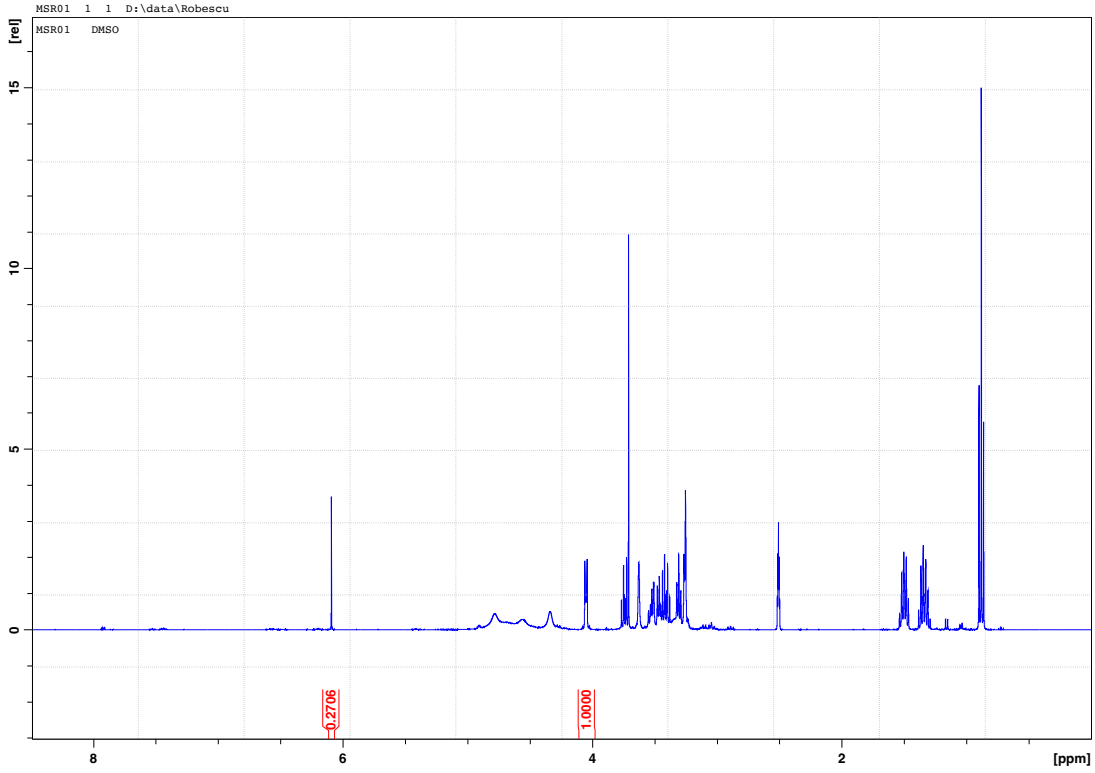

Figure S15. ^{1}H-NMR of BuGal isolated by L/L extraction and elution from XAD with 1-BuOH (Entry 9, Table 1).

Reaction #1: Transglycosylation of WP in ternary system & flash chromatography

The pH of WP (45 g/L) was adjusted to 4.5 with HCl 1 M. WP (20 mL, corresponding to 2.6 mmol of lactose), 1-BuOH (50 mL), and acetone (30 mL) were mixed in a round bottom flask. The immobilized GalAo (2 g, 2 IU) was added to the reaction and the mixture was maintained under magnetic stirring at 30 °C for 2.5 hours. The reaction was stopped by filtration of the enzyme, and then the immobilized enzyme was washed with 1-BuOH (10 mL). The filtrate was evaporated under reduced pressure, the crude was solubilized in MeOH (10 mL) and added with silica (≈ 2.5 g) to adsorb the components of the reaction mixture. The suspension was dried under reduced pressure and dried silica was loaded on top of a packed silica column for flash chromatography purification (DCM/MeOH, 90:10). The product was obtained as a light-yellow powder (yield: 40%, 250 mg) (Table 1, Entry 1).[1]

Reaction #2: Transglycosylation of CWP in ternary system & flash chromatography

Before setting up the transglycosylation reactions, CWP (126 g/L lactose) was filtered by vacuum filtration using a sintered glass filter to remove a white precipitate that was observed after its thawing and to obtain a clear yellow solution. The pH of CWP (pH 6.7) was adjusted to 4.5 by adding 85% H_3PO_4 (~40 μL). CWP (20 mL, corresponding to 7.4 mmol of lactose), 1-BuOH (50 mL) and acetone (30 mL) were mixed in a round bottom flask. The immobilized GalAo (2 g, 2 IU) was added to the reaction and the mixture was maintained under magnetic stirring at 30 °C for 17 h hours. The reaction was stopped by filtration of the enzyme, and then the immobilized enzyme was washed with 1-BuOH (10 mL). The filtrate was evaporated under reduced pressure, the crude was solubilized in MeOH and added with silica (≈ 2.5 g) to adsorb the components of the reaction mixture. The suspension was dried under reduced pressure and dried silica was loaded on top of a packed silica column for flash chromatography purification (DCM/MeOH, 90:10). The product was obtained as a light-yellow powder (yield: 30%, 525 mg) (Table 1, Entry 2).

Reaction #3: Transglycosylation of CWP in biphasic system & flash chromatography

Before setting up the transglycosylation reactions, CWP (126 g/L lactose) was filtered by vacuum filtration using a sintered glass filter to remove a white precipitate that was observed after its thawing and to obtain a clear yellow solution. The pH of CWP (pH 6.7) was adjusted to 4.5 by adding 85% H_3PO_4 (~40 μL). CWP (20 mL, corresponding to 7.4 mmol of lactose) and 1-BuOH (80 mL) were mixed in a round bottom flask. The immobilized GalAo (2 g, 2 IU) was added to the reaction and the mixture was maintained under magnetic stirring at 30 °C for 17 h hours. The reaction was stopped by filtration of the enzyme, and then the immobilized enzyme was washed with 1-BuOH (10 mL). The filtrate was evaporated under reduced pressure, the crude was solubilized in MeOH and added with silica (≈ 2.5 g) to adsorb the components of the reaction mixture. The suspension was dried under reduced pressure and dried silica was loaded on top of a packed silica column for flash chromatography purification (DCM/MeOH, 90:10). The product was obtained as a light-yellow powder (yield: 28%, 485 mg) (Table 1, Entry 3).

