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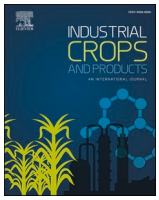

Upcycling agro-industrial biomass molasses into D-mannitol via an enzymatic cascade with in situ cofactor regeneration

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ABSTRACT

Molasses, a sugar industry by-product rich in sucrose, D-glucose, and D-fructose, remains underutilized in high-value biomanufacturing. In this study, we present an efficient enzymatic cascade for the direct conversion of molasses into D-mannitol, a valuable sugar alcohol widely used in the food and pharmaceutical sectors. The cascade integrates three enzymes: invertase for sucrose hydrolysis, mannitol dehydrogenase (MDH) for D-fructose reduction, and glucose dehydrogenase (GDH) for in situ NADH regeneration via D-glucose oxidation, which simultaneously generates D-gluconolactone as a value-added coproduct. Two reaction formats were evaluated: a two-step system that sequentially optimizes enzyme-specific conditions, and a one-pot system enabling operational simplicity. The two-step method yielded 137 ± 13 mM D-mannitol, corresponding to ~92 % conversion, while the one-pot system produced 123.1 ± 1.3 mM, corresponding to ~95 % conversion under molasses conditions within 24 h. The molasses pretreatment was not essential for D-mannitol production. Enzymes maintained high catalytic activity in the complex molasses matrix, and glucose supplementation improved cofactor regeneration, eliminating residual D-fructose. This integrated strategy offers a sustainable and scalable platform for agro-industrial waste valorization by co-producing D-mannitol and D-gluconolactone, aligning with circular bioeconomy principles and advancing green bioprocessing technologies.

1. Introduction

As the global population grows and human activities increase, natural resources are being increasingly depleted. To address this challenge, resource utilization within the framework of a circular economy is gaining significant attention. Unlike the traditional linear economy, which follows a “take-make-dispose” model, the circular economy emphasizes the reuse of waste to minimize environmental impact and maximize resource efficiency through recycling and upcycling strategies (Brydges, 2021; Calisto Friant et al., 2020; Castro et al., 2022; Iacovidou et al., 2020; Reuter et al., 2019). Among the major sources of industrial waste are agro-industrial processes, which generate substantial volumes of underutilized by-products (Ataei et al., 2025; Barcelos et al., 2020; Feng et al., 2024; Saxena et al., 2025).

Although agro-industrial waste is produced in large quantities annually, a significant portion remains unmanaged or discarded without further valorization (Ataei et al., 2025; Barcelos et al., 2020; Perwez and Al Asheh, 2025; Singh et al., 2022). Notably, approximately 37.36 % is landfilled, and 32.18 % is openly dumped, indicating that over half of all agro-industrial waste is discarded—contributing to both environmental pollution and inefficient resource use (Kee et al., 2021). Sugar refining processes represent a major segment of the agro-industrial sector and are known to generate substantial quantities of by-products, including molasses, bagasse, pulp, and filter mud (Akbar and Ali, 2017; Lima and Beacorn, 2022).

Molasses is a thick, viscous by-product of sugar refining, composed primarily of sucrose, D-glucose, and D-fructose, along with minerals and organic acids (Jin et al., 2025; Jung et al., 2013). Due to its high sugar content, molasses is considered a promising feedstock for bioconversion processes (Jung et al., 2013; Sharma et al., 2016). However, its current utilization remains limited to low-value applications such as bioethanol production, animal feed, and conventional fermentations (Lima and

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Beacorn, 2022). Expanding the upcycling of molasses into higher-value products is essential to advancing circular resource utilization.

Among potential high-value products derived from molasses, D-mannitol has broad applications in the pharmaceutical, food, and nutraceutical industries, primarily as a low-calorie sweetener (Ghoreishi and Shahrestani, 2009a; Xu et al., 2020). Its desirable physicochemical properties—including antioxidant activity, osmotic pressure regulation, and non-metabolizable behavior—have contributed to increasing market demand (Xu et al., 2020). The global market value of D-mannitol was USD 209.4 million in 2015 and has been steadily increasing over the past decade (Ruiz Rodríguez et al., 2017).

