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Characterization of thermostable β-glucosidase immobilized on linen fabric

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ABSTRACT

In this study, a thermostable β-glucosidase derived from a hot spring metagenome was successfully immobilized on linen fabric to create an eco-friendly and reusable biocatalyst for lactose hydrolysis. The enzyme is derived from a metagenome of the high-temperature Pisciarelli hot springs, an extreme environment known to yield robust biocatalysts with industrial relevance. Two immobilization strategies were employed: direct covalent binding (Lf-β-glucosidase) and binding via a spacer arm (LfEG-β-glucosidase). The latter exhibited superior enzymatic performance, retaining 76.6% of its initial activity and demonstrating enhanced thermal and operational stability. Both free and immobilized forms of enzyme showed similar pH and temperature optima, with the immobilized form exhibiting significantly improved long-term thermostability and storage stability—retaining full activity after 1 yr at 4°C. The LfEG-β-glucosidase biocatalyst was effectively applied in the enzymatic treatment of cheese whey, retaining 90% activity after 15 reuse cycles, yielding more than 6.4 g of glucose. These findings highlight the promising use of biodegradable, low-cost linen fabric as a carrier for thermostable enzymes in food biotechnology. The high efficiency and reusability of the developed biocatalyst offer a compelling solution for lactose hydrolysis and valorization of dairy industry by-products, particularly whey, within a circular and environmentally conscious production model.

Key words: thermostable β-glucosidase, enzyme immobilization, linen fabric carrier, lactose hydrolyzes, whey valorization

INTRODUCTION

Lactose hydrolysis plays a critical role in the dairy industry, particularly for producing lactose-free products and valorizing whey, a byproduct rich in lactose. After the discovery of lactose intolerance among people, the hydrolysis of milk lactase became of key importance in the field of the dairy industry. The use of enzymes to reduce lactose content in milk and dairy products offers a valuable opportunity to create products that are highly suitable for lactose-intolerant adults and children (Panesar et al., 2010). In recent times, there has been a growing interest in utilizing whey as an economical cultivation medium for microbial growth (Chaparro et al., 2021; Crament et al., 2024). Additionally, there is active consideration for enzymatic hydrolysis of lactose to enhance its accessibility for the cultivation of a diverse array of bacteria (Poladyan et al., 2023). β-Galactosidase, commonly named lactase, is one of the most important enzymes used for this purpose. Various β-galactosidases isolated from mesophilic microorganisms have been described so far (Brandão et al., 1987; Joshi et al., 1989; Stred'anský et al., 1993; Hoyoux et al., 2001; Nagy et al., 2001; Mahoney, 2002; El-Gindy, 2003), however, β-galactosidase of *Kluyveromyces lactis* still remains the major commercially usable one despite its thermolability (Ganeva et al., 2001). The application of known β-galactosidases is, however, partly hampered because of the moderate thermal stability and narrow pH profile as well as the significant inhibition by D-galactose. From an alternative perspective, β-glucosidases able to hydrolyze lactose can be used for this purpose (Li et al., 2013). Thus, thermostable β-glucosidases could help to overcome at least some of these problems and reduce the lactose content while functioning under pasteurization conditions. Most importantly, it is possible to combine the process of pasteurization and lactose reduction in milk or whey using thermostable enzymes.

It has been shown that a thermostable β-glucosidase isolated from a hydrothermal spring metagenome hydrolyzes lactose into its monomers, glucose and galactose, and has a potential for applications in lactose/lactose-containing food processing industry (Schröder et al., 2014). This enzyme has been successfully used for enzymatic treatment of whey-based side streams to develop it as a cultivation medium for biomass and enzyme production (Poladyan et al., 2023; Ghevondyan et al., 2024).

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

These industrial processes would benefit from the use of immobilized enzyme systems that allow several reuses of the enzyme and avoid contamination of the product by enzyme preparation. The key points in enzyme immobilization are a suitable carrier and an immobilization method. Recent innovations in immobilized β-galactosidases have improved the performance of the immobilized enzymes and broadened their applications in the fields of food, energy, and medicine (Duan et al., 2022). Various methods have been reported also for β-glucosidase immobilization, including adsorption, covalent binding, and entrapment (Libertino et al., 2008; Figueira et al., 2013; Wei et al., 2013; Zheng et al., 2013; Tan and Lee, 2015; Javed et al., 2016). Up to now, the development of a cost-efficient way to reduce the leakage of β-glucosidase from carrier after immobilization during operation, enhance the separation after the reaction, and improve the reuse times and operational stability remains very challenging.

Immobilization of enzymes by covalent coupling usually leads to very stable preparations compared with the ones obtained by other immobilization procedures. There is a large number of carriers that can be used to immobilize enzymes, but medical and nutritional requirements greatly limit the number of carriers that can be used to immobilize enzymes for food purposes. The carriers must be nontoxic, noncarcinogenic, and in no way harmful to the biological environment.

Cellulose is the most common natural polymer that is strong, hydrophilic, insoluble in water, resistant to chemicals, safe for living bodies, reproducible, recyclable, and biodegradable. Depending on the nature of the oxidizing agent and the oxidation conditions, chemical modification with oxidizing agents leads to the formation of aldehydes, ketones, and carboxyl groups in cellulose. Oxidation of cellulose with periodate leads to the formation of a compound, which is dialdehyde cellulose (**DAC**; Varma and Kulkarni, 2002; Potthast et al., 2009). The resulting dialdehyde cellulose can be used to immobilize proteins by reacting with their amino groups to form a Schiff bond (Varavinit et al., 2001). One significant advantage of periodate over other oxidizing agents is that the reaction proceeds under mild conditions, which minimizes degradation and retains the mechanical and morphological properties of the starting oxidized material (Husemann, 1965).

In this work, we reported the oxidation of linen fabric with sodium periodate solution to obtain a carrier for subsequent immobilization of β-glucosidase. Linen, as the natural form of cellulose, was chosen due to the numerous advantages over cotton. These include: (1) a degree of polymerization in flax fiber cellulose that is 2 to 3 times higher than that of cotton, resulting in superior strength; (2) excellent physical properties such as high hygroscopicity (up to 12%), heat resistance (withstanding temperatures up to 170°C), and light resistance (twice that of cotton). Moreover, a comparative analysis of the chemical composition of linen and cotton fibers highlights unique components in linen cellulose and their quantitative ratios, which contribute to its enhanced biostability, greater hygroscopicity, and higher moisture capacity when compared with cotton cellulose.

This study aimed to immobilize thermophilic β-glucosidase on linen fabric and characterize the immobilized catalyst. The immobilization conditions and the catalytic properties of immobilized β-glucosidase, such as optimal pH and temperature values, kinetic parameters of the lactose degradation reaction, and thermal, operational, and storage stability, were investigated.

The obtained biocatalyst was able to hydrolyze whey lactose into more valuable and easily metabolizable sugars, thereby addressing 2 key issues: reducing whey waste and generating substrates for food and biotechnological applications. Enzyme-based hydrolysis, particularly with thermostable catalysts such as immobilized β-glucosidase, offers the possibility of integrating lactose removal into existing industrial heat treatment steps, such as pasteurization, thereby streamlining processes and lowering production costs.