Reaction #4: Transglycosylation of CWP in biphasic system & simplified work-up (elution with EtOH)

Before setting up the transglycosylation reactions, CWP (120 g/L lactose) was filtered by vacuum filtration using a sintered glass filter to remove a white precipitate that was observed after its thawing and to obtain a clear yellow solution. The pH of CWP (pH 6.7) was adjusted to 4.5 by adding 85% H_3PO_4 (~40 μL). CWP (20 mL, corresponding to 7.2 mmol of lactose) and 1-BuOH (80 mL) were mixed in a round bottom flask. The immobilized GalAo (2 g, 2 IU) was added to the reaction and the mixture was maintained under magnetic stirring at 30 °C for 17 h hours. The reaction was stopped by filtration of the enzyme, and then the immobilized enzyme was washed with 1-BuOH (10 mL). The filtrate was transferred into a separatory funnel, washed with a 1% w/v NaCl solution (10 mL) and then it was left to settle overnight to achieve phase separation. The two phases were then recovered separately. The aqueous phase (15 mL), once the reaction was scaled-up, was used for cell growth. The organic phase was dried under reduced pressure, 1-BuOH was recovered to be reused, and the crude was resolubilized in H_2O (20 mL). Subsequently, Amberlite® XAD4 polymeric adsorbent resin was washed with H_2O to eliminate the NaCl and Na_2CO_3 used as preservatives. The wet resin (2 g x 7 batches) was added to the solution under mechanical stirring. Product adsorption onto the resin was monitored by HPLC-ELSD until its almost complete removal from the solution. The resin was then filtered and subjected to five consecutive washings with EtOH (following supplier indications) (10 mL/batch) under mechanical stirring at room temperature until no product was anymore detected in the filtrate by HPLC-ELSD analysis. 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 mL) and vacuum. The product was obtained as a yellow powder (804 mg_{powder}) (Table 1, Entry 4). The powder contained: 73% BuGal (586.92 mg; yield: 35%), 27% others (Glc, Gal and inorganic salts) (217.88 mg).

Reaction #5–#6: Transglycosylation of CWP in biphasic system & simplified work-up (elution with EtOH) 1 L scale

Before setting up the transglycosylation reactions, CWP (123 g/L lactose) was filtered by vacuum filtration using a sintered glass filter to remove a white precipitate that was observed after its thawing and to obtain a clear yellow solution. The pH of CWP (pH 6.7) was adjusted to 4.5 by adding 85% H_3PO_4 (~400 μL). CWP (200 mL, corresponding to 71.8 mmol of lactose) and 1-BuOH (800 mL) were mixed in a round bottom flask. The immobilized GalAo (20 g, 20 IU) was added to the reaction and the mixture was maintained under magnetic stirring at 30 °C for 17 h hours. The reaction was stopped by filtration of the enzyme, and then the immobilized enzyme was washed with 1-BuOH (100 mL). The filtrate was transferred into a separatory funnel, washed with a 1% w/v NaCl solution (100 mL) and then it was left to settle overnight to achieve phase separation. The two phases were then recovered separately. The aqueous phase (168 mL) containing 63.3 g/L – 73.8 g/L Glc and 27.1 g/L – 48.2 g/L Gal (Table 3) was used for cell growth. The organic phase was dried under reduced pressure, 1-BuOH (1007 mL) was recovered to be reused, and the crude was resolubilized in H_2O (50 mL). Subsequently, Amberlite® XAD4 polymeric adsorbent resin was washed with H_2O to eliminate the NaCl and Na_2CO_3 used as preservatives. The wet resin (10 g x 7 batches) was added to the solution under mechanical stirring. Product adsorption onto the resin was monitored by HPLC-ELSD until its almost complete removal from the solution. The resin was then filtered and subjected to five consecutive washings with EtOH (following supplier indications) (50 mL/batch) under mechanical stirring at room temperature until no product was anymore detected in the filtrate by HPLC-ELSD analysis. 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 (50 mL) and vacuum. This reaction was performed in duplicate. The product was obtained as a yellow powder (7.03 – 7.8 g_{powder}) (Table 1, Entries 5 – 6). The powder contained: 63% – 56.4% BuGal (4.4 g; yield: 14 – 17%), 37 –43.6% others (Glc, Gal and inorganic salts) (2.62 – 3.4 g).

Reaction #7: Transglycosylation of CWP in biphasic system & simplified work-up (elution with EtOH) 1.5 L scale & recycled 1-BuOH

Before setting up the transglycosylation reactions, CWP (142 g/L lactose) was filtered by vacuum filtration using a sintered glass filter to remove a white precipitate that was observed after its thawing and to obtain a clear yellow solution. The pH of CWP (pH 6.7) was adjusted to 4.5 by adding 85% H_3PO_4 (~600 μL). CWP (300 mL, corresponding to 124.5 mmol of lactose) and 1-BuOH recovered from the previous reactions (1200 mL) were mixed in a round bottom flask. The immobilized GalAo (30 g, 30 IU) was added to the reaction and the mixture was maintained under magnetic stirring at 30 °C for 17 h hours. The reaction was stopped by filtration of the enzyme, and then the immobilized enzyme was washed with recycled 1-BuOH (250 mL). The filtrate was transferred into a separatory funnel, without washing with a 1% w/v NaCl solution since phase separation was immediately visible probably due to the water content present in the recycled 1-BuOH. The two phases were then recovered separately. The aqueous phase (300 mL) containing 69.0 g/L Glc and 56 g/L Gal (Table 3) was used for cell growth. The organic phase was dried under reduced pressure, 1-BuOH was recovered to be reused, and the crude was resolubilized in H_2O (50 mL). Subsequently, Amberlite® XAD4 polymeric adsorbent resin was washed with H_2O to eliminate the NaCl and Na_2CO_3 used as preservatives. The wet resin (10 g x 7 batches) was added to the solution under mechanical stirring. Product adsorption onto the resin was monitored by HPLC-ELSD until its almost complete removal from the solution. The resin was then filtered and subjected to six consecutive washings with EtOH (following supplier indications) (50 mL/batch) under mechanical stirring at room temperature until no product was anymore detected in the filtrate by HPLC-ELSD analysis. 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 (50 mL) and vacuum. The product was obtained as a yellow powder (7.20 g_{powder}) (Table 1, Entry 7). The powder contained: 50% BuGal (3.6 g; yield: 12%), 50% others (Glc, Gal and inorganic salts) (3.6 g).