D-mannitol production from molasses has been explored using various microbial fermentation processes. Lactic acid bacteria such as *Leuconostoc*, *Lactobaillus*, and *Fructobacillus* have been widely employed due to their ability to convert D-fructose—a major component if molasses—into D-mannitol via mannitol dehydrogenase (MDH) activity (Baeshen et al., 2015; Ghoreishi and Shahrestani, 2009b; Molina et al., 2023; Wisselink et al., 2002). However, microbial fermentation of molasses to D-mannitol has inherent potential limitations. In microbial fermentation, D-fructose serves not only as the substrate for D-mannitol synthesis but also as a carbon and energy source for microbial growth and maintenance. As a result, a portion of D-fructose is consumed for biomass production and energy, challenging complete conversion to D-mannitol. Reported yields typically range from 60 % to 90 % of the theoretical maximum (Li et al., 2022; Ortiz et al., 2015; Zhang et al., 2018). Furthermore, microbial metabolism generated various by-products such as lactic acid, acetic acid, ethanol, which reduce the selectivity for D-mannitol and complicate downstream purification.

The purified enzyme cascade strategy a potential to address these limitations. In this approach, purified enzymes are used to convert D-fructose directly into D-mannitol. In these cell-free systems, all available D-fructose is directed toward D-mannitol synthesis, enabling conversion efficiencies approaching 100 % under optimized conditions. The absence of living cells also eliminates the formation of by-products such as organic acid, ethanol, and others, simplifying product recovery. Furthermore, the system operates under mild, non-sterile conditions and does not require complex nutrient supplementation or waste management associated with microbial fermentation.

Although sucrose—abundant in molasses—is one of the most accessible carbon sources from renewable resources, few sucrose-derived products currently have commercial applications due to challenges in reaction control involving its numerous hydroxyl groups (Mathlouthi and Reiser, 1995; Queneau et al., 2004). To enhance its utility as a feedstock, sucrose must first be hydrolyzed into reducing sugars—D-glucose and D-fructose—a process that can be catalyzed by invertase (Cadena et al., 2010; Yang and Montgomery, 2007). Following hydrolysis, D-fructose can be reduced to D-mannitol by MDH, an NADH-dependent oxidoreductase (Brünker et al., 1997; Slatner et al., 1998). However, the requirement for NADH poses economic and operational limitations due to the cost and continuous consumption of the cofactor (Cha et al., 2023; Wang et al., 2017). In enzymatic systems involving oxidoreductases such as MDH, cofactor regeneration is typically achieved through the use of an auxiliary enzyme pair (Bachosz et al., 2023; Cha et al., 2025).

For MDH, we selected an enzyme that preferentially catalyzes the reduction of D-fructose to D-mannitol, rather than the oxidation of D-mannitol, making it more suitable for D-mannitol production. To enable efficient NADH regeneration, we selected a glucose dehydrogenase (GDH) that specifically utilizes D-glucose as a substrate. D-glucose is not only inexpensive but also present in molasses at a level comparable to D-fructose, making it an ideal and cost-effective choice for our coupled enzymatic system. Importantly, the role of D-glucose in this cascade is not as a direct product precursor, but as a cofactor-regenerating component that enables a self-sufficient and economically favorable conversion of D-fructose to D-mannitol. This eliminates the need for external NADH supply and thereby improves process sustainability and cost efficiency.

Notably, unlike glucose oxidase (GOx), the NAD^+-dependent GDH does not utilize molecular oxygen as an electron acceptor and therefore does not generate hydrogen peroxide (H_2O_2) as a byproduct. Instead, the reaction couples the oxidation of D-glucose directly to the reduction of NAD^+, enabling efficient cofactor regeneration without generating oxidative stress. As a result, concerns regarding enzyme inhibition due to H_2O_2 accumulation—an issue frequently associated with GOx-based systems—are not relevant to this process. This mode of cofactor regeneration supports both the efficiency and safety of the enzymatic cascade system.

For the enzymatic cascade, we employed MDH originated from *Pseudomonas fluorescens* (EC 1.1.1.67) and GDH originated from *Bacillus megaterium* (EC 1.1.1.47) both of which were recombinantly expressed in *Escherichia. coli*. For experimental validation, we focused on microbial enzymes that could be efficiently produced in *E. coli*, which offers advantages such as rapid growth, low cost, high protein yield, and a well-established, standardized expression system (Rosano and Ceccarelli, 2014; Terpe, 2006).