Due to its high operational stability, immobilized β-glucosidase provides the dairy sector with a reusable, safe, and scalable tool for the sustainable production of lactose-free products and the efficient valorization of whey side streams. This approach not only mitigates the environmental burden associated with whey disposal but also contributes to the development of functional and value-added dairy products, supporting the broader goals of a circular bioeconomy.

MATERIALS AND METHODS

Chemicals, Microbial Strains, Enzymes

Sodium periodate was supplied by VWR International, Leuven, Belgium. Potassium borohydride, D-lactose, D-glucose, isopropyl β-D-thiogalactoside (**IPTG**), and ampicillin were purchased from Sigma Chemicals (Sigma-Aldrich Corp., St. Louis). The linen fabric was produced at the TD Orshanski Lnokombinat factory in Orsha, Belarus. Glucose oxidase (**GOX**) from the *Penicillium chrysogenum* MDC 8358 strain (Microbial Depository Center of SPC “Armbiotechnology” NAS of RA) was isolated and purified by us, yielding a specific activity of 123 μmol/min/mg (**U/mg**; Paloyan et al., 2023). Peroxidase (**POX**) was isolated and purified from horseradish, with an activity of 1,020 U/mL (unpublished data). The pQE-80L::bgl1 plasmid was kindly provided by colleagues from the Center for Biobased Solutions (**CBBS**), TUHH (Hamburg, Germany). *Escherichia coli*

BL21(DE3) was used for the expression of β-glucosidase. Profinity IMAC Ni-charged resin was purchased from Bio-Rad. All other reagents were of analytical grade. All solutions were prepared using distilled water and filtered before use.

Expression and Purification of the Recombinant β-glucosidase

For gene expression, *E. coli* BL21 (DE3) cells harboring the recombinant pQE-80L::bgl1 plasmid were first inoculated into 10 mL of Luria-Bertani agar (**LB**) medium supplemented with ampicillin (100 µg/mL) and incubated overnight at 37 °C with shaking at 180 rpm. The overnight culture was then transferred into 1 L of fresh LB medium containing ampicillin, and incubation was continued under the same conditions. When the optical density at 600 nm reached 0.6, 1 m*M* IPTG was added to the culture medium to induce recombinant protein expression. After incubation at 30°C and 180 rpm for 12 h, the cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C. The resulting pellets were stored at −20°C for further experiments.

Five grams of cells were resuspended in 10 mL of ice-cold lysis buffer (50 m*M* KH_2PO_4 pH 8.0; 300 m*M* NaCl; 10 m*M* imidazole) and were sonicated at middle intensity for 10 min (30 s pulse, 30 s interval) using a sonicator (Labsonic 2000, B. Braun Germany). The sonicated cell lysate was centrifuged at 1,000 × *g* and 4°C for 40 min. The supernatant containing recombinant thermostable β-glucosidase was incubated at 70°C for 15 min, followed by centrifugation at 1,000 × *g* for 40 min at 4°C. Afterward, a hexa-histidine-tagged β-glucosidase was purified to homogeneity using nickel affinity chromatography Profinity IMAC Ni-charged resin (Bio-Rad). The column containing 1 mL of resin was equilibrated with wash buffer (50 m*M* KH_2PO_4; pH 8.0; 300 m*M* NaCl; 10 m*M* imidazole). The supernatant was transferred to the column and incubated for 60 min at 4°C. After removal of the flow-through from the column, the resin was washed twice with 4 mL of washing buffer, and the bound protein was eluted 4 times with 1.5 mL of elution buffer (50 m*M* KH_2PO_4, pH 8.0, 300 m*M* NaCl; 500 m*M* imidazole). All fractions were analyzed by SDS-PAGE (Laemmli, 1970).

The elution fractions containing the targeted protein were combined and buffer exchanged to remove the imidazole, then concentrated by 10 kDa cut-off protein concentrators (Merck Amicon) for further analyses. The protein concentration was determined by the Bradford method using BSA as a standard (Bradford, 1976).

Enzyme Immobilization

Linen fabric (**Lf**) was selected as the carrier, sodium periodate (**Sp**) as the oxidizing agent, and ethylenediamine and glutaraldehyde (**EG**) as spacer arms for immobilization. A sample of Lf with a surface area of 3.61 cm^2 (1.9 × 1.9 cm) and a weight of 0.0872 ± 0.013 g was treated with a hot sodium hydroxide solution, washed several times with distilled water to remove the alkali, and then dried.

The obtained regenerated carrier was oxidized by soaking in 50 m*M* sodium periodate solution at room temperature for overnight in the dark. To remove the oxidant, the activated carrier was washed with distilled water several times and used for immobilization of β-glucosidase. Two types of biocatalysts were generated. The first type involved the direct attachment of the β-glucosidase to the surface of linen fabric (**Lf-β-glucosidase**). The second type of biocatalyst employed was EG, positioned between the surface of the linen fabric and the enzyme (**LfEG-β-glucosidase**). For the preparation of Lf-β-glucosidase, the protein was directly immobilized onto the activated carrier by incubation with a 3 mL solution containing 10 mg of β-glucosidase in 50 m*M* phosphate buffer (pH 6.0) at 4°C overnight. For the biocatalyst with a spacer arm (LfEG-β-glucosidase), the activated carrier was first incubated with a 2.5% ethylenediamine solution for 2 h at 4°C. The excess ethylenediamine was removed by washing the activated carrier several times with distilled water, followed by treatment with a 2% glutaraldehyde solution for 1 h at 4°C. After washing the carrier 4 times with distilled water, the protein was immobilized using the same procedure, resulting in the formation of the LfEG-β-glucosidase biocatalyst. Uncross-linked proteins were removed by washing with distilled water 4 times. The washing solutions were collected to detect the amount of uncrossed-linked enzyme. The amount of β-glucosidase being successfully immobilized onto the carrier was calculated as the difference between the initial and the unbound enzyme. To finish the protein immobilization process, all immobilized preparations were reduced with sodium borohydride, as indicated below.

Activity Assay

The β-glucosidase activity in both the solution and immobilized states was determined using a method based on the initial rate of lactose hydrolysis. Immobilized biocatalyst was incubated in a 3 mL substrate solution consisting of 100 m*M* lactose in 50 m*M* phosphate buffer at pH 6.0 in a batch reactor at 70°C. After a 10-min process, an aliquot was taken. β-glucosidase activity was measured by determining the liberated D-glucose in the coupled glucose oxidase-peroxidase reaction using the modified Trinder method (Trinder, 1969). The final volume (1.5 mL) of the reaction mixture contained: 0.3 m*M* 4-aminoantipyrine, 5 m*M* phenol, 1 to 2 U/mL horseradish POX, 1 to 2 U/mL of GOX, and 100 m*M* Na-acetate at pH 5.8 was incubated at 30°C for 5 min. The reaction was initiated by adding an aliquot of the sample to determine D-glucose, resulting from the hydrolysis of the lactose. The amount of D-glucose (μmol) that is formed from lactose degradation under the action of 1 mg of free or attached β-glucosidase per minute was taken as a unit of free and immobilized biocatalyst enzymatic specific activity—U/mg. This unit of activity was used for calculations in experiments comparing the free enzyme with the immobilized enzyme. The following unit of activity was also used to characterize the immobilized biocatalyst—μmol/min/g_{cat} (**U/$\mathbf{g_{cat}}$**).

