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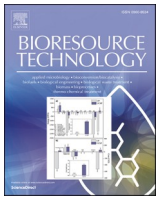

Sustainable production of a biotechnologically relevant β-galactosidase in *Escherichia coli* cells using crude glycerol and cheese whey permeate

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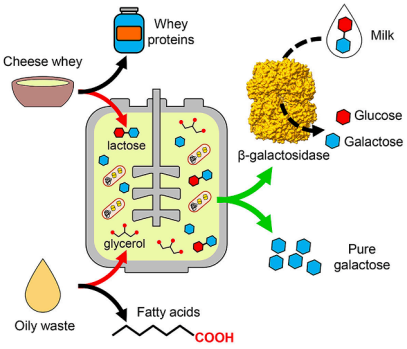
HIGHLIGHTS

- Cheese whey permeate (CWP) and crude glycerol (CG) are agro-food derived biomasses.
- M-βGal is a β-galactosidase capable of hydrolyzing lactose in complex matrices.
- CWP and CG are combined in a *E. coli* fed-batch culture to produce recombinant M-βGal.
- Galactose, a by-product of the culture, can be extracted from the culture supernatant.
- Recombinant enzymes and galactose can be efficiently produced from agro-food wastes.

GRAPHICAL ABSTRACT



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ABSTRACT

Responsible use of natural resources and waste reduction are key concepts in bioeconomy. This study demonstrates that agro-food derived-biomasses from the Italian food industry, such as crude glycerol and cheese whey permeate (CWP), can be combined in a high-density fed-batch culture to produce a recombinant β-galactosidase from *Marinomonas* sp. ef1 (M-βGal). In a small-scale process (1.5 L) using 250 mL of crude glycerol and 300 mL of lactose-rich CWP, approximately 2000 kU of recombinant M-βGal were successfully produced along with 30 g of galactose accumulated in the culture medium. The purified M-βGal exhibited high hydrolysis efficiency in lactose-rich matrices, with hydrolysis yields of 82 % in skimmed milk at 4 °C and 94 % in CWP at 50 °C, highlighting its biotechnological potential. This approach demonstrates the effective use of crude glycerol and CWP in sustainable and cost-effective high-density *Escherichia coli* cultures, potentially applicable to recombinant production of various proteins.

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1. Introduction

The growing demand for sustainable industrial processes has led to a significant increase in the use of enzymes as biocatalysts (Alcántara et al., 2022; Bell et al., 2021; Maghraby et al., 2023; Woodley, 2020). However, the widespread employment of enzymes in industrial applications is hampered by their low stability under process conditions and their high production costs (Bell et al., 2021). Industrial enzymes are mainly produced as recombinant proteins in two main hosts, namely *Pichia pastoris—Komagataella phaffii* (Barone et al., 2023; Gasset et al., 2022) and *Escherichia coli* (Rosano et al., 2019; Rosano and Ceccarelli, 2014). *E. coli* BL21(DE3) cells and their derivatives are the most widely used microbial cell factory because they are easy to manipulate and provide high yields of recombinant proteins (Rosano et al., 2019; Rosano and Ceccarelli, 2014; Studier, 2005; Studier and Moffatt, 1986). In BL21-pET systems, recombinant proteins are usually produced using complex media (e.g. Lennox, Luria-Bertani, Terrific broth, and Zym5052) and inducers such as isopropyl β-D-thiogalactopyranoside (IPTG) or pure lactose (Rosano et al., 2019; Rosano and Ceccarelli, 2014; Studier, 2005). Albeit these methods support high yields, they also imply significant costs, mainly due to the nitrogen (e.g. tryptone, yeast extract, soy peptone) and carbon (e.g. glucose and glycerol) sources in the media formulation, and the use of expensive inducers, especially IPTG (Cardoso et al., 2020; Ferreira et al., 2018; Fong and Wood, 2010).

The use of residual biomasses to replace traditional nitrogen and carbon sources, as well as inducers, is a promising strategy for reducing production costs, improving process sustainability, and promoting a circular economy approach. Studies have shown that residual biomasses, including crude glycerol, algae extracts, molasses, glutamate, and exhausted media from mammalian cell cultures, can serve as alternative sources of carbon and nitrogen, supporting the growth of *E. coli* cells and the production of high yields of recombinant proteins in combination with traditional inducers (Chiang et al., 2022, 2020; Lynch and O'Connell, 2022; Rechtin et al., 2014; Yaman and Çalık, 2017). In this context, residual streams from the dairy industry, notably cheese whey or cheese whey permeate (CWP), proved to be valuable alternative inducers due to their lactose-rich composition (de Divitiis et al., 2023; De León-Rodríguez et al., 2006; Gennari et al., 2023; Hausjell et al., 2019; Viitanen et al., 2003). However, the effects of combining different residual biomasses on *E. coli* growth and recombinant protein production in fed-batch systems remain poorly understood.

This study investigates the potential of combining two agro-food derived biomasses, namely crude glycerol and CWP for the recombinant production of a β-galactosidase from *Marinomonas* sp. ef1 (M-βGal) in high-density *E. coli* cultures. Crude glycerol was obtained from a company specializing in the hydrolytic production of fatty acids from agro-food oil and fat wastes, while CWP was obtained from a company processing cheese whey derived from the production of Grana Padano and Parmigiano Reggiano. M-βGal is active over a wide range of temperatures (4–55 °C) and pH (pH 5–7.5) and exhibits high thermal stability (Mangiagalli et al., 2021), making it attractive for various biotechnological applications. β-galactosidases are industrially-relevant enzymes known to hydrolyze β-galactosidic bonds in lactose or oligosaccharides (Mangiagalli and Lotti, 2021; Tabachnikov and Shoham, 2013) and to catalyze the transfer of galactose moieties under anhydrous conditions (Lu et al., 2020). They find applications in the production of lactose-free dairy products, the valorization of residual biomasses, and the synthesis of galactosyl building blocks for the food, cosmetic, and pharmaceutical industries (Lu et al., 2020; Mangiagalli and Lotti, 2021; Tabachnikov and Shoham, 2013).