Reaction #8–#9: Transglycosylation of CWP in biphasic system & simplified work-up (elution with 1-BuOH) 1 L scale

Before setting up the transglycosylation reactions, CWP (113 – 119 g/L lactose) was filtered by vacuum filtration using a sintered glass filter to remove a white precipitate that was observed after its thawing and to obtain a clear yellow solution. The pH of CWP (pH 6.7) was adjusted to 4.5 by adding 85% H_3PO_4 (~400 μL). CWP (200 mL, corresponding to 66.0 – 69.5 mmol of lactose) and 1-BuOH (800 mL) were mixed in a round bottom flask. The immobilized GalAo (20 g, 20 IU) was added to the reaction and the mixture was maintained under magnetic stirring at 30 °C for 17 h hours. The reaction was stopped by filtration of the enzyme, and then the immobilized enzyme was washed with 1-BuOH (100 mL). The filtrate was transferred into a separatory funnel, washed with a 1% w/v NaCl solution (100 mL) and then it was left to settle overnight to achieve phase separation. The two phases were then recovered separately. The aqueous phase

(155 – 158 mL) containing 56.6 g/L – 67.9 g/L Glc and 36.2 g/L – 38.8 g/L Gal (Table 3) was used for cell growth. The organic phase was dried under reduced pressure, 1-BuOH was recovered to be reused, and the crude was resolubilized in H_2O (50 mL). Subsequently, Amberlite® XAD4 polymeric adsorbent resin was washed with H_2O to eliminate the NaCl and Na_2CO_3 used as preservatives. The wet resin (10 g x 7 batches) was added to the solution under mechanical stirring. Product adsorption onto the resin was monitored by HPLC-ELSD until its almost complete removal from the solution. The resin was then filtered and washed with 5 mL of 1-BuOH in the first elution step, then 20 mL 1-BuOH and finally 50 mL 1-BuOH for 5 times. In this case the first two volumes eluted were of water (and thus discarded), the third was a mixture of water and 1-BuOH and from the fourth volume just 1-BuOH was recovered. The 1-BuOH fractions containing the product were then collected and dried under reduced pressure. After drying an off white solid was obtained which required some washings with diethyl ether (50 mL) and vacuum. This reaction was performed in duplicate. The product was obtained as a white powder (3.14 – 3.30 g_{powder}) (Table 1, Entries 8 – 9). The powder contained: 74.5 – 82% BuGal (2.3 – 2.7 g; yield: 14 – 17%), 25.5 – 18% others (Glc, Gal and inorganic salts) (0.84 – 0.60 g).

E-factor calculations

For flash chromatography purification the subsequent assumptions were used: 1 L solvent and 100 g silica gel per gram of product.

Reaction #1

REACTION
WP pH (6.0-6.5) was adjusted to 4.5 by adding HCl 1 M
20 mL of WP (45 g/L lactose)
50 mL 1-BuOH
30 mL acetone
2 g β-galactosidase immobilized on glyoxyl-Sepabeads
30 °C for 2.5 h
WORKUP
Filtration under vacuum
Washing with 1-BuOH (10 mL)
Evaporation under reduced pressure
CHROMATOGRAPHY
Crude added to silica (≈ 2.5 g) and MeOH (10 mL)
Drying under reduced pressure
Added to column
Flash chromatography (25 g silica gel)
DCM/MeOH 90/10 (250 mL)
YIELD
250 mg (40%)

Calculation details

Step	Calculation	Mass (g)
Lactose in CWP	0.02 L x 45 g/L	0.9
Lactose waste	0.9 g – 0.250 $g_{product}$	0.65
Water from WP	20 mL x 1 g/mL	20
1-BuOH (reaction)	50 mL x 0.81 g/mL	40.5
Acetone	30 mL x 0.79 g/mL	23.7
HCl 1 M	-	n.d.
Immobilized β-galactosidase	Given	2
1-BuOH (wash)	10 mL x 0.81 g/mL	8.1
MeOH (pre-chromatography)	10 mL x 0.79 g/mL	7.9
Silica gel (pre-chromatography)	Given	2.5
Silica gel (chromatography)	0.25 g x 100 $g_{product}$	25
Chromatography DCM	225 mL x 1.33 g/mL	299.25
Chromatography MeOH	25 mL x 0.79 g/mL	19.75
Product mass	Given isolated product	**0.250**
Total Waste (reaction)		**86.85**
Total waste (reaction + purification)		**449.35**

E-factor (considering only the reaction)= $\frac{86.85\ \mathrm{g\ (waste)}}{0.250\ \mathrm{g\ (product)}} = 347.6$

E-factor (considering reaction and purification)= $\frac{449.35\ \mathrm{g\ (waste)}}{0.250\ \mathrm{g\ (product)}} = 1797.4$

Reaction #2

REACTION
CWP pH (6.7) was adjusted to 4.5 by adding 40 µL H_3PO_4 85%
20 mL of CWP (126 g/L lactose)
50 mL 1-BuOH
30 mL acetone
2 g β-galactosidase immobilized on glyoxyl-Sepabeads
30 °C for 17 h
WORKUP
Filtration under vacuum
Washing with 1-BuOH (10 mL)
Evaporation under reduced pressure
CHROMATOGRAPHY
Crude added to silica (≈ 2.5 g) and MeOH (10 mL)
Drying under reduced pressure
Added to column
Flash chromatography (52.5 g silica gel)
DCM/MeOH 90/10 (525 mL)
YIELD
525 mg (30%)