Effects of pH and Temperature on β-glucosidase Activity

To determine pH optimum of free enzyme and Lf-β-glucosidase, the above-mentioned reaction medium was used, changing only the initial buffer pH. Additionally, 50 m*M* citrate-, phosphate-, and Tris-containing buffers were used, the pH of buffers was adjusted by concentrated solutions of HCl or NaOH. The pH range between 2.8 and 8.5 was examined.

To determine the optimal temperature for free enzyme and Lf-β-glucosidase, enzymatic activity was measured in the temperature range of 40 to 100°C at pH 6.0. To assess thermal stability, the samples of enzymes (free and immobilized) were incubated at temperatures ranging from 50 to 90°C, at pH 6.0, for 60 min, followed by cooling on ice. Afterward, the residual enzyme activity (%) was measured as described above.

Kinetics and Inhibition

The apparent Michaelis constant ($\boldsymbol{K_m}$) and maximum reaction rate ($\boldsymbol{V_{max}}$) were determined by measuring Lf-β-glucosidase activity at different substrate concentrations (10–250 m*M*). The reaction mechanism was determined graphically by the Lineweaver-Burk plot. The kinetic parameters, namely, K_m and V_{max}, and their standard errors, were calculated for both the free enzyme and Lf-β-glucosidase biocatalyst by the method of multivariate linear regression (Cornish-Bowden, 1976), using the program written in Gauss 4.0.

To study the mechanism of inhibition of β-glucosidase by D-glucose, the activity of Lf-β-glucosidase was measured at different concentrations of the substrate (5–50 m*M*) and inhibitor (0–50 m*M*). The inhibition mechanism was determined graphically by plotting 1/V as a function of [I]—inhibitor concentration and [S]/V as a function of [I] and was predicted. The inhibition constant K_i, the apparent K_m, V_{max}, and their standard errors were calculated as described by Cornish-Bowden (1976) with some modifications (Hambardzumyan, 2018).

Operational Stability of Lf-β-glucosidase and LfEG-β-glucosidase

The operational stability of Lf-β-glucosidase and LfEG-β-glucosidase was tested during the batch reaction process of lactose hydrolysis. Biocatalysts were incubated in 3 mL of the substrate solution consisting of 100 m*M* lactose in 50 m*M* phosphate buffer at pH 6.0 in a batch reactor. The process was started at 70°C for 10 min, samples were taken for measuring glucose concentration and calculation of activity. Afterward, the temperature lowered to the level of 60°C until the end of the cycle. After 3 h of use, the biocatalyst was removed from the reactor, washed, and used in the next cycle. For each cycle, the activities of both biocatalysts and the amount of glucose produced were calculated.

The operational stability of LfEG-β-glucosidase was further evaluated for lactose hydrolysis in curd whey. The curd whey substrate was pretreated by adjusting its pH to 6.5 using NaOH, followed by heating at 70°C. The mixture was cooled overnight, and the resulting protein precipitate was removed through centrifugation and filtration until a clear solution was obtained. After precipitation, the pH was adjusted to 5.6.

The immobilized enzyme was incubated in 3 mL of the pretreated substrate. The process began at 70°C for 10 min, during which samples were collected to measure glucose concentration and calculate enzyme activity. Subsequently, the temperature was reduced to 60°C and maintained until the end of the cycle.

Storage Stability of Free Enzyme and Lf-β-glucosidase

The stability of the free enzyme and Lf-β-glucosidase under steady-state conditions was determined by measuring enzyme activity after 1 yr of storage. Samples of Lf-β-glucosidase and free enzyme were placed in 50 m*M* phosphate buffer, pH 6.0 (storage solution), at 4°C. Storage stability of samples is given as relative activity. Immobilized enzyme activity is related to the activity of the same sample immediately after immobilization (initial activity of immobilized enzyme), and free enzyme activity of stored β-glucosidase solution is related to the activity of freshly prepared enzyme solution (initial activity of free enzyme).

To study the β-glucosidase leakage from the carrier, the protein concentration in the storage solution was measured as described above.

Statistical Analysis

All presented data are the arithmetic means of at least 3 independent measurements.

RESULTS

Expression and Purification of Recombinant Enzyme

The β-glucosidase gene derived from hot spring metagenomes was successfully cloned and characterized, and the pQE-80L::bgl1 construct has been transferred to us for study as a suitable enzyme for lactose hydrolysis. The construct was transformed to *Escherichia coli* BL21 (DE3) expression host for heterologous expression. Induction of β-glucosidase was performed at 30°C for 12 h. Because β-glucosidase is known for its thermostability, the supernatant obtained after ultrasonication was subjected to a temperature treatment process to effectively eliminate thermolabile proteins. Afterward, the enzyme was purified by nickel affinity chromatography and analyzed by SDS-PAGE (Supplemental Figure S1, see Notes). All 4 elution fractions were combined, buffer exchanged, and concentrated. The purified β-glucosidase, with a protein concentration of 3.2 mg/mL, and a specific activity of 270 U/mg measured at 70°C and pH 6.0, was used for immobilization experiments.

Immobilization of β-glucosidase on Linen Fabric

The objective of this study was to find a suitable method for the efficient and reproducible coupling of β-glucosidase to a support suitable for multiple and long-term use, inexpensive, easy to use, and environmentally friendly in the recycling process. Linen fabric, as the purest natural product, was chosen for surface coupling of purified β-glucosidase. Here, we applied a covalent binding method for immobilization. This method involved establishing strong and durable chemical bonds between the enzyme and the fabric surface, ensuring stable and long-lasting enzyme activity for various applications. To obtain aldehyde groups capable of interacting with the amino groups of β-glucosidase, we employed an oxidation step by $NaIO_4$ to generate cellulose aldehyde groups that can be further employed as binding sites (Figure 1A).

The Lf-β-glucosidase biocatalyst was obtained by directly attaching β-glucosidase to the oxidized linen carrier surface (Figure 1A). In this case, the catalyst retained 57.2% (154.4 ± 1.2 U/mg, equal to 478 ± 2.2 U/g_{cat}, which corresponds to 1.77 $mg_{protein}/g_{cat}$) of the initial enzyme activity. In this case, the catalyst retained 57.2% of the initial enzyme activity (478 ± 2.2 U/g_{cat}), which corresponds to 1.77 mg of protein per gram of catalyst.

To investigate the effect of a spacer arm, a second type of biocatalyst named LfEG-β-glucosidase was developed, which contains EG between the surface of the linen fabric and the enzyme (Figure 1B).