Overall, data indicate that crude glycerol and CWP support high-density cell growth and the production of two valuable products: recombinant M-βGal and galactose, a by-product of lactose hydrolysis. Galactose accumulates in the culture medium as it is not metabolized by *E. coli* BL21(DE3) cells. The biotechnological potential of M-βGal for lactose hydrolysis has been evaluated on a small laboratory scale (25 mL), providing conversion yields comparable to commercial enzymes. This process offers several advantages, including water conservation, reduced production costs, and the valorization of two low-cost, high-organic-content agro-food derived-biomasses.

2. Materials and methods

2.1. Strains and materials

The recombinant production of M-βGal (NCBI: WP_100635792) was performed using *E. coli* BL21(DE3) cells carrying the plasmid pET21 [M-βGal] (Mangiagalli et al., 2021). M9 minimal medium, lactose, glucose and *ortho*-nitrophenyl-β-galactoside (ONPG) were purchased from Merck (Merck Darmstadt, Germany); pure glycerol, sodium carbonate, sodium chloride, imidazole, sodium phosphate monobasic and dibasic were purchased from Carlo Erba (Milano, Italy). 1,3- and 1,6-β-D-galactosyl-D-glucose were purchased from Megazyme International (Bray, Ireland) and Biosynth Carbosynth (Staad, Switzerland), respectively. Ambrogio Pagani S.p.A. (Bergamo, Italy) and Serum Italia (Cazzago San Martino, Brescia, Italy) kindly provided crude glycerol and CWP, respectively.

2.2. Characterization of crude glycerol

The analysis of crude glycerol was performed by combining high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy. Chromatographic analysis was carried out using a Jasco HPLC system (Jasco Europe, Cremella, Lecco, Italy) equipped with a refractive index detector (Jasco Europe, Cremella, Lecco, Italy) and an Aminex-HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). The chromatographic separation of glycerol samples (20 μL) was performed isocratically as described in (de Divitiis et al., 2023). The ^{1}H NMR spectrum of crude glycerol was acquired after dilution in 90 % D_2O employing the Bruker noesygppr d pulse sequence with 8 scans, an acquisition time of 2.5 s and a relaxation delay (d1) of 60 s at 300 K and 600 MHz using an Avance III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA).

2.3. Recombinant production of M-βGal in shake-flask cultures

Luria-Bertani medium (LB, see supplementary material) modified with the addition of 5 g/L pure or crude glycerol was employed to produce recombinant M-βGal. To evaluate the role and importance of glycerol as a carbon source, cultures in LB medium without glycerol were also conducted. All Cultures were performed in 500 mL shake flasks containing 150 mL LB as previously described in (de Divitiis et al., 2023). Briefly, heterologous expression was induced by adding 20 g/L pure lactose or 18.2 mL of CWP (final lactose concentration: 20 g/L) to cultures with an optical density at 600 nm (OD_{600}) of 0.8–1.0. The cultures were then incubated at 25 °C for 48 h.

2.4. Recombinant production of M-βGal in fed-batch bioreactors

Fed-batch cultures were performed in a 2-L glass bench-top bioreactor equipped with a digital control unit for bioprocess automation (BIOSTAT A system, Sartorius Stedim Biotech, Göttingen, Germany). The temperature was maintained at 37 °C (biomass growth phases) and 25 °C (induction phase), the pH was adjusted to 7.0 by adding 16 % (v/v) NH_4OH or 16 % (v/v) H_3PO_4, and the initial air flow was 1 L/min. A polarographic oxygen electrode (Hamilton, Bonaduz Switzerland) was used to measure the dissolved oxygen concentration, which was automatically maintained above 20 % by gradually increasing the stirring speed.

Cultures were initiated in 1 L of synthetic medium (SM) prepared as detailed in supplementary material. The initial batch phase in SM (Phase I) lasted for 16 h. In the subsequent phase (Phase II), a feeding solution containing 500 g/L of crude glycerol and 15 g/L $MgSO_4{\cdot}7H_2O$ was added at a rate conducive to exponential growth, with a dilution rate (μ)

of 0.2 h^{-1}. Phase III began upon reaching OD_{600} of 75, when the temperature was lowered to 25 °C and heterologous expression was induced by linear co-feeding with a glycerol solution at a constant rate of 0.09 mL/min and either lactose solution (165 g/L lactose) or CWP at a constant rate of 0.2 mL/min. Following a 18-hour induction, co-feeding ceased and growth parameters were monitored for additional 24 h (Phase IV).

2.5. Sample collection and preparation

Time course experiments were performed according to the methods described in (de Divitiis et al., 2023) with the following modifications. Cell pellets corresponding to 40 OD_{600} were washed twice with physiological solution (0.9 % NaCl) and resuspended in 1 mL of lysis buffer (see supplementary material). Total protein concentration and β-galactosidase activity were determined on clarified crude cell extracts. Bradford protein assay (Bio-Rad, Hercules, CA, USA) with a calibration curve obtained with bovine serum albumin (1–10 μg/mL) was employed to determine the total protein content. β-galactosidase activity was monitored in phosphate buffer, pH 7.0, at 25 °C using ONPG as a substrate at a concentration of 10 mM, as described in (Mangiagalli et al., 2021). M-βGal production was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Crude cell extracts, together with cytoplasmic soluble and insoluble protein fractions from *E. coli* cells, were analyzed on 10 % or 14 % acrylamide gels (Laemmli, 1970). After electrophoresis, the gels were washed with Milli-Q water and stained with Coomassie Brilliant Blue R-250 solution (Bio-Rad, Hercules, CA, USA).

To determine the dried weight of cells at the end of fed-batch cultures, 1 mL of cell culture was collected in a pre-weighed Eppendorf tube by centrifugation at 1000 *g* for 10 min. The cell pellets were washed three times with 1 mL milliQ water and dried in a lyophilizer (Heto FD1.0, Gemini BV, Apeldoorn, the Netherlands) overnight to remove all traces of water. All dry weight measurements were performed with an analytical balance (Gibertini Elettronica, Novate Milanese, Italy) in quadruplicate. For bioreactor experiments, an OD_{600} of 1 corresponds to 0.3 g/L of cell dry weight.