In this study, we developed an enzymatic cascade process for the upcycling of molasses into D-mannitol. Among the two main types of industrial molasses—sugarcane and sugar beet molasses—we selected sugarcane molasses, which is the more abundant and widely available variety globally. Sucrose in molasses was first hydrolyzed by invertase into D-glucose and D-fructose. Subsequently, MDH catalyzed the reduction of D-fructose to D-mannitol, while GDH simultaneously oxidized D-glucose to D-gluconolactone, thereby regenerating NADH and maintaining the redox balance of the system (Kaswurm et al., 2013). This integrated enzymatic system established a self-sufficient redox cycle that enhanced both the feasibility and efficiency of D-mannitol production. To assess the process flexibility, we evaluated both: (i) a two-step method—comprising sucrose hydrolysis followed by D-fructose reduction, and (ii) a one-pot method, in which all enzymatic reactions occur concurrently (Fig. 1).

This study demonstrates high-efficiency D-mannitol production directly from molasses via a self-sufficient enzymatic cascade. We systematically evaluated both two-step and one-pot reaction formats, optimizing enzyme ratios and reaction conditions for maximal yield and operational practicality. This work highlights a viable route for transforming low-cost industrial residues into high-value products and supports the broader adoption of bio-based circular manufacturing systems.

2. Materials and methods

2.1. Materials

Bacto™ Tryptone (#211705) and yeast extract (#212750) were obtained from BD Biosciences (San Jose, CA, USA). Ampicillin and kanamycin sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Invertase (EC 3.2.1.26, #9001–57–4) was sourced from purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Hydroxybenzhydrazide was obtained from Sigma-Aldrich. Strep-Tactin® XT 4Flow resin was supplied by IBA Lifesciences (Göttingen, Germany), and biotin was purchased from Deajung (Deajeon, Republic of Korea). Polypropylene columns (1 mL) were obtained from Qiagen (Hilden, Germany). PD-10 desalting columns (#GE17–0851–01) and Vivaspin 500 centrifugal concentrators with a 10 kDa molecular weight cut-off (MWCO) were procured from Cytiva (Uppsala, Sweden). Wholesome Organic Blackstrap sugarcane molasses was purchased from Wholesome Sweeteners, Inc. (Sugar Land, TX, USA). High-performance liquid chromatography (HPLC) analysis was performed using an Agilent 1260 Infinity II LC system (Agilent Technologies, CA, USA) equipped with a Sugar-Pak™ I column (6.5 × 300 mm, 10 μm; Waters Corp., Milford, MA, USA) for sugar separation and quantification. Unless otherwise specified, all other reagents and chemicals were obtained from Sigma-Aldrich.