The effect of varying concentrations of ethylenediamine in the immobilization reaction was studied. The results revealed that reaching a concentration of ethylenediamine of 400 m*M* led to a remarkable outcome, with 76.6% retention of the initial enzyme activity yield (206.82 ± 1.1 U/mg, equal to 642.4 ± 3.2 U/g_{cat}, which corresponds to 1.77 $mg_{protein}/g_{cat}$; Figure 2). In this case, the catalyst retained 76.6% of the initial enzyme activity (642.4 ± 3.2 U/g_{cat}), which corresponds to 2.38 mg of protein per gram of catalyst.

Characterization of Free and Immobilized β-glucosidase

The effect of pH and temperature on both free β-glucosidase and Lf-β-glucosidase biocatalyst activity is shown in Figure 3. The results indicate that the free enzyme (orange line) and the Lf-β-glucosidase biocatalyst (blue line) exhibit their highest activity in the pH range of 5.5 to 6.0 (Figure 3A).

Regarding temperature, both the free enzyme and Lf-β-glucosidase display similar temperature-activity curves. The maximum activity for both forms is observed at ~90°C (Figure 3B). This finding suggests that the optimal pH and temperature of the enzyme did not change during immobilization.

A study of the thermal stability of Lf-β-glucosidase (blue line) biocatalyst showed that whereas maximum enzyme activity was observed at 90°C, prolonged exposure to this temperature resulted in a significant loss of activity (78% decrease; Figure 4A). At 70°C, the biocatalyst showed only a slight decrease of ~10% in activity, indicating better stability at this temperature. Notably, at 60°C, the activity of the catalyst remained unchanged. Up to 80°C, the thermal stability of both free enzyme (orange line) and Lf-β-glucosidase was found to be similar, whereas free enzyme was more stable at 90°C compared with Lf-β-glucosidase in the case of a short time of incubation (Figure 4A). During long-time incubation at a temperature of 70°C, the Lf-β-glucosidase biocatalyst demonstrates significantly enhanced thermal stability when compared with its free form. After 6 h of incubation at this temperature, the biocatalyst retains 68.1% of its original activity, whereas in the case of the free enzyme, only 11.1% of the original activity remains (Figure 4B).

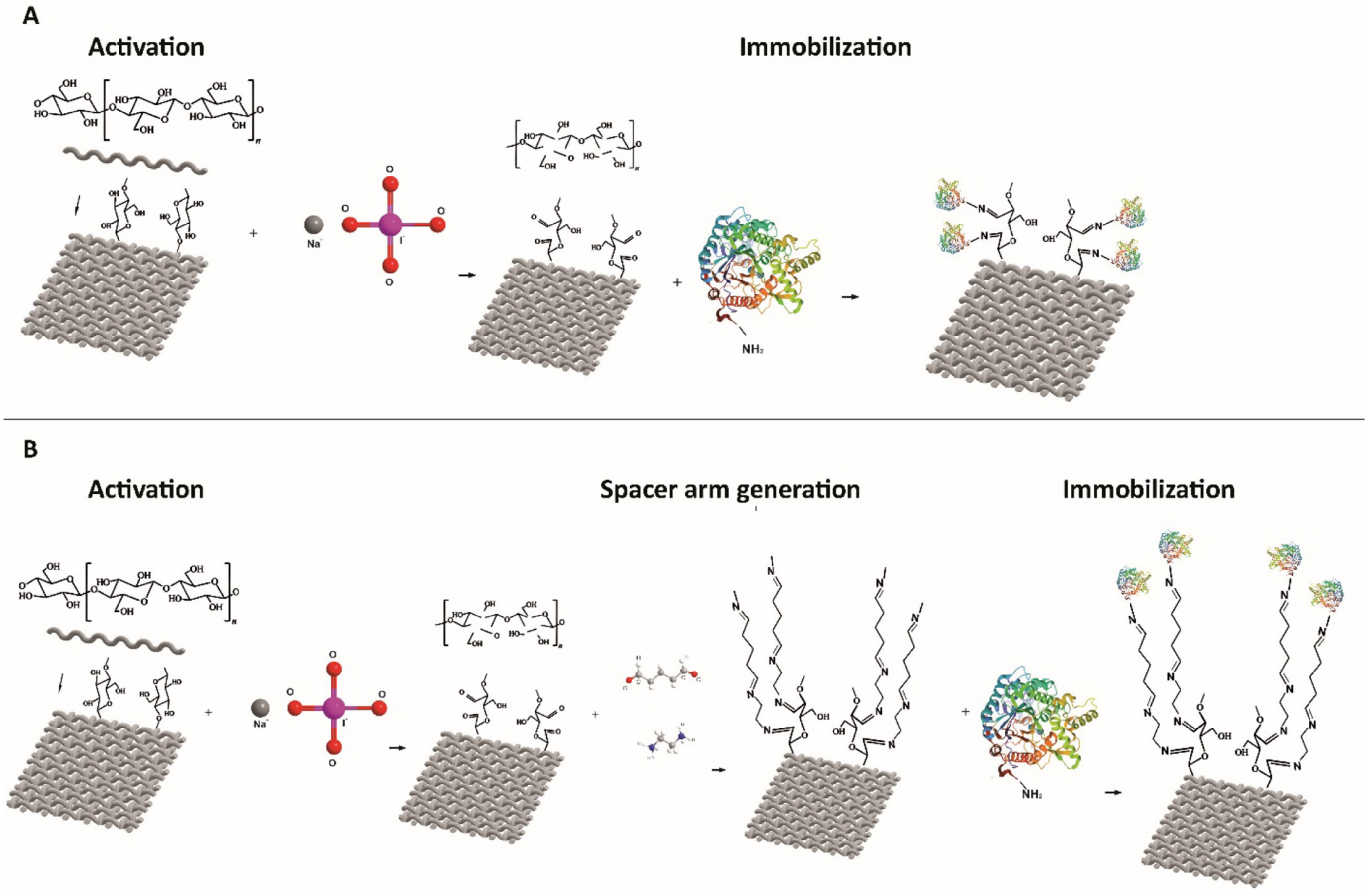

Figure 1. Schematic picture of immobilization of β-glucosidase to obtain Lf-β-glucosidase biocatalyst (A) and LfEG-β-glucosidase (B).

The effect of substrate concentration on the reaction velocity was investigated for both the free enzyme and Lf-β-glucosidase. In these studies, lactose was used as the substrate at concentrations ranging from 10 to 250 m*M* (Supplemental Figure S2, see Notes).

The inhibitory effect of D-glucose on β-glucosidase was investigated, consistent with the previously published data indicating the inhibition of β-glucosidase by D-glucose. In this study, a more detailed analysis of the inhibitory mechanism was conducted for free enzyme as well as for Lf-β-glucosidase. The graphical analysis (Supplemental Figure S3, see Notes) indicates the competitive type of inhibition by D-glucose. The program used also revealed a competitive type of inhibition, which corresponds to the results of the graphical analysis. The calculated values of the kinetic parameters are presented in Supplemental Table S2, see Notes. Thus, values of $V_{\max}$ and K_m for Lf-β-glucosidase obtained from 2 different experiments (Michaelis-Menten kinetics and inhibition kinetics) are close enough and are within the range of dispersion of the parameters.