2.6. Purification of recombinant M-βGal

Cells from CWP-induced fed-batch cultures (end of Phase IV) were harvested by centrifugation at 2000 *g* for 30 min and stored at −20 °C until use. The supernatant was collected and stored at −20 °C for galactose recovery (see details below). 0.6 g dry weight cells (4.0 g wet weight cell) were thawed at room temperature, resuspended in 50 mL of lysis buffer and lysed using a cell disruptor (Constant Systems Ltd, Daventry, UK) at 25 kPSI. Crude extracts were clarified by centrifugation at 11,000 *g* for 30 min at 4 °C and filtered with a 0.22 μm filter. Recombinant M-βGal was purified using a 5 mL HIS-TRAP high performance column (Cytiva, Marlborough, US) and a NGC Quest Plus Chromatography System (Bio-Rad). The mobile phase comprised a lysis buffer (hereafter referred to as buffer A, see supplementary material) and a buffer B (see supplementary material). The clarified crude extract was applied to the pre-equilibrated column (100 % A) using a sample pump at 2.4 mL/min flow rate. Wash conditions involved isocratic 100 % A (10 column volumes − CV) and a linear gradient from 0 to 4 % B (10 CV). Elution proceeded with isocratic 50 % B (2 CV) and 100 % B (2 CV) at a flow rate of 2.4 mL/min. Following the first purification, a second purification was carried out by re-equilibrating the column in buffer A, reloading the flow-through and repeating the previously described steps. For each purification, 20 fractions were collected and tested for β-galactosidase activity. Fractions with the highest activity were pooled, concentrated using Amicon Ultra-15 centrifugal filters (Merck-Millipore, MW cut-off: 30 kDa). The concentrated fractions were then buffer-exchanged in a 10 mM phosphate buffer, pH 7.0 (PB) using a PD10 column and stored at 4 °C. Protein concentrations and β-galactosidase activity were determined as detailed in paragraph 2.5.

2.7. Galactose purification and analysis

50 mL of the supernatant from CWP-induced fed-batch cultures was deproteinized by heating at 65 °C for 4 h and then clarified by centrifugation at 6000 *g* for 20 min at 25 °C. The protein content of the sample was measured before and after deproteinization using the Bradford assay. The clarified supernatant was then heated to 65 °C and 35 mL of pure ethanol was added. The mixture was incubated at 65 °C for 10 min with magnetic stirring. The reaction was then transferred to an ice bath and incubated at 4 °C for 24 h. The galactose crystals were filtered using a buckner system, washed with an ice-cold ethanol solution (85 % *v/v*) and dried at room temperature. The amount of precipitated galactose was determined using an analytical balance (Gibertini Elettronica, Novate Milanese, Italy) and its purity was compared with pure commercial galactose used as a standard in HPLC chromatograms and Fourier transform infrared (FTIR) spectra.

For HPLC analysis, 15 mg of galactose were resuspended in 1 mL of distilled water. The analysis was carried out by a HPLC system (Jasco Europe, Cremella, Lecco, Italy) equipped with an Aminex-HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). This column enabled the analysis of sugars, alcohols, ketones and short-chain organic acids. The chromatographic setup and conditions are the same as those described in paragraph 2.5.

For FTIR analysis in the mid-IR spectral range, galactose powder was deposited onto the single reflection diamond crystal of the attenuated total reflection (ATR) device (Quest Specac, UK). Absorption spectra were recorded by a Varian 670-IR spectrometer (Varian, Australia Pty Ltd., Mulgrave VIC, Australia) as detailed in supplementary material.

2.8. Substrate specificity of M-βGal

M-βGal substrate specificity was determined using the following colorimetric substrates: ONPG, *para*-nitrophenyl-β-glucoside (PNPG), *para*-nitrophenyl-β-xyloside (PNPX) and *para*-nitrophenyl-β-mannoside (PNPM). Reactions were carried out at 50 °C in phosphate buffer 25 mM, pH 6.0, in a final volume of 500 μL. After 3 min of incubation, 500 μL of 1 M sodium carbonate buffer, pH 11 were added to stop the reaction. The absorbance of the reactions containing PNPG, PNPX, and PNPM was recorded at 405 nm (ε: 18.6 $mM^{-1}cm^{-1}$), while that containing ONPG was measured at 420 nm (ε: 4.6 $mM^{-1}cm^{-1}$) using a Jasco V-730 UV/NIR spectrophotometer (JASCO Europe, Cremella, Lecco, Italy).

To evaluate the activity of M-βGal towards different galactosidic bonds, lactose, 1,3-β-D-galactosyl-D-glucose, and 1,6-β-D-galactosyl-D-glucose were used as substrates at a concentration of 1 % *w/v*. The reaction conditions were the same as described above. The reaction was started by adding 50 U of enzyme, as determined with ONPG, and performed at a mixing speed of 400 rpm in a thermal shaker (Eppendorf, Hamburg, Germany). As a negative control, the substrate was incubated under the same conditions without enzyme. The reaction products were analyzed on an HPLC system as previously described.

2.9. Hydrolysis of skimmed milk and cheese whey permeate with M-βGal

The ability of M-βGal to hydrolyze the lactose contained in complex matrices was studied using skim milk and CWP. Commercial skimmed milk (Régilait, Saint-Martin-Belle Roche, Macon, France) was prepared as described by the customer, using sterile water to resuspend the milk powder. CWP was sterilized by filtration through a 0.22 μm filter. 25 mL of skimmed milk or CWP were transferred to a previously sterilized glass reactor (DISA Raffaele, Sesto San Giovanni, Milan, Italy). Reactions were carried out for 24 h at 4 °C for skimmed milk and 25 °C and 55 °C for CWP, with magnetic stirring in the presence of 125 U of M-βGal per mL of milk/CWP, determined by the ONPG assay. At each time point, 200 μL of the reactions were withdrawn and analyzed using FTIR

spectroscopy as described in (Cocciardi et al., 2004).