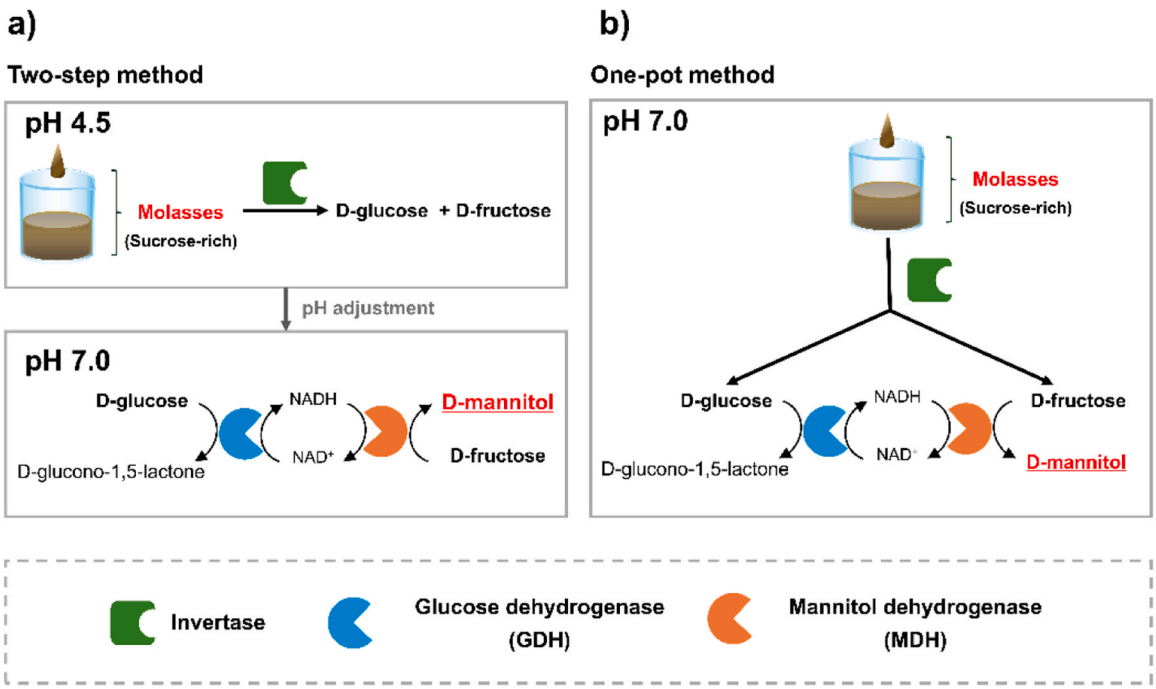

Fig. 1. Schematic illustration of the enzymatic conversion of molasses to D-mannitol. Sucrose present in molasses is hydrolyzed by invertase into D-glucose and D-fructose. D-fructose is subsequently reduced to D-mannitol via NADH-dependent reactions catalyzed by GDH and MDH. a) Two-step method, b) One-pot method.

2.2. Expression and purification of proteins

The construction of pET-24a(+)-Strep TagII-GDH and pQE80-Strep TagII-MDH plasmids has been previously described (Cha et al., 2023). These plasmids were transformed into *E. coli* BL21(DE3) for protein expression, and large-scale production of GDH and MDH was carried out in a 7 L fermenter (MARADO-05S-XS, CNS, Daejeon, Korea).

For the large-scale production of GDH, 40 mL of BL21(DE3)[pET-24 (+)-Strep tagII-GDH] was pre-cultured in Luria broth (LB) medium containing 50 μg/mL kanamycin. The pre-culture was inoculated with the prepared 7 L terrific broth (TB) medium. The fermenter pH was controlled between 6.9 and 7.0 by automatic injection of 1 M HCl and 1 M NaOH. 1 mL antifoam was added to control foaming. The pre-culture was inoculated into the fermenter and cultivated at 37 ℃ with 300 rpm, until the OD_{600} reached 3.0. At the point, the temperature was lowered 30 ℃, and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. When OD_{600} reached 30, cells were collected by centrifugation at 6000 ×g for 10 min and stored at – 80 ℃. The expression conditions for Strep-tagged MDH were consistent with those previously reported (Bak et al., 2024).

Cell pellets containing Strep-tagged MDH and GDH were resuspended in 50 mM potassium phosphate (KPi) buffer (pH 7.0) containing 2 M NaCl for purification. Cell disruption was achieved via sonication using 1-second pulses with 2-second rests at 28 % amplitude (500 W, 20 kHz) for a total of 25 min. The lysate was clarified by centrifugation at 13,000 × g for 10 min at 4 °C, and the supernatant was incubated with Strep-Tactin XT 4Flow resin at 4 °C for 40 min. The resin was then packed into a gravity-flow polypropylene column, washed with 50 mM KPi buffer (pH 7.0) containing 2 M NaCl, and the proteins were eluted with 50 mM biotin in 50 mM KPi buffer (pH 7.0) containing 2 M NaCl. Residual biotin was removed via buffer exchange using a PD-10 column, and proteins were transferred into fresh 50 mM KPi buffer (pH 7.0) containing 2 M NaCl.

Protein concentrations were determined by measuring absorbance at 280 nm using a Synergy microplate reader (BioTek, USA). Based on amino acid sequence calculations, the extinction coefficients of Strep-tagged GDH and MDH were 119,640 $M^{-1} \cdot cm^{-1}$ and 60,850 $M^{-1} \cdot cm^{-1}$, respectively (Cha et al., 2023; Gill and von Hippel, 1989). The amino acid sequences of the recombinant GDH and MDH in this study are provided in the Supporting information (Table S1)

2.3. Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) analysis

The purified GDH and MDH were desalted using Zeba™ spin desalting columns (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The eluted proteins were then mixed at a 1:1 (v/v) ratio with a sinapinic acid-saturated matrix solution consisting of 30 % acetonitrile and 0.1 % trifluoroacetic acid (TFA). The mixtures were analyzed by MALDI-TOF mass spectrometry using an Autoflex Speed instrument (Bruker Corporation, Billerica, MA, USA).