Calculation details

Step	Calculation	Mass (g)
Lactose in CWP	0.02 L x 126 g/L	2.52
Lactose waste	2.52 g – 0.525 $g_{product}$	1.99
Water from CWP	20 mL x 1 g/mL	20
1-BuOH (reaction)	50 mL x 0.81 g/mL	40.5
Acetone	30 mL x 0.79 g/mL	23.7
H_3PO_4 acid	0.04 mL x 1.685 g/mL	0.067
Immobilized β-galactosidase	Given	2
1-BuOH (wash)	10 mL x 0.81 g/mL	8.1
MeOH (pre-chromatography)	10 mL x 0.79 g/mL	7.9
Silica gel (pre-chromatography)	Given	2.5
Silica gel (chromatography)	0.525 g x 100 $g_{product}$	52.5
Chromatography DCM	472.5 mL x 1.33 g/mL	628.4
Chromatography MeOH	52.5 mL x 0.79 g/mL	41.5
Product mass	Given isolated product	**0.525**
Total Waste (reaction)		**88.26**
Total waste (reaction + purification)		**829.16**

E-factor (considering only the reaction)= $\frac{88.26\ \mathrm{g\ (waste)}}{0.525\ \mathrm{g\ (product)}} = 168.11$

E-factor (considering reaction and purification) = $\frac{829.16\ \mathrm{g\ (waste)}}{0.525\ \mathrm{g\ (product)}} = 1579.35$

Reaction #3

REACTION
CWP pH (6.7) was adjusted to 4.5 by adding 40 µL H_3PO_4 85%
20 mL of CWP (126 g/L lactose)
80 mL 1-BuOH
2 g β-galactosidase immobilized on glyoxyl-Sepabeads
30 °C for 17 h
WORKUP
Filtration under vacuum
Washing with 1-BuOH (10 mL)
Evaporation under reduced pressure
CHROMATOGRAPHY
Crude added to silica (≈ 2.5 g) and MeOH (10 mL)
Drying under reduced pressure
Added to column
Flash chromatography (48.5 g silica gel)
DCM/MeOH 90/10 (485 mL)
YIELD
485 mg (28%)

Calculation details

Step	Calculation	Mass (g)
Lactose in CWP	0.02 L x 126 g/L	2.52
Lactose waste	2.52 g – 0.485 $g_{product}$	2.03
Water from CWP	20 mL x 1 g/mL	20
1-BuOH (reaction)	80 mL x 0.81 g/mL	64.8
H_3PO_4 acid	0.04 mL x 1.685 g/mL	0.067
Immobilized β-galactosidase	Given	2
1-BuOH (wash)	10 mL x 0.81 g/mL	8.1
MeOH (pre-chromatography)	10 mL x 0.79 g/mL	7.9
Silica gel (pre-chromatography)	Given	2.5
Silica gel (chromatography)	0.485 g x 100 $g_{product}$	48.5
Chromatography DCM	436.5 mL x 1.33 g/mL	580.5
Chromatography MeOH	48.5 mL x 0.79 g/mL	38.3
Product mass	Given isolated product	**0.485**
Total Waste (reaction)		**88.90**
Total waste (reaction + purification)		**774.70**

E-factor (considering only the reaction)= $\frac{88.90\ \text{g (waste)}}{0.485\ \text{g (product)}} = 183.30$

E-factor (considering reaction and purification) = $\frac{774.70\ \text{g (waste)}}{0.485\ \text{g (product)}} = 1597.32$

Reaction #4

REACTION
CWP pH (6.7) was adjusted to 4.5 by adding 40 µL H_3PO_4 85%
20 mL of CWP (120 g/L lactose)
80 mL 1-BuOH
2 g β-galactosidase immobilized on glyoxyl-Sepabeads
30 °C for 17 h
WORKUP
Filtration under vacuum
Washing with 1-BuOH (10 mL)
Washing with NaCl solution 1% w/v (10 mL)
Phase separation
Catch and release purification
Organic phase drying under reduced pressure
Crude solubilization in H_2O (20 mL)
Product adsorption on XAD4 resin (14 g)
Product elution with EtOH (50 mL)
Drying under reduced pressure
Washing with diethyl ether (5 mL) + vacuum
YIELD
804 mg powder (Entry 4, Table 1): 586.92 mg BuGal (73% purity) (considering mean of % purity & amount obtained) 217.88 mg others

Calculation details

Step	Calculation	Mass (g)
Lactose in CWP	0.02 L x 120 g/L	2.4
Lactose waste	2.4 g – 0.587 $g_{product}$	1.81
Water from CWP	20 mL x 1 g/mL	20
1-BuOH (reaction)	80 mL x 0.81 g/mL	64.8
H_3PO_4 acid	0.04 mL x 1.685 g/mL	0.067
Immobilized β-galactosidase	Given	2
1-BuOH (wash)	10 mL x 0.81 g/mL	8.1
NaCl solution 1% w/v (wash)	10 mL x 1 g/mL	10
	1% w/v NaCl	0.1
Water (crude solubilization)	20 mL x 1 g/mL	20
XAD resin	Given	14
EtOH (elution)	50 mL x 0.789 g/mL	39.45
Diethyl ether (washing)	5 mL x 0.713 g/mL	3.56
Product mass	Given isolated product	0.587
Total Waste (reaction)		**88.68**
Total waste (reaction + purification)		**183.89**

E-factor (considering only the reaction)= $\frac{88.68\ \text{g (waste)}}{0.587\ \text{g (product)}} = 151.07$

E-factor (considering reaction and purification) = $\frac{183.89\ \text{g (waste)}}{0.587\ \text{g (product)}} = 313.27$