For FTIR analysis, 20 μL of each sample were deposited onto the nine-reflection diamond crystal of the ATR device (DuraSamplIR II, Smith Detection, USA) and the FTIR spectra were recorded using a Varian 670-IR spectrometer (Varian Australia Pty Ltd., Mulgrave VIC, Australia). The measurement details and parameters are reported in supplementary material. The absorption spectra of the sample solutions were corrected for PB absorption, and the 1200–950 cm^{-1} region was employed to determine the concentration of lactose, glucose, and galactose. To this aim, a partial least squares (PLS) calibration curve was constructed using standard solutions with varying concentrations of these sugars (Cocciardi et al., 2004). The PLS analysis was performed

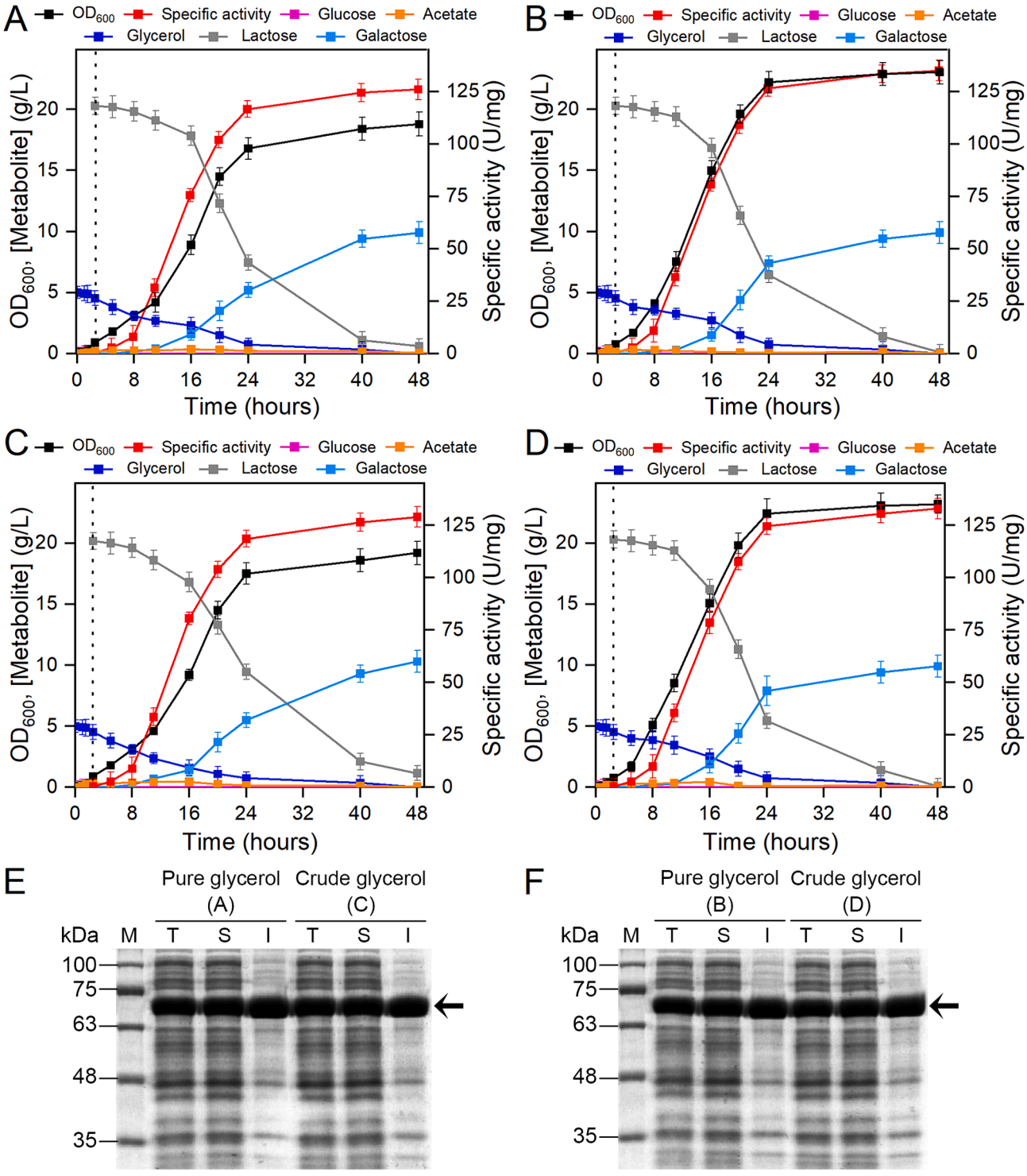

Fig. 1. Shake-flask cultures of *E. coli* BL21(DE3) cells over-expressing M-βGal. Growth curves of *E. coli* BL21(DE3) cells harboring pET21 [M-βGal] in 5 g/L crude (A, B) and pure (C, D) glycerol. Induction was performed with 20 g/L pure lactose (A-C) and CWP (B-D). The addition of the inducer is indicated by the vertical dashed line. Error bars indicate the standard deviation of three independent replicates (n = 3). E-F) Production of M-βGal monitored by SDS-PAGE analysis. After 48 h of induction with either pure lactose (E) or CWP (F), cells corresponding to an OD_{600} of 40 were harvested by centrifugation and lysed with glass beads. M: molecular weight marker, T: total protein fraction, S: soluble protein fraction, I: insoluble protein fraction. The letter in brackets indicates the corresponding panel of this figure.

using the GRAMS/32 software (Galactic, Salem, NH, USA). Conversion yields of hydrolytic reactions have been calculated as the ratio of the combined mole of the products (*i.e.* glucose and galactose) to the initial mole of the substrate (*i.e.* lactose) before enzymatic treatment, expressed as a percentage.

2.10. Statistical analysis

The experiments were conducted in triplicate and statistical analysis was performed using OriginLab software (OriginLab Corporation, Northampton, USA). The results are presented as the mean of independent replicates with standard deviation. The statistical significance of different growth conditions was determined using an unpaired two-tailed *t*-test.