Operational and Storage Stability

The operational stability of Lf-β-glucosidase and LfEG-β-glucosidases (obtained by using 400 m*M* of ethylenediamine) was studied by subjecting them to consecutive runs of lactose hydrolysis lasting 3 h each (Figure 5). In the initial 3 runs, Lf-β-glucosidase achieved a lactose conversion rate of 69.9% ± 5.3%, whereas LfEG-β-glucosidase exhibited a slightly higher conversion rate of 71.7% ± 7.2%, indicating comparable catalytic performance during early cycles. However, the stability and life span of the biocatalysts differed significantly upon repeated use. The Lf-β-glucosidase exhibited a sharp decline in activity after 8 cycles, with conversion efficiency dropping drastically to just 10% by the final cycle. This rapid reduction suggests limited operational stability under these conditions. In contrast, LfEG-β-glucosidase displayed greater resilience, maintaining relatively stable lactose conversion rates of ~71% during the first 4 cycles before exhibiting a gradual decline in performance. Even after 15 cycles, LfEG-β-glucosidase achieved a lactose

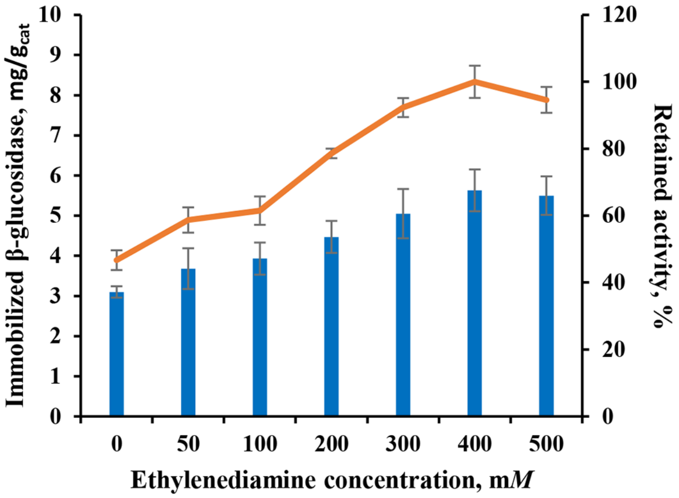

Figure 2. Impact of ethylenediamine concentration on the immobilization yield and enzymatic activity. The blue columns show the amount of protein (β-glucosidase enzyme) bounded in 1 g of linen fabric, orange line shows retained activity after immobilization, when different concentrations (0–500 m*M*) of ethylenediamine were used. The 0 corresponds to Lf-β-glucosidase biocatalyst, which is without spacer arm. In all cases, 3 mL of enzyme preparation (containing ~10 mg of protein) with the activity of 270 U/mg was used for 0.0872 g (3.61 cm^2) linen fabric. The maximum retained activity of the immobilized catalyst 206.82 U/mg ± 1.1 equal to 642.4 ± 3.2 U/g$_{cat}$ at an ethylenediamine concentration of 400 m*M* was taken as 100%.

conversion rate of 41%, which is significantly higher than the rate recorded for Lf-β-glucosidase after just 6 cycles. The LfEG-β-glucosidase had a longer life span before a noticeable decrease in its catalytic efficiency. Thus, the use of a spacer for immobilization of β-glucosidase yielded superior outcomes compared with directly binding the enzyme to the surface of linen.

In the subsequent phase, the performance of LfEG-β-glucosidase was evaluated in a batch reactor for its reusability in lactose degradation in curd whey (Figure 6). Interestingly, the biocatalyst exhibited a slight activity increase of up to 20% during the first 3 runs before stabilizing. This enhancement may be attributed to the activating effect of compounds present in whey, particularly salts.

Notably, even after multiple cycles, the biocatalyst's activity declined by only 10%, highlighting its high stability and efficiency during extended usage. Despite this, the lactose conversion yield remained consistent across 15 cycles, with an average glucose concentration of ~70 m*M*, underscoring the enzyme's robustness and suitability for prolonged applications.

The storage stability of both free enzyme and Lf-β-glucosidase was studied during long-term storage at 4°C. The findings revealed notable differences in their stability over time. After 2 mo of storage, the free enzyme lost 88% of its initial activity. In contrast, the Lf-β-glucosidase showed improved durability, with minimal decrease in activity even after 1 yr of storage (Figure 7). The Lf-β-glucosidase demonstrated a remarkable ability to retain its activity at the initial level throughout the storage period, whereas the free enzyme was irreversibly inactivated.

The study of the enzyme leakage from the carrier showed that after 1 yr of storing of the Lf-β-glucosidase, no protein was detected in the storage solution. This suggests that the stable covalent bonding restricts conformational changes and prevents enzyme leakage.

DISCUSSION

Sustainability, eco-friendliness, and circularity have emerged as foundational strategies across modern industries. Consequently, the development of a circular industrial model that achieves zero waste production is increasingly recognized as a critical priority in today's economic and environmental landscape.

The results of this study demonstrate the successful immobilization of thermostable β-glucosidase onto linen fabric, highlighting its potential for repeated and sustained use in lactose hydrolysis and whey treatment processes. β-Glucosidase enzyme was identified through metagenomic analysis of samples collected from the Pisciarelli hot springs, located in the Campi Flegrei volcanic area near Naples, Italy. The researchers extracted metagenomic DNA from environmental samples obtained at Pisciarelli, which is known for its high-temperature hydrothermal activity. They successfully cloned, expressed, and characterized a β-glucosidase enzyme that demonstrated significant thermostability and activity at elevated temperatures. These properties suggest potential applications in industrial processes that require robust enzymes capable of functioning under extreme thermal conditions (Schröder et al., 2014).

We have used linen fabric as a carrier, which is a sustainable and environmentally friendly material, it can be easily recycled or reused, or both. Covalent binding via the periodate method enabled the firm attachment of the enzyme to the carrier. To obtain a sufficient amount of bound enzyme on linen fabric and maintain the strength of the oxidizable carrier, the optimal concentration of periodate selected by us was 50 m*M* (1.1%), and the optimal oxidation time was 16 h. For comparison, in the literature data the optimal parameters for the oxidation of cotton fabric were 0.4% and 6 h of exposure (Kim et al., 2000; Nikolic et al., 2010). Thus, more stringent oxidation conditions were required to process linen fabric, which is associated with the specific properties of linen fabric compared with cotton fabric. Additionally, the use of an ethylenediamine-glutaraldehyde spacer significantly enhanced the enzyme's operational properties.