Reaction #5–#6

REACTION
CWP pH (6.7) was adjusted to 4.5 by adding 400 µL H_3PO_4 85%
200 mL of CWP (123 g/L lactose)
800 mL 1-BuOH
20 g β-galactosidase immobilized on glyoxyl-Sepabeads
30 °C for 17 h
WORKUP
Filtration under vacuum
Washing with 1-BuOH (100 mL)
Washing with NaCl solution 1% w/v (100 mL)
Phase separation
Catch and release purification
Organic phase drying under reduced pressure
Crude solubilization in H_2O (50 mL)
Product adsorption on XAD4 resin (70 g)
Product elution with EtOH (250 mL)
Drying under reduced pressure
Washing with diethyl ether (50 mL) + vacuum
YIELD
7.0 g (Entry 5, Table 1) **– 7.8 g** (Entry 6, Table 1) **powder**: 4.4 g BuGal (63% – 56.4% purity) (considering mean of % purity & amount obtained) 2.62 – 3.4 g others

Calculation details

Step	Calculation	Mass (g)
Lactose in CWP	0.2 L x 123 g/L	24.6
Lactose waste	24.6 g – 4.4 $g_{product}$	20.2
Water from CWP	200 mL x 1 g/mL	200
1-BuOH (reaction)	800 mL x 0.81 g/mL	648
H_3PO_4 acid	0.4 mL x 1.685 g/mL	0.67
Immobilized β-galactosidase	Given	20
1-BuOH (wash)	100 mL x 0.81 g/mL	81
NaCl solution 1% w/v (wash)	100 mL x 1 g/mL	100
	1% w/v NaCl	1
Water (crude solubilization)	50 mL x 1 g/mL	50
XAD resin	Given	70
EtOH (elution)	250 mL x 0.789 g/mL	197.25
Diethyl ether (washing)	50 mL x 0.713 g/mL	35.65
Product mass	Given isolated product	4.4
Total Waste (reaction)		**888.87**
Total waste (reaction + purification)		**1423.77**

E-factor (considering only the reaction)= $\frac{888.87\ \text{g (waste)}}{4.4\ \text{g (product)}} = 202.01$

E-factor (considering reaction and purification) = $\frac{1423.77\ \text{g (waste)}}{4.4\ \text{g (product)}} = 323.58$

An additional E-factor was calculated not considering as waste the recovered and reused materials:
- Glc and Gal recovered in the aqueous phase were not included in the calculations (see **Table 3**);
- the volume of 1-BuOH used for reaction (800 mL) and for enzyme washing (100 mL) was not included in the calculations since it was recovered and recycled in a subsequent reaction. A loss of 10% was considered as waste due to its slight miscibility with water;
- the volume of water from CWP (200 mL) was mostly recovered (168 mL) and used for cell growth, just the unrecovered volume (32 mL) was considered as waste;
- Amberlite XAD4 was not included as waste since it is recovered and it can be regenerated and reused.

Calculation details

Step	**Calculation**	**Mass (g)**
Lactose in CWP	0.2 L x 123 g/L	24.6
Glc in CWP	24.6 g/2	12.3
Gal in CWP unreacted	(24.6 g/2) – 4.4 $g_{product}$	7.9
Glc in aq. phase (average 68.5 g/L)	68.5 g/L x 0.168 L	11.51
Gal in aq. phase (average 37.6 g/L)	37.6 g/L x 0.168 L	6.32
Glc waste	12.3 g – 11.51 g	0.79
Gal waste	7.9 g – 6.32 g	1.58
Water from CWP	(200 mL -168 mL) x 1 g/L	32
1-BuOH (reaction + washing))	[(800 mL x 0.81 g/mL) x 10]/100	64.8
H_3PO_4 acid	0.4 mL x 1.685 g/mL	0.67
Immobilized β-galactosidase	Given	20
1-BuOH (wash)	[(100 mL x 0.81 g/mL) x 10]/100	8.1
NaCl solution 1% w/v (wash)	100 mL x 1 g/mL	100
	1% w/v NaCl	1
Water (crude solubilization)	50 mL x 1 g/mL	50
EtOH (elution)	250 mL x 0.789 g/mL	197.25
Diethyl ether (washing)	50 mL x 0.713 g/mL	35.65
Product mass	Given isolated product	**4.4**
Total waste (reaction + purification considering recovered materials) 511.84		

E-factor (considering the recovered materials)= $\frac{511.84\ \text{g (waste)}}{4.4\ \text{g (product)}} = 116.32$

Reaction #7

REACTION
CWP pH (6.7) was adjusted to 4.5 by adding 600 µL H_3PO_4 85%
300 mL of CWP (142 g/L lactose)
1200 mL 1-BuOH (recycled)
30 g β-galactosidase immobilized on glyoxyl-Sepabeads
30 °C for 17 h
WORKUP
Filtration under vacuum
Washing with recycled 1-BuOH (250 mL)
Phase separation
Catch and release purification
Organic phase drying under reduced pressure
Crude solubilization in H_2O (50 mL)
Product adsorption on XAD4 resin (70 g)
Product elution with EtOH (300 mL)
Drying under reduced pressure
Washing with diethyl ether (50 mL) + vacuum
YIELD
7.2 g powder (Entry 7, Table 1) 3.6 g BuGal (50% purity) (considering mean of % purity & amount obtained) 3.6 g others

Calculation details

Step	Calculation	Mass (g)
Lactose in CWP	0.3 L x 142 g/L	42.6
Water from CWP	300 mL x 1 g/mL	300
1-BuOH recycled (reaction)	1200 mL x 0.81 g/mL	972
H_3PO_4 acid	0.6 mL x 1.685 g/mL	1.01
Immobilized β-galactosidase	Given	30
1-BuOH recycled (wash)	250 mL x 0.81 g/mL	202.5
Water (crude solubilization)	50 mL x 1 g/mL	50
XAD resin	Given	70
EtOH (elution)	300 mL x 0.789 g/mL	236.7
Diethyl ether (washing)	50 mL x 0.713 g/mL	35.65
Product mass	Given isolated product	**3.6**
Total Waste (reaction)		**1345.61**
Total waste (reaction + purification)		**1940.46**

E-factor (considering only the reaction)= $\frac{1345.61\ \text{g (waste)}}{3.6\ \text{g (product)}} = 373.78$

E-factor (considering reaction and purification) = $\frac{1940.46\ \text{g (waste)}}{3.6\ \text{g (product)}} = 539.02$

An additional E-factor was calculated not considering as waste the recovered and reused materials:
- Glc and Gal recovered in the aqueous phase were not included in the calculations (see **Table 3**);
- the volume of 1-BuOH used for reaction (1200 mL) and for enzyme washing (250 mL) was not included in the calculations since it was recovered and can be recycled in a subsequent reaction. A loss of 10% was considered as waste due to its slight miscibility with water;
- the volume of water from CWP (300 mL) was totally recovered (300 mL) and used for cell growth, thus was not considered as waste;
- Amberlite XAD4 was not included as waste since it is recovered and it can be regenerated and reused.