3. Results and discussion

3.1. Crude glycerol and cheese whey permeate support the growth of *E. coli* BL21(DE3) cells and the recombinant production of M-βGal in shake-flask cultures

The CWP used in this study derives from the same batch analyzed in a previous study. Its composition comprises lactose (165 g/L), organic acids such as citrate (4.8 g/L), acetate (1.2 g/L), and lactate (5.0 g/L), and micronutrients including vitamins and coenzymes (de Divitiis et al., 2023). Analysis of the crude glycerol obtained from the hydrolysis of oil and fat wastes was carried out by HPLC and NMR spectroscopy (see supplementary material). HPLC analysis revealed a single peak eluted at 16.4 min (see supplementary material) with an estimated concentration of 605.6 ± 11.1 g/L. Consistently with the HPLC analysis, the ^{1}H NMR spectrum indicated glycerol as the major component of this biomass with a purity of 95 % (see supplementary material).

To evaluate the potential interference of crude glycerol on *E. coli* growth and recombinant M-βGal production, preliminary shake-flask cultures were conducted as follows: two cultures were initiated in LB supplemented with 5 g/L crude glycerol and induced with either 20 g/L pure lactose or CWP (final lactose concentration: 20 g/L). In addition, two independent cultures were initiated in LB supplemented with 5 g/L pure glycerol and induced with the same inducers. Cultures were incubated at 37 °C until an OD_{600} of ~ 1 was reached, followed by induction of M-βGal expression and further incubation at 25 °C for 48 h.

In the presence of crude glycerol and lactose (Fig. 1**A**), the growth curve showed a sigmoidal shape, reaching a final OD_{600} of 18.8 ± 0.7 after 48 h. Glycerol and lactose concentrations gradually decreased during growth and were completely depleted after 24 and 40 h, respectively. During the induction phase, the specific activity of recombinant β-galactosidase increased, reaching 126.9 ± 4.5 U/mg at the end of the incubation (red line in Fig. 1**A**). When replacing lactose with CWP, cell biomass increased by ~ 20 %, (Fig. 1**B**), without significantly affecting the specific activity of the recombinant enzyme (132.4 ± 5.5 U/mg). This increase in biomass accumulation with CWP is attributed to micronutrients, such as vitamins and organic acids, alleviating cellular stress associated with recombinant protein production (de Divitiis et al., 2023). Similar results were observed in the presence of pure glycerol (*p-value* > 0.05, Fig. 1**C and D**), indicating that contaminants do not affect cell growth or recombinant β-galactosidase production or activity. On the other hand, in the absence of glycerol, cell biomass is ~ 25 % lower than that observed in the presence of pure or crude glycerol, indicating that glycerol plays a pivotal role in supporting the growth of *E. coli* cells (see supplementary material). To assess M-βGal solubility and propensity to form inclusion bodies, SDS-PAGE analysis was performed on soluble and insoluble protein fractions obtained at the end of the fermentation. The results show an even distribution of M-βGal between soluble and insoluble fractions of the cell extracts under all tested conditions (Fig. 1**E-F**).

Overall, these preliminary results suggest that crude glycerol supports *E. coli* cell growth comparably to pure glycerol, thus establishing it as a sustainable carbon source. Combining crude glycerol and CWP yields a comparable amount of recombinant M-βGal as obtained with pure reagents, indicating that these agro-food derived biomasses can serve as alternative carbon sources and expression inducers.

3.2. Crude glycerol and cheese whey permeate enable the sustainable production of recombinant M-βGal in high-cell-density fed-batch cultures

High-cell-density fed-batch cultures were employed to exploit crude glycerol and CWP for the recombinant production of M-βGal. This strategy allows precise control of metabolic rates and supports high-cell densities by utilizing large amounts of nutrients compared to traditional batch growth methods (Krause et al., 2016). This experimental design compares fed-batch cultures utilizing crude glycerol as the primary carbon source and either lactose or CWP as both the carbon source and inducer.

In this process, the cell biomass was produced at 37 °C through two distinct phases: a 14-hour batch growth phase with an initial glucose concentration of 10 g/L (refer to Phase I in Fig. 2**A and B**), followed by a fed-batch growth phase where crude glycerol (500 g/L) is continuously added imposing a specific growth rate (μ) of 0.2 h^{-1} (refer to Phase II in Fig. 2**A and B**). The primary objective of Phase I is to rapidly accumulate an adequate cell biomass, which serves as the foundation for initiating the subsequent exponential feeding phase. In this phase, glucose is easily metabolized and at concentration above 1 g/L represses the expression system, thus alleviating the metabolic stress associated with heterologous gene expression (Donovan et al., 1996; Snoeck et al., 2024). Phase II, on the other hand, aims to produce cell biomass using crude glycerol as primary carbon source. Completion of the batch growth resulted in a cell biomass with OD_{600} of ~ 12, achieved by complete glucose consumption. During Phase II, no glycerol (blue line in Fig. 2**A and B**) and no secondary metabolites accumulated in the culture medium, as expected when cells maintain their growth rate by consuming incoming glycerol while maintaining aerobic metabolism (oxygen level indicated by purple line in Fig. 2**A and B**). At the end of this phase, a cell biomass with an OD_{600} of ~ 75 was reached, consuming 52.5 g of glycerol (equivalent to 105 mL of the glycerol feed solution).

Heterologous expression of M-βGal was induced at 25 °C by linear co-feeding of crude glycerol (0.09 mL/min) and CWP (0.2 mL/min), or an equivalent lactose solution (refer to Phase III in Fig. 2**A and B**). During the induction phase, lactose concentration ranged between 1.8 to 3.2 g/L (gray line in Fig. 2**A and B**). Simultaneously, the galactose concentration increased (light blue line in Fig. 2**A and B**), confirming the inability of BL21(DE3) cells to metabolize galactose, consistent with prior findings (Viitanen et al., 2003). The concentration of glycerol in the medium remained negligible due to its rapid consumption (see Fig. 2**A and B**). The specific activity of M-βGal showed a parallel increase in the presence of both lactose and CWP (red line in Fig. 2**A and B**). This increase in enzyme specific activity was linear and predominant during Phase III, with much less pronounced effects in Phase IV. In the final phase, feeding is stopped to allow the cells to completely consume the residual lactose in the culture medium (Phase IV in Fig. 2**A and B**).