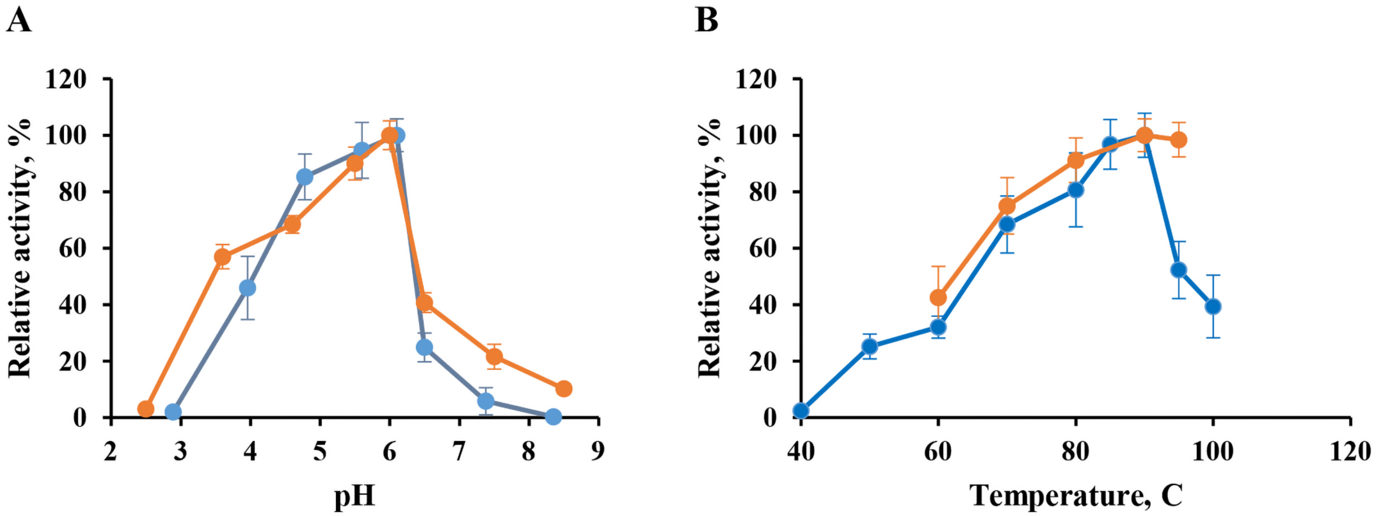

Figure 3. Activity of free and immobilized β-glucosidase at varying pH and temperatures. The activities of the free enzyme (orange line) were assessed across a pH range of 2.5 to 8.5, whereas the activity of Lf-β-glucosidase (blue line) was evaluated in the pH range of 2.9 to 8.35, at 70°C (A). Additionally, the temperature effects were measured from 60 to 95°C for the free enzyme (orange line) and from 40 to 100°C for Lf-β-glucosidase (blue line; B). The activity of samples is given as relative activity. In pH optimum experiments, 100% corresponds to 271.1 ± 5.1 U/mg and 470 ± 5.2 U/g_{cat}, which were obtained at pH 6.0 for free and immobilized enzymes, respectively. The maximum activity of the free and immobilized enzyme at the optimal temperature at 90°C corresponds to 358.2 ± 5.8 U/mg and 582.8 ± 8.8 U/ g_{cat} for free and immobilized enzymes at pH 6.0, were taken as 100%, respectively. Experiments were performed with 2 technical replicates each from 2 biological replicates.

It was observed that the amount of coupled protein in per unit surface increased more than 1.5 times when using 400 m*M* ethylenediamine (Supplemental Table S1, see Notes). Similar results have been obtained by Tan and Lee (2015). They have shown that the concentration of polyethyleneimine plays a critical role in immobilization yield. These results can be explained by the increase of aldehyde groups available to bind with the amine groups of the enzyme with a covalent bond. At higher concentrations of polyethyleneimine, more amine groups will be available in support to react with glutaraldehyde.

Two types of biocatalysts, Lf-β-glucosidase and LfEG-β-glucosidase, were obtained and studied. The use of the spacer arm preserved 76% of the enzyme's initial activity, whereas without it, only 57% of the initial activity was retained. To the best of our knowledge, these are the first reported results on the immobilization of β-glucosidases onto linen fabric. Compared with other β-galactosidase

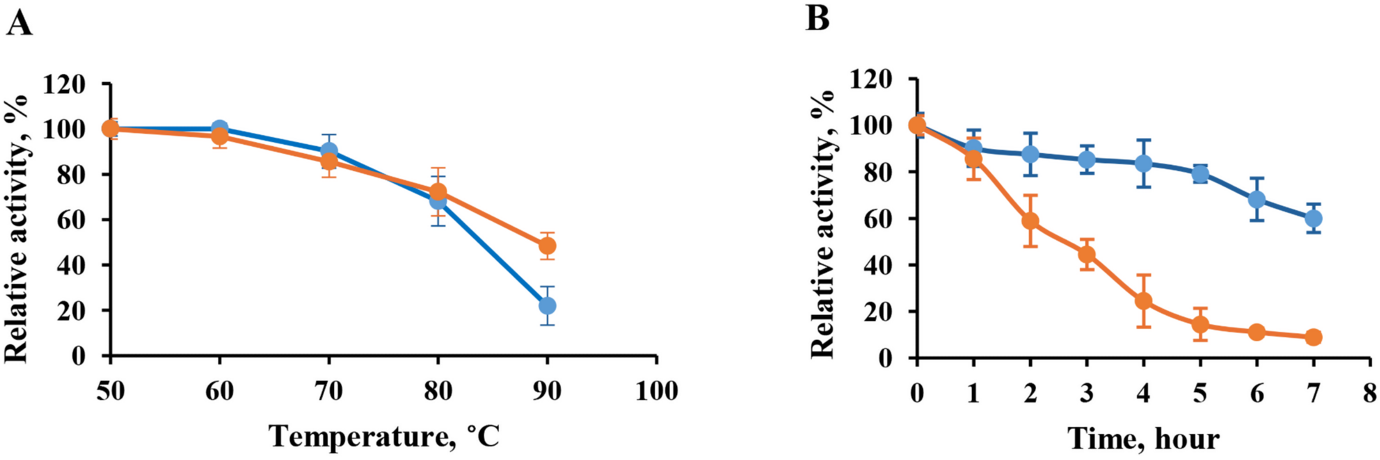

Figure 4. Temperature-dependent stability of free and immobilized β-glucosidase. Samples of free enzyme (orange line) and Lf-β-glucosidase (blue line) were preincubated in the temperature range between 50 and 90°C for 1 h (A); and at 70°C from 1 to 7 h (B), followed by cooling. The residual activity was assayed at the standard assay condition. For thermostability experiments, samples were prepared in 50 m*M* phosphate buffer, pH 6.0. The activity of samples is given as relative activity. Experiments were performed with 2 technical replicates each from 2 biological replicates.