Step	**Calculation**	**Mass (g)**
Lactose in CWP	0.3 L x 142 g/L	42.6
Glc in CWP	42.6 g/2	21.3
Gal in CWP	(42.6 g/2) – 3.6 $g_{product}$	17.7
Glc in aq. phase (69.0 g/L)	69.0 g/L x 0.3 L	20.7
Gal in aq. phase (56 g/L)	56 g/L x 0.3 L	16.8
Glc waste	21.3 g – 20.7 g	0.6
Gal waste	17.7 g – 16.8 g	0.9
1-BuOH recycled (reaction)	[(1200 mL x 0.81 g/mL) x 10]/100	97.2
H_3PO_4 acid	0.6 mL x 1.685 g/mL	1.01
Immobilized β-galactosidase	Given	30
1-BuOH recycled (wash)	[(250 mL x 0.81 g/mL) x 10]/100	20.25
Water (crude solubilization)	50 mL x 1 g/mL	50
EtOH (elution)	300 mL x 0.789 g/mL	236.7
Diethyl ether (washing)	50 mL x 0.713 g/mL	35.65
Product mass	Given isolated product	**3.6**
Total waste (reaction + purification considering recovered materials)		**472.31**

E-factor (considering the recovered materials)= $\frac{472.31\ \mathrm{g\ (waste)}}{3.6\ \mathrm{g\ (product)}} = 131.2$

Reaction #8–#9

REACTION
CWP pH (6.7) was adjusted to 4.5 by adding 400 µL H_3PO_4 85%
200 mL of CWP (113 – 119 g/L lactose)
800 mL 1-BuOH
20 g β-galactosidase immobilized on glyoxyl-Sepabeads
30 °C for 17 h
WORKUP
Filtration under vacuum
Washing with 1-BuOH (100 mL)
Washing with NaCl solution 1% w/v (100 mL)
Phase separation
Catch and release purification
Organic phase drying under reduced pressure
Crude solubilization in H_2O (50 mL)
Product adsorption on XAD4 resin (70 g)
Product elution with 1-BuOH (275 mL)
Drying under reduced pressure
Washing with diethyl ether (50 mL) + vacuum
YIELD
3.14 g (Entry 8, Table 1) **– 3.3 g** (Entry 9, Table 1) **powder** 2.3 – 2.7 g BuGal (74.5- 82% purity) (considering mean of % purity & amount obtained) 0.84 – 0.60 g others

Calculation details

Step	Calculation	Mass (g)
Lactose in CWP (mean)	0.2 L x 116 g/L	23.2
Lactose waste	23.2 g – 2.5 $g_{product}$	20.7
Water from CWP	200 mL x 1 g/mL	200
1-BuOH (reaction)	800 mL x 0.81 g/mL	648
H_3PO_4 acid	0.4 mL x 1.685 g/mL	0.67
Immobilized β-galactosidase	Given	20
1-BuOH (wash)	100 mL x 0.81 g/mL	81
NaCl solution 1% w/v (wash)	100 mL x 1 g/mL	100
	1% w/v NaCl	1
Water (crude solubilization)	50 mL x 1 g/mL	50
XAD resin	Given	70
1-BuOH (elution)	275 mL x 0.81 g/mL	222.75
Diethyl ether (washing)	50 mL x 0.713 g/mL	35.65
Product mass	Given isolated product (mean)	**2.5**
Total Waste (reaction)		**889.37**
Total waste (reaction + purification)		**1449.77**

E-factor (considering only the reaction)= $\frac{889.37\ \text{g (waste)}}{2.5\ \text{g (product)}} = 355.75$

E-factor (considering reaction and purification) = $\frac{1449.77\ \text{g (waste)}}{2.5\ \text{g (product)}} = 579.91$

An additional E-factor was calculated not considering as waste the recovered and reused materials:
- Glc and Gal recovered in the aqueous phase were not included in the calculations (see **Table 3**);
- the volume of 1-BuOH used for reaction (800 mL) and for enzyme washing (100 mL) was not included in the calculations since it was recovered and can be recycled in a subsequent reaction. A loss of 10% was considered as waste due to its slight miscibility with water;
- the volume of water from CWP (250 mL) was mostly recovered (155 – 158 mL) and used for cell growth, thus was not considered as waste;
- Amberlite XAD4 was not included as waste since it is recovered and it can be regenerated and reused.

Step	**Calculation**	**Mass (g)**
Lactose in CWP (mean)	0.2 L x 116 g/L	23.2
Glc in CWP	23.2 g/2	11.6
Gal in CWP	(23.2 g/2) – 2.5 $g_{product}$	9.1
Glc in aq. phase (average 62.25 g/L)	62.25 g/L x 0.1565 L	9.74
Gal in aq. phase (average 37.5 g/L)	37.5 g/L x 0.1565 L	5.87
Glc waste	11.6 g – 9.74 g	1.86
Gal waste	9.1 g – 5.87 g	3.23
Water from CWP	(200 mL – 156.5 mL) x 1 g/mL	43.5
1-BuOH (reaction)	[(800 mL x 0.81 g/mL) x 10]/100	64.8
H_3PO_4 acid	0.4 mL x 1.685 g/mL	0.67
Immobilized β-galactosidase	Given	20
1-BuOH (wash)	[(100 mL x 0.81 g/mL) x 10]/100	8.1
NaCl solution 1% w/v (wash)	100 mL x 1 g/mL	100
	1% w/v NaCl	1
Water (crude solubilization)	50 mL x 1 g/mL	50
1-BuOH (elution)	275 mL x 0.81 g/mL	222.75
Diethyl ether (washing)	50 mL x 0.713 g/mL	35.65
Product mass	Given isolated product (mean)	**2.5**
Total waste (reaction + purification considering recovered materials)		**551.56**