Regarding M-βGal solubility, SDS-PAGE analysis revealed a uniform distribution of the protein across the soluble and insoluble fractions of *E. coli* cytoplasmic proteins under both tested conditions (Fig. 2**C**). This distribution aligns with findings from shake-flask cultures, indicating that the solubility of this enzyme remains unaffected by the fed-batch process conditions.

In summary, the biomass and M-βGal production yields obtained from the fed-batch process with CWP are comparable with those achieved using lactose (Table 1, *p-value* > 0.5). The entire production process used 125 g crude glycerol (equivalent to 250 mL crude glycerol solution) and 49.5 g lactose (300 mL CWP) to produce 52.9 ± 2.2 g dry cell weight, 1980.0 ± 135.9 kU recombinant soluble M-βGal, and 20 g/L galactose. These components are the primary products of the process. Note that both crude glycerol and CWP are aqueous solutions and that their use saves about 500 mL of water per 1.5 L fermentation.

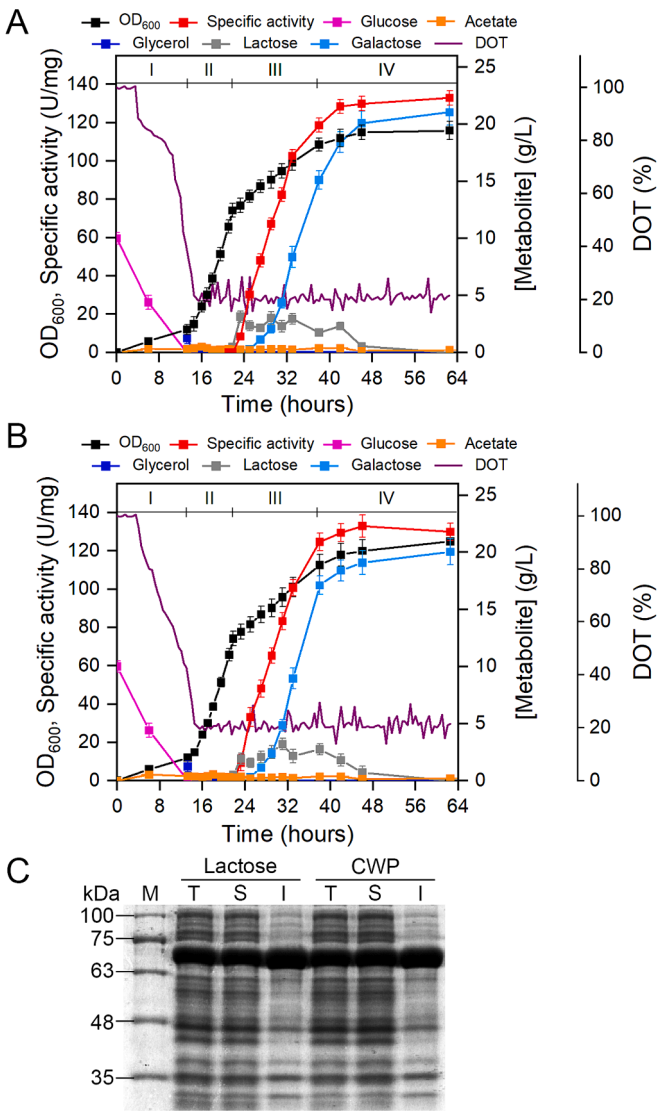

Fig. 2. Fed-batch cultures of *E. coli* BL21(DE3) cells overexpressing M-βGal. Growth curves of *E. coli* BL21 (DE3) cells harboring pET21 [M-βGal] induced with pure lactose (A) and CWP (B). Biomass growth was performed at 37 °C in 2 phases: (I) batch growth with 10 g/L glucose as carbon source; (II) exponential feeding with crude glycerol (50 % *v/v*) at a μ: 0.2. Heterologous expression was induced at 25 °C in 2 phases: (III, 18 h) expression was initiated by a linear feed (0.2 mL/min) of crude glycerol (50 % *v/v*) and lactose (165 g/L) obtained from commercial powder or CWP; (IV, 24 h) co-feeding was stopped, allowing the cell culture to consume lactose. Error bars indicate the standard deviation of three independent replicates (n = 3). C) Production of M-βGal monitored by SDS-PAGE analysis. After 48 h of induction with pure lactose or CWP, cells corresponding to an OD_{600} of 40 were harvested by centrifugation and lysed with glass beads. M: molecular weight marker, T: total protein fraction, S: soluble protein fraction, I: insoluble protein fraction.

Table 1
Products obtained at the end of fed-batch cultures.

Inducer	Dry cell weight (g)	Activity (kU)	Galactose (g)
Lactose	48.3 ± 2.4	1848.0 ± 120.7	33.8 ± 1.8
CWP	52.9 ± 2.2	1980.0 ± 135.9	31.2 ± 2.1

Activity units refer to assays carried out on ONPG. Means and standard deviations are based on three independent experiments. No significant differences were observed between the two culture conditions (p-value > 0.05).

Overall, the results of this fed-batch process pave the way for developing sustainable production processes using agro-food residual biomasses characterized by high organic content and difficult disposal, such as CWP. In addition, crude glycerol and CWP are relatively inexpensive compared to traditional carbon sources and inducers, resulting in reduced production costs. The latter aspect is particularly impactful in the context of industrial enzymes, which are typically less expensive than therapeutic proteins (Puetz and Wurm, 2019). Besides, to increase the versatility of this approach, crude glycerol could be derived from various industrial processes (e.g. biodiesel production) (He et al., 2017; Monteiro et al., 2018). However, the main limitations of this fed-batch process stem from the small scale used in this study; scale-up to medium/large scale is required to assess its applicability.Furthermore, in order to improve the sustainability of the process and to further reduce the production costs, the glucose used in the batch phase (Phase I) should be replaced by cruder, less expensive molasses.

3.3. Purification of M-βGal and galactose from fed-batch cultures

As a proof of concept, both recombinant M-βGal and galactose were purified from cells and culture broth, respectively, obtained from CWP-induced fed-batch cultures. Affinity chromatography was employed to purify M-βGal from 0.6 g dry cell weight, yielding 20.8 ± 1.7 kU of pure enzyme (equivalent to 39.4 ± 2.3 mg) with a purification yield of 64.6 ± 1.7 % (Table 2 and supplementary material).