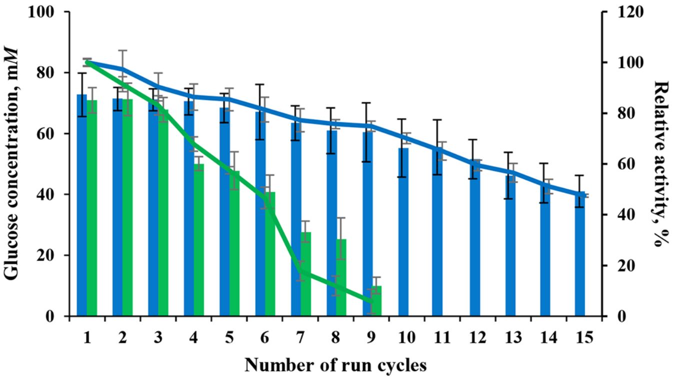

Figure 5. Operational stability and productivity of Lf-β-glucosidase and LfEG-β-glucosidase. The Lf-β-glucosidase (containing 3.1 mg/mg β-glucosidase, 478 U/gcat) and LfEG-β-glucosidase (5.63 mg/g_{cat} β-glucosidase, 642.4 U/g_{cat}) were incubated in a 3 mL substrate solution containing 0.1 *M* lactose at pH 6.0 in a batch reactor at 70°C. After 10 min, an aliquot was taken for activity measurement. The temperature was then lowered to 60°C, and the process continued for up to 3 h. Enzyme activity of 478.0 U/mg was considered 100%.

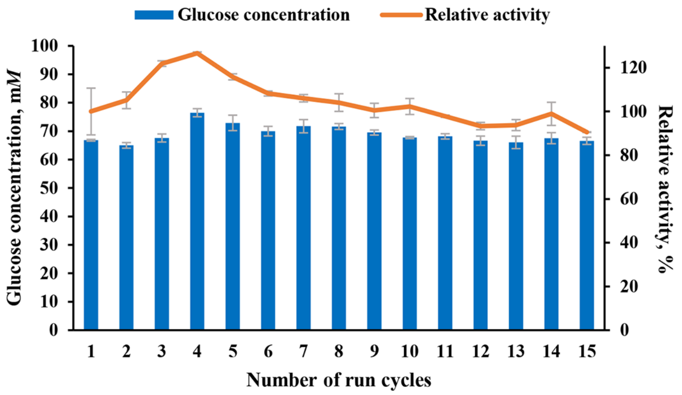

Figure 6. Stability and productivity of LfEG-β-glucosidase during repeated cycles of whey lactose hydrolysis. The blue bars represent glucose concentration in millimolar, while the orange line denotes the relative activity of LfEG-β-glucosidase during the runs. The LfEG-β-glucosidase at a concentration of 5.63 mg/g_{cat} β-glucosidase, 642.4 U/g_{cat} were taken as 100%. The biocatalyst was incubated in 3 mL of curd whey containing 0.135 *M* lactose at pH 5.6 in a batch reactor at 70°C. An aliquot was taken after 10 min for activity measurement. Subsequently, the temperature was lowered to 60°C, and the process continued for up to 4 h.

and β-glucosidase systems immobilized on traditional supports, the linen-based biocatalyst demonstrated superior stability and reusability, underlining its potential for sustainable industrial applications. Meanwhile, various β-glucosidases and β-galactosidases have been successfully covalently immobilized onto a range of support materials and applied in diverse bioconversion processes. Thus, β-glucosidase from *Thermotoga maritima* was covalently immobilized onto magnetic nanoparticles functionalized with chitin, retaining 79% of its original activity and yielding 31% galacto-oligosaccharides after 10 repeated cycles (Alnadari et al., 2020). Similarly, β-glucosidase from *Bacillus subtilis* PS was immobilized on SiO_2 nanoparticles with an immobilization efficiency of 52% and a product yield of 14.1%. This immobilized enzyme was further applied in the treatment of sugarcane juice, resulting in a reduction in juice density and viscosity, along with an increase in reducing sugar content (Agrawal et al., 2016). Another example is β-glucosidase from *Aspergillus niger*, which was immobilized on a κ-carrageenan hybrid matrix via polyethyleneimine and glutaraldehyde. The immobilized enzyme was successfully applied to hydrolyze pretreated macroalgae cellulosic residue (Tan and Lee, 2015).

In the case of β-galactosidase, the enzyme from *K. lactis* was covalently immobilized onto functionalized silicon dioxide nanoparticles using glutaraldehyde as a cross-linker. The immobilized enzyme retained over 50% of its initial activity after 11 cycles, with maximum lactose hydrolysis achieved within 8 h (Verma et al., 2012). This enzyme was also immobilized in calcium alginate spheres, achieving a 79.8% initial activity yield (Argenta et al., 2021), and on a silver-reduced graphene oxide nanocomposite, with a 96.8% initial activity yield (Shafi et al., 2021). Similarly, β-galactosidase from *Aspergillus oryzae* was covalently immobilized onto glass beads via 3-aminopropyl triethoxysilane as a cross-linker, resulting in a biocatalyst that retained ~80% of its initial activity. This immobilized enzyme was effectively used for the synthesis of galacto-oligosaccharides, achieving a 56.4% lactose conversion rate (Eskandarloo and Abbaspourrad, 2018). Interestingly, whereas enzyme immobilization often leads to a decrease in residual activity, an opposite

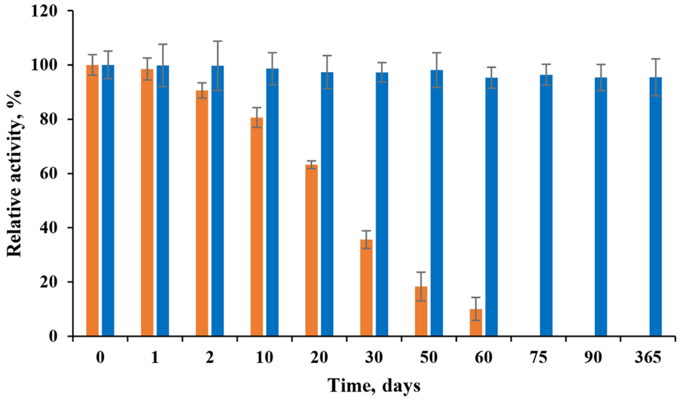

Figure 7. The stability of free enzyme (orange bars) and Lf-β-glucosidase (blue bars) at long-term storage. Both forms of enzyme preparations were stored in 0.05 *M* phosphate buffer, pH 6.0 at 4°C for 1 yr. Throughout this time, the specific activity of both enzyme forms was periodically assessed using the assay conditions mentioned earlier. Additionally, 100% corresponds to 270 U/mg for free enzyme and 478 U/g_{cat} Lf-β-glucosidase.

effect was observed for β-galactosidase from *A. oryzae* when immobilized on magnetic maghemite nanoparticles using glutaraldehyde and pentaethylenehexamine as cross-linkers (Križnik et al., 2018). These findings highlight the influence of immobilization methods and supports on enzyme activity and stability. In a comparable study the effect of pH and temperature on the catalytic activity of immobilized Lf-β-glucosidase was examined. Notably, both the free and immobilized forms of the enzyme exhibited identical activity profiles across the pH and temperature ranges tested. Similar observations have been reported for β-glucosidase from *Bacillus subtilis* PS, whereas in the cases of *T. maritima* (Alnadari et al., 2020) and *A. niger* (Tan and Lee, 2015) immobilization resulted in a slight shift in optimal pH.