E-factor (considering the recovered materials)= $\frac{551.56\ \mathrm{g\ (waste)}}{2.5\ \mathrm{g\ (product)}} = 220.62$

E^+-factor calculations

The E^+-factor calculations were performed considering only the reaction unit. The CO_2 equivalency was calculated taking into account the current equivalency in Europe equal to ~240 g CO_2/kWh.[2]

Reaction #1

The approximate energy consumption calculation for stirring plus heating at 30 °C for 2.5 hours was calculated by taking into consideration the following parameters:

Parameter	**Calculations**	**Value**
Magnetic stirrer motor power	Given for a typical lab stirrers	20 W
Heating plate power	Given assuming heat cycles to maintain 30°C, less than max 300 W	150 W (average)
Total operation time	Given	2.5 h
Total power combined	20 + 150	170 W
Energy consumption	0.170 kW x 2.5 h	0.425 kWh
CO_2 equivalency	0.425 kWh x 240 g CO_2/kWh	102 g CO_2
Waste mass	From previous E-factor calculations (total inputs reaction unit)	86.85 g
Total waste including energy equivalent = 102 g CO_2 + 86.85 g = 188.85 g		

$$\textbf{E}^+\textbf{-factor} = \frac{188.85\ \text{g (waste+energy)}}{0.250\ \text{g (product)}} = 755.40$$

Reaction #2

The approximate energy consumption calculation for stirring plus heating at 30 °C for 17 hours was calculated by taking into consideration the following parameters:

Parameter	**Calculations**	**Value**
Magnetic stirrer motor power	Given for a typical lab stirrers	20 W
Heating plate power	Given assuming heat cycles to maintain 30°C, less than max 300 W	150 W (average)
Total operation time	Given	17 h
Total power combined	20 + 150	170 W
Energy consumption	0.170 kW x 17 h	2.89 kWh
CO_2 equivalency	2.89 kWh x 240 g CO_2/kWh	693.6 g CO_2
Waste mass	From previous E-factor calculations (total inputs reaction unit)	88.26 g
Total waste including energy equivalent = 693.6 g CO_2 + 88.26 g = 781.86 g		

$$\textbf{E}^+\textbf{-factor} = \frac{781.86\ \text{g (waste+energy)}}{0.525\ \text{g (product)}} = 1489.25$$

Reaction #3

The approximate energy consumption calculation for stirring plus heating at 30 °C for 17 hours was calculated by taking into consideration the following parameters:

Parameter	Calculations	Value
Magnetic stirrer motor power	Given for a typical lab stirrers	20 W
Heating plate power	Given assuming heat cycles to maintain 30°C, less than max 300 W	150 W (average)
Total operation time	Given	17 h
Total power combined	20 + 150	170 W
Energy consumption	0.170 kW x 17 h	2.89 kWh
CO_2 equivalency	2.89 kWh x 240 g CO_2/kWh	693.6 g CO_2
Waste mass	From previous E-factor calculations (total inputs reaction unit)	88.90 g
Total waste including energy equivalent = 693.6 g CO_2+ 88.90 g = 782.5 g		

E⁺-factor= $\frac{782.5\ \text{g (waste+energy)}}{0.485\ \text{g (product)}} = 1613.40$

Reaction #4

The approximate energy consumption calculation for stirring plus heating at 30 °C for 17 hours was calculated by taking into consideration the following parameters:

Parameter	Calculations	Value
Magnetic stirrer motor power	Given for a typical lab stirrers	20 W
Heating plate power	Given assuming heat cycles to maintain 30°C, less than max 300 W	150 W (average)
Total operation time	Given	17 h
Total power combined	20 + 150	170 W
Energy consumption	0.170 kW x 17 h	2.89 kWh
CO_2 equivalency	2.89 kWh x 240 g CO_2/kWh	693.6 g CO_2
Waste mass	From previous E-factor calculations (total inputs reaction unit)	88.68 g
Total waste including energy equivalent = 693.6 g CO_2+ 88.68 g = 782.28 g		

E⁺-factor= $\frac{782.28\ \text{g (waste+energy)}}{0.586\ \text{g (product)}} = 1334.94$

Reaction #5–#6

The approximate energy consumption calculation for stirring plus heating at 30 °C for 17 hours was calculated by taking into consideration the following parameters:

Parameter	Calculations	Value
Magnetic stirrer motor power	Given for a typical lab stirrers	20 W
Heating plate power	Given assuming heat cycles to maintain 30°C, less than max 300 W	150 W (average)
Total operation time	Given	17 h
Total power combined	20 + 150	170 W
Energy consumption	0.170 kW x 17 h	2.89 kWh
CO_2 equivalency	2.89 kWh x 240 g CO_2/kWh	693.6 g CO_2
Waste mass	From previous E-factor calculations (total inputs reaction unit)	888.87 g
Total waste including energy equivalent = 693.6 g CO_2+ 888.87 g = 1582.47 g		

E⁺-factor= $\frac{1587.42\ \text{g (waste+energy)}}{4.4\ \text{g (product)}} = 359.65$

Reaction #7

The approximate energy consumption calculation for stirring plus heating at 30 °C for 17 hours was calculated by taking into consideration the following parameters:

Parameter	Calculations	Value
Magnetic stirrer motor power	Given for a typical lab stirrers	20 W
Heating plate power	Given assuming heat cycles to maintain 30°C, less than max 300 W	150 W (average)
Total operation time	Given	17 h
Total power combined	20 + 150	170 W
Energy consumption	0.170 kW x 17 h	2.89 kWh
CO_2 equivalency	2.89 kWh x 240 g CO_2/kWh	693.6 g CO_2
Waste mass	From previous E-factor calculations (total inputs reaction unit)	1345.61 g
Total waste including energy equivalent = 693.6 g CO_2+ 1345.61 g = 2039.21 g		