To recover galactose, the experimental plan schematized in supplementary material was applied on 50 mL of culture supernatant. First, the culture broth was deproteinized by heat treatment at 65 °C for 3 h to remove ∽ 90 % of the proteins (see supplementary material). The deproteinized culture supernatants were then treated with pure ethanol (final concentration: 35 % *v/v*) for cold precipitation. Following a 24-hour incubation at 4 °C, galactose powder was recovered by filtration, washed with cold 75 % *v/v* ethanol, and air-dried at room temperature. The resulting galactose powder weighed 0.91 ± 0.13 g, with a recovery yield of 55 ± 6.2 %. The purity of the galactose powder was confirmed using a combination of HPLC analysis and FTIR spectroscopy (see supplementary material). The HPLC analysis identified a single peak eluted at 11.9 min, identical to that of a pure, analytical grade galactose commercial standard. The FTIR spectrum of the powder overlapped with that of the pure galactose standard, displaying a particularly high absorption between ~ 1200–900 cm^{-1}, mainly due to C-O, C-C and C-OH vibrational modes (Kačuráková and Mathlouthi, 1996). The low IR absorption between ~ 1750–1500 cm^{-1} indicates that the galactose powder does not contain significant amounts of organic acids (C = O: ~ 1750–1700- cm^{-1}) or proteins (amide I and II peaks: ~ 1700–1600, ~ 1600–1500 cm^{-1}, respectively) (Tamm and Tatulian, 1997).

Although carried out on a small scale, the purification procedures yielded satisfactory results in terms of the amount and purity of recombinant M-βGal and galactose. Optimization of these procedures could improve the purification yields and scalability, allowing testing on larger culture volumes.

In conclusion, the recovery of galactose from the culture medium allows the complete valorization of agro-food derived biomass used in the process. This highly pure sugar can be directly marketed or further chemically/enzymatically modified into sugar building blocks such as galactaric acid and galactoaldehydes (Parikka et al., 2015; Pasquale et al., 2024). In addition, the galactose-rich supernatant could serve as a medium for culturing oleaginous yeasts, such as those producing galactitol (Jagtap et al., 2019) and lipids (Lazar et al., 2015; Wang et al., 2021).

3.4. M-βGal application for lactose hydrolysis in milk and cheese whey permeate

The substrate specificity of M-βGal was tested on various colorimetric substrates with distinct glycosidic bonds, under optimal catalytic

Table 2
Purification table.

Steps	Volume (mL)	Total proteins (mg)	Activity (kU)	Specific activity (U/mg)	Yield (%)
Crude extract	49.9 ± 1.7	210.3 ± 7.5	32.2 ± 2.9	153.3 ± 4.6	100
IMAC Chromatography	1.7 ± 0.1[a]	39.4 ± 2.3	20.8 ± 1.5	506.8 ± 8.3	64.6 ± 1.7

Values in the table refer to purifications carried out on 0.6 g dry weight cells. Units of activity refer to assays carried out on ONPG. Means and standard deviations are based on three independent experiments.

[a] IMAC fractions containing M-βGal were pooled and concentrated with Amicon Ultra-15 centrifugal filters.

conditions (pH 6.5, 55 °C). Notably, hydrolytic activity was observed only on ONPG (specific activity: 1121.5 ± 78.4), suggesting that M-βGal exhibits a narrow substrate specificity. To evaluate the preference of M-βGal for specific chemical bonds, regioselectivity was studied on 1,3-β-D-galactosyl-D-glucose, lactose (β-1–4 galactosidic bond) and allolactose (β-1–6 galactosidic bond). M-βGal showed broad regioselectivity, hydrolyzing all the tested disaccharides, albeit with different conversion yields (Fig. 3**A-C**). Within a 4-hour reaction, lactose was completely hydrolyzed into glucose and galactose (Fig. 3**A**), while 1,3-β-D-galactosyl-D-glucose and allolactose showed conversion yields of 94.3 ± 1.7 % and 83.2 ± 2.3 %, respectively (Fig. 3**B and C**). Overall, these results indicate that M-βGal is exclusively active on β-galactosidic bond and can hydrolyze lactose and allolactose, which is consistent with the behavior of other GH42s (Karan et al., 2013; Wang et al., 2020). Conversely, the ability to hydrolyze the β(1–3) glycosidic bond seems to be a distinctive feature of M-βGal which, to our knowledge, is shared only with a cold-active GH42 from *Marinomonas* sp. BSi20414 (Ding et al., 2017).

Investigation of the catalytic properties of M-βGal is crucial to assess its biotechnological potential, *i.e.* in lactose hydrolysis from complex matrices such as milk and CWP. M-βGal was applied in a 25-mL hydrolysis reaction on skimmed milk, carried out for 24 h at 4 °C to prevent microbial spoilage and simulate the industry-standard batch process (Dekker et al., 2019). ATR-FTIR analysis revealed a conversion yield of 70 % in the first two hours, reaching a final yield of 82.5 ± 3.2 %