The thermostability of the enzyme following immobilization represents a key criterion in the present study. Short-term thermal stability assays demonstrated that the immobilization process did not significantly alter the enzyme's stability. However, during prolonged incubation at 70°C, the immobilized enzyme exhibited markedly enhanced thermal stability compared with its free form (Figure 4). This improvement indicates that immobilization confers increased resistance to thermal denaturation, thereby supporting the enzyme’s reusability and functional retention under elevated temperature conditions. An improvement in temperature resistance by 10°C was also observed in immobilized β-glucosidase of *A. niger* (Tan and Lee, 2015) as well as for β-glucosidase of *B. subtilis* PS which shows ~288-fold increase in the thermal stability at 65°C compared with free enzyme.

The Lf-β-glucosidase performs lactose hydrolysis, which follows Michaelis-Menten kinetics with the competitive product (D-glucose) inhibition with values of kinetic parameters: K_{m} = 92.5 mM, $V_{\max}$ = 106.3 μmol/min. The $V_{\max}$ of Lf-β-glucosidase was lower than that of free β-glucosidase due to the lower diffusion rate near the catalyst surface, in the absence of agitation. The value of the inhibition constant in both cases is very close and is equal to K_{i} = 35.1 mM for Lf-β-glucosidase (Supplemental Table S2).

The characteristics of the immobilized biocatalyst suggest that lactose hydrolysis with immobilized thermostable β-glucosidase is preferable at a lower substrate concentration and a temperature well below the optimum. Temperature regulation can also serve as a tool to balance the enzymatic reaction rate and the decline in biocatalyst activity (Dyukova, 2018), thereby enhancing process productivity.

Two types of biocatalysts, Lf-β-glucosidase and LfEG-β-glucosidase, were evaluated for lactose hydrolysis at 60°C, pH 6.0. The LfEG-β-glucosidase biocatalyst exhibited 2.6 times greater productivity before losing ~60% of its initial activity. Calculations showed that with an initial activity of 642.4 U/gcat, LfEG-β-glucosidase remained effective for 15 reaction cycles, hydrolyzing 10.57 g of lactose and yielding over 5.5 g of glucose in 45 h. In contrast, Lf-β-glucosidase produced only 2.2 g of glucose in 6 reaction cycles (18 h), after which its continued use became economically unfeasible due to significant activity loss (Figure 5). Meanwhile, immobilized β-glucosidase of *A. niger* retained ~75% of its initial activity after 12 runs (Tan and Lee, 2015).

Whey, a byproduct of cheese and cottage cheese production, remains a significant waste stream, and its effective biotechnological valorization continues to be a topic of considerable interest (Buchanan et al., 2023). In the enzymatic treatment of cheese whey, the LfEG-β-glucosidase biocatalyst demonstrated high stability, retaining 90% of its initial activity even after 15 cycles. Interestingly, enzyme activity gradually increased over the first few cycles, likely due to the stabilizing effects of whey components. Calculations indicate that after 15 cycles, 1 g of biocatalyst can enzymatically process over 0.5 L of whey, yielding more than 6.4 g of glucose. Moreover, the data suggest that the biocatalyst may remain effective for additional cycles beyond this threshold (Figure 6). For comparison, β-galactosidase from *K. lactis* immobilized on calcium alginate was applied for whey lactose hydrolysis over 15 cycles, retaining 86.5% of its initial activity by the end of the treatment (Argenta et al., 2021). In a separate study, the same enzyme immobilized on a silver-reduced graphene oxide nanocomposite demonstrated similarly high operational stability, maintaining 85% activity after 10 reuse cycles during the whey processing.

The Lf-β-glucosidase was found to be much more stable when stored in a storage solution at 4°C than a native enzyme. During 1 yr of storage, its activity remained at the same level (Figure 7). In comparison, the storage stability of other immobilized β-galactosidase preparations was notably lower. For instance, β-galactosidase immobilized in calcium alginate spheres retained 63.4% of its initial activity after 90 d at 4°C, whereas the free enzyme retained only 48.7% under the same conditions (Argenta et al., 2021). A better result was observed for β-galactosidase immobilized on a silver-reduced graphene oxide nanocomposite, which maintained ~85% of its initial activity after 60 d at 4°C, compared with ~50% for the free enzyme (Shafi et al., 2021). Similarly, β-glucosidase immobilized on magnetic nanoparticles retained 91% of its initial activity after 30 d at 4°C (Alnadari et al., 2020). In contrast, significantly lower stability was reported for β-glucosidase immobilized on silicon oxide nanoparticles, which preserved only 10%

of its initial activity after 9 d of storage at 2°C (Agrawal et al., 2016).

The increased reusability, higher pH, improved thermal stability during prolonged incubation, and storage stability of the immobilized β-glucosidase compared with the free enzyme will be important for its sustainable use and the economic viability of the biosynthetic process.

The results of this study provide a practical solution for the dairy industry, where stable, reusable, and food-compatible enzyme preparations are urgently needed. The developed LfEG-glucosidase biocatalyst demonstrated excellent reusability, retaining high activity for at least 15 cycles of whey treatment. This level of operational stability surpasses many conventional immobilization systems and makes the process economically attractive by reducing enzyme costs. Moreover, the use of linen—a biodegradable, food-safe carrier—ensures compatibility with industrial food processing standards and supports sustainability goals. By transforming whey from a waste problem into a valuable carbohydrate resource, the approach aligns with circular bioeconomy principles and could be adopted by dairies to lower waste management costs, develop lactose-free product lines, and create added value through the production of fermentation-ready substrates.

Therefore, the resulting composition of thermostable β-glucosidase with linen has potential for food industry application.

CONCLUSIONS

This work introduces a robust and eco-friendly biocatalyst based on thermostable β-glucosidase immobilized on linen fabric. The enzyme, sourced from a metagenomic library from the Pisciarelli hot springs, retained high activity and thermal stability following covalent immobilization via a periodate-based method. The inclusion of an ethylenediamine-glutaraldehyde spacer further enhanced immobilization efficiency and preserved enzymatic activity. Among the 2 developed biocatalyst systems, LfEG-β-glucosidase exhibited superior operational stability, retaining over 90% activity across 15 reuse cycles and efficiently converting whey lactose to glucose. Comparative data suggest that linen-based immobilization performs as well as, or better than, many traditional supports in terms of enzyme retention, reusability, and storage stability, while offering clear advantages in biodegradability and environmental sustainability. Overall, these findings demonstrate that linen is a promising sustainable support material for enzyme immobilization, with strong potential to improve the efficiency and circularity of biocatalytic processes in dairy and food industry applications.

NOTES

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Nonstandard abbreviations used: CBBS = Center for Biobased Solutions; DAC = dialdehyde cellulose; EG = ethylenediamine and glutaraldehyde; GOX = glucose oxidase; IPTG = isopropyl β-D-thiogalactoside; Km = Michaelis constant; LB = Luria-Bertani agar; Lf = linen fabric; Lf-β-glucosidase = β-glucosidase attached to linen fabric surface; LfEG-β-glucosidase = EG, positioned between the surface of the linen fabric and the enzyme; POX = peroxidase; Sp = sodium periodate; U/gcat = µmol/min/gcat; U/mg = µmol/min/mg; Vmax = maximum reaction rate.

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