E⁺-factor= $\frac{2039.21\ \text{g (waste+energy)}}{3.6\ \text{g (product)}} = 566.47$

Reaction #8–#9

The approximate energy consumption calculation for stirring plus heating at 30 °C for 17 hours was calculated by taking into consideration the following parameters:

Parameter	Calculations	Value
Magnetic stirrer motor power	Given for a typical lab stirrers	20 W
Heating plate power	Given assuming heat cycles to maintain 30°C, less than max 300 W	150 W (average)
Total operation time	Given	17 h
Total power combined	20 + 150	170 W
Energy consumption	0.170 kW x 17 h	2.89 kWh
CO_2 equivalency	2.89 kWh x 240 g CO_2/kWh	693.6 g CO_2
Waste mass	From previous E-factor calculations (total inputs reaction unit)	889.37 g
Total waste including energy equivalent = 693.6 g CO_2+ 889.37 g = 1582.97 g		

E⁺-factor= $\frac{1582.97\ \text{g (waste+energy)}}{2.5\ \text{g (product)}} = 633.20$

Eco-impact

GSK Eco-Impact composite scores used for the calculations are reported below from Ferrara et al.[3]

Solvent	Score and eco-impact (per kg solvent)
H_2O	29
1-BuOH	171
acetone	168
DCM	10000
MeOH	232
EtOH	90
Diethyl ether	10000

Reaction #1

Solvent	Composite score	Mass (kg)	Eco-impact contributions
1-BuOH (reaction)	171	0.0405	6.92
acetone	168	0.0237	3.98
1-BuOH (wash)	171	0.0081	1.38
MeOH (pre-chromatography)	232	0.0079	1.83
DCM (chromatography)	10000	0.29925	2992.5
MeOH (chromatography)	232	0.01975	4.582
		Total eco-impact = 3011.19	

Eco-impact/kg$_{BuGal}$= $$\frac{3011.19}{0.000250\ \mathrm{kg\ (product)}} = 12044760$$

Reaction #2

Solvent	Composite score	Mass (kg)	Eco-impact contributions
1-BuOH (reaction)	171	0.0405	6.92
acetone	168	0.0237	3.98
1-BuOH (wash)	171	0.0081	1.38
MeOH (pre-chromatography)	232	0.0079	1.83
DCM (chromatography)	10000	0.6284	6284
MeOH (chromatography)	232	0.0415	9.63
		Total eco-impact = 6307.74	

Eco-impact/kg$_{BuGal}$= $$\frac{6307.74}{0.000525\ \mathrm{kg\ (product)}} = 12014742.9$$

Reaction #3

Solvent	Composite score	Mass (kg)	Eco-impact contributions
1-BuOH (reaction)	171	0.0648	11.08
1-BuOH (wash)	171	0.0081	1.38
MeOH (pre-chromatography)	232	0.0079	1.83
DCM (chromatography)	10000	0.5805	5805
MeOH (chromatography)	232	0.0383	8.9
			Total eco-impact = 5828.19

Eco-impact/kg$_{BuGal}$= $\frac{5828.19}{0.000485\ \mathrm{kg\ (product)}} = 12016886.6$

Reaction #4

Solvent	Composite score	Mass (kg)	Eco-impact contributions
1-BuOH (reaction)	171	0.0648	11.08
1-BuOH (wash)	171	0.0081	1.38
H_2O (crude solubilization)	29	0.02	0.58
EtOH (elution)	90	0.03945	3.55
Diethyl ether (washing)	10000	0.00356	35.6
			Total eco-impact = 52.19

Eco-impact/kg$_{BuGal}$= $\frac{52.19}{0.000587\ \mathrm{kg\ (product)}} = 88909.7$

Reaction #5–#6

Solvent	Composite score	Mass (kg)	Eco-impact contributions
1-BuOH (reaction)	171	0.648	110.81
1-BuOH (washing)	171	0.081	13.85
H_2O (crude solubilization)	29	0.05	1.45
EtOH (elution)	90	0.197	17.75
Diethyl ether (washing)	10000	0.0356	356.5
			Total eco-impact = 500.36

Eco-impact/kg$_{BuGal}$= $\frac{500.36}{0.0044\ \mathrm{kg\ (product)}} = 113718.2$

Reaction #7

Solvent	Composite score	Mass (kg)	Eco-impact contributions
1-BuOH (reaction)	171	0.972	166.2
1-BuOH (washing)	171	0.202	34.63
H_2O (crude solubilization)	29	0.05	1.45
EtOH (elution)	90	0.237	21.33
Diethyl ether (washing)	10000	0.0356	356
			Total eco-impact = 579.61

Eco-impact/kg$_{BuGal}$= $\frac{579.61}{0.0036\ \mathrm{kg\ (product)}} = 161002.8$

Reaction #8–#9

Solvent	Composite score	Mass (kg)	Eco-impact contributions
1-BuOH (reaction)	171	0.648	110.8
1-BuOH (washing)	171	0.081	13.8
H_2O (crude solubilization)	29	0.05	1.45
1-BuOH (elution)	171	0.2227	38.09
Diethyl ether (washing)	10000	0.0356	356
		Total eco-impact = 520.14	

Eco-impact/kg$_{BuGal}$= $\frac{520.14}{0.0025\ \mathrm{kg\ (product)}} = 208056$

References

1 R. Semproli, L. Cassano, G. Ballabio, G. Cappelletti, G. Speranza, S. Donzella, C. Compagno, D. Ubiali and M. S. Robescu, *Appl. Microbiol. Biotechnol.*, 2025, **109**, 270.
2 P. Dominguez de María, *Curr. Opin. Green Sustain. Chem.*, 2025, **52**, 101003.
3 M. Ferrara, F. DellaNegra and M. Verzini, *Org. Process Res. Dev.*, 2025, **29**, 1110−1124.