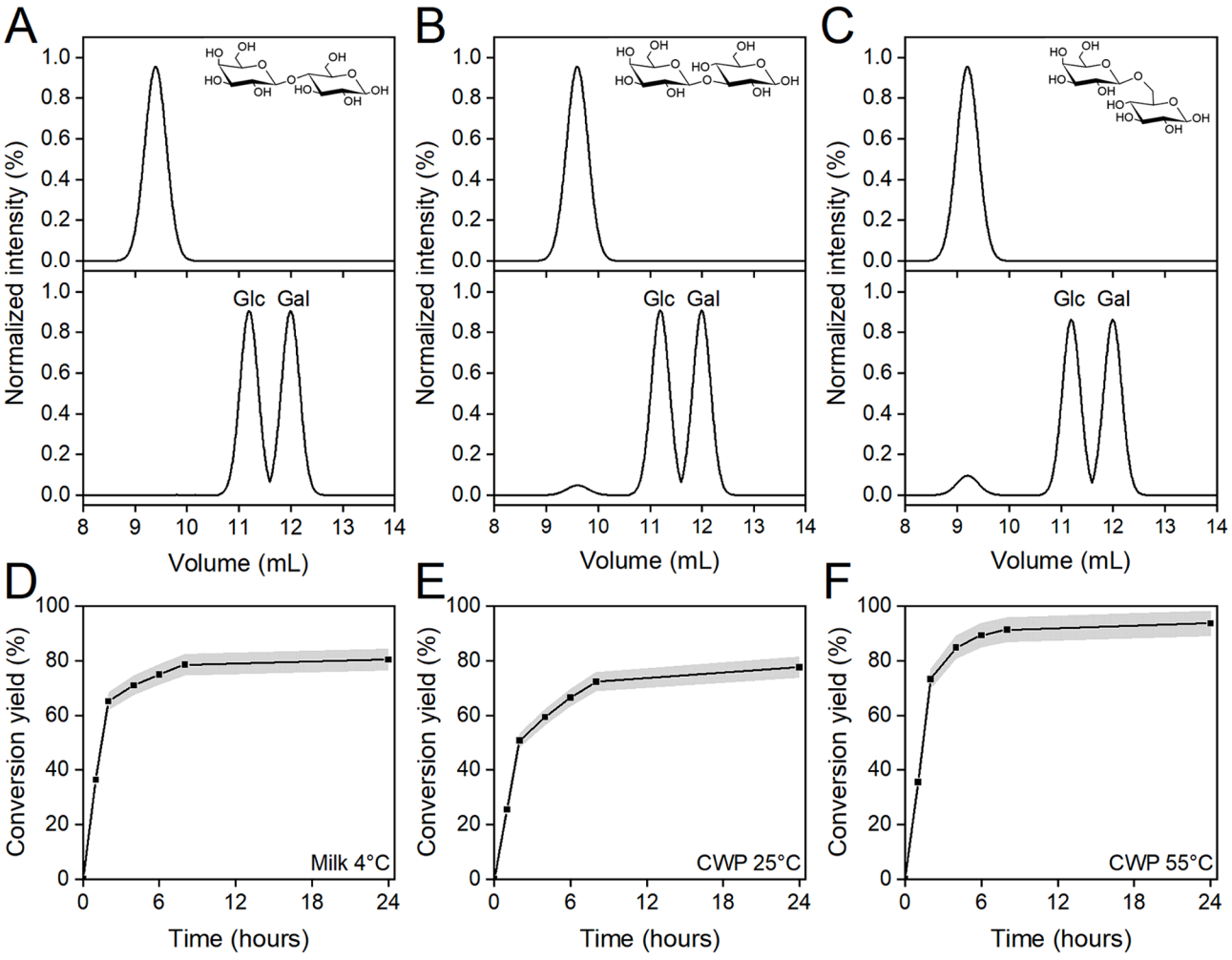

Fig. 3. Substrate specificity of M-βGal and lactose hydrolysis in skimmed milk and CWP matrices. Hydrolysis of lactose (A), 1,3-β-D-galactosyl-D-glucose (B) and 1,6-β-D-galactosyl-D-glucose (C). Upper panels: HPLC chromatograms of the reaction substrates before enzymatic treatment. Bottom panels: HPLC chromatograms of the hydrolysis products obtained with 10 U of M-βGal. Enzymatic reactions were performed in the presence of 1 % (*w/v*) disaccharide in PB at 50 °C for 4 h. One of three independent experiments is shown. D-F) Kinetics of lactose hydrolysis in skimmed milk and CWP matrices monitored by ATR-FTIR spectroscopy. Hydrolysis reactions were carried out on 25 mL of each matrix with 125 U M-βGal per mL, for 24 h. Reactions were performed on skimmed milk at 4 °C (D), CWP at 25 °C (E) and CWP at 50 °C (F). The shaded area indicates the standard deviation of three independent replicates.

(Fig. 3**D**). This extent of lactose hydrolysis is comparable to that reported for commercially available lactases from *Kluyveromyces fragilis* (LYL, 83 %) and *Aspergillus oryzae* (EYL, 85 %) under similar conditions, but lower than that of commercial lactases from *Kluyveromyces lactis* (approximately 100 %), which are commonly used in the production of lactose-free products (Horner et al., 2011).

Similarly, reactions carried out for 24 h at 25 °C and 50 °C on filter-sterilized CWP were monitored by ATR-FTIR spectroscopy. As expected, the reaction was slower with lower conversion yields at 25 °C (final conversion yield: 77.7 ± 4.1 %) compared to 50 °C (final conversion yield: 94. 5 ± 3.7 %) (Fig. 3**E and F**). These results suggest that M-βGal can be used in the enzymatic pretreatment of CWP, expanding its use as a microbial growth medium, since only a limited number of natural or recombinant microorganisms can convert lactose into value-added products such as polyhydroxyalkanoates (Amaro et al., 2019).

Overall, substrate specificity and regioselectivity, together with proficiency in lactose hydrolysis across diverse matrices and temperature conditions, make M-βGal a versatile catalyst, adaptable to various processes and environments, with potential for further enhancement through protein or process engineering.

4. Conclusion

This work introduces a sustainable method for producing a recombinant hydrolytic enzyme along with galactose. M-βGal efficiently hydrolyzes lactose in complex matrices, making it applicable for biotechnological purposes. Substantial amounts of cell biomass, recombinant proteins, and galactose are obtained from agro-food streams such as crude glycerol and CWP by application of fed-batch culture techniques. This approach promotes sustainability by saving water and other growth media components, optimizing their use into a promising biotechnological process for potential high-value products. In conclusion, this work outlines a path for integrating traditional industrial processes with innovative ones, paving the way for a zero-waste economy.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Stefania Brocca reports financial support was provided by Cariplo Foundation. Marco Mangiagalli reports financial support was provided by Cariplo Foundation. Antonino Natalello reports financial support was provided by Cariplo Foundation. Marina Lotti reports financial support was provided by Cariplo Foundation. Greta Bianchi reports financial support was provided by Cariplo Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131063.

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