2.4. Enzyme kinetics

Commercially available invertase was used in this study. Its hydrolytic activity was quantified using a colorimetric assay based on p-hydroxybenzoic acid hydrazide (PAHBAH). Reducing sugars generated during sucrose hydrolysis by invertase react with PAHBAH to form chromogenic derivatives. Specifically, 900 μL of 200 mM PAHBAH solution in 2 M NaOH was mixed with 100 μL of the sample solution. The mixture was incubated at 95 °C for 10 min, then cooled on ice for 5 min. PAHBAH reacts with reducing sugars to form yellow-colored osazone derivatives. Following the reaction, the mixture was diluted 1:10 with distilled water, and absorbance was measured at 430 nm to quantify reducing sugar such as D-glucose and D-fructose. For standardization, a glucose standard solution was prepared in 0.2 M KPi buffer (pH 4.5) with 2 M NaCl and used to generate a standard calibration curve.

The catalytic activity of MDH was assessed by monitoring the NADH-dependent reduction of D-fructose to D-mannitol. The reaction was initiated by adding 50 μL of NADH (final concentration: 0–1 mM) to 150 μL of reaction mixture containing 10 nM MDH and varying concentrations of D-fructose (final concentration 0–100 mM) in 0.2 M KPi buffer (pH 7.0) with 2 M NaCl. The decrease in absorbance at 340 nm, corresponding to NADH oxidation, was used to monitor the reaction.

Similarly, the activity of GDH was evaluated by measuring the NAD^+-dependent oxidation of D-glucose to D-glucono-1,5-lactone. The reaction was started by adding 50 μL of NAD^+ (final concentration: 0–1 mM) to 150 μL of reaction mixture containing 2 nM GDH and D-glucose (final concentration 0–100 mM) in 0.2 M KPi buffer (pH 7.0) with 2 M NaCl. NADH formation was monitored by measuring the increase in absorbance at 340 nm.

All GDH and MDH kinetic assays were performed in triplicate using a 96-well microplate, and absorbance changes were recorded at 1-min intervals over 10 min using a microplate reader. Kinetic parameters were determined by fitting the data to the Michaelis–Menten equation.

2.5. Molasse pretreatment

To remove turbidity and precipitate crude proteins that could interfere with downstream analyses, molasses samples were pretreated using the Carrez clarification method (Palmonari et al., 2020). Carrez Solution I (15 % $K_4[Fe(CN)_6]\cdot 3H_2O$) and Carrez Solution II (30 % $ZnSO_4\cdot 7H_2O$) were freshly prepared for this purpose. Due to the high viscosity of molasses, samples were initially diluted with deionized water (DW) at a 1:1 wt ratio. For clarification, Carrez Solutions I and II were sequentially added at one-ten the volume of the diluted molasses mixture, with thorough mixing after each addition.

After reagent addition, the pH was measured and adjusted to approximately 8.0 using 6 M NaOH, as necessary, to facilitate protein precipitation. The resulting precipitate was removed by centrifugation at 13,000 × g for 10 min, and the clear supernatant was collected. The pH of the supernatant was then adjusted to pH 4.5 and pH 7.0, the required condition for subsequent enzymatic reactions. Finally, the solution was filtered through a syringe filter to remove any remaining particulates or interfering substances.

2.6. High performance liquid chromatography (HPLC)

Molasses containing concentrations of compounds were determined using high-performance liquid chromatography (HPLC) with an Agilent 1260 Infinity II system (Agilent Technologies, USA). The system was equipped with a Sugar-Pak™ I column (Waters Corp., Milford, MA, USA), a refractive index detector (RID), and an autosampler (Khandekar et al., 2014). The mobile phase consisted of DW, which was filtered through a 0.45 μm membrane, autoclaved, and degassed prior to use. Chromatographic separation was performed under isocratic conditions at a flow rate of 0.5 mL/min. The column temperature was maintained at 85 °C to optimize resolution, and the RID was operated at 50 °C. The resulting chromatograms enabled precise quantification of sugars and sugar alcohols present in the samples.

2.7. Two-step D-mannitol production

The first step of two-step D-mannitol production was conducted in sealed vials. For the first step, 200 μL of 500 μg/mL invertase was added to 1.8 mL of reaction solution containing 200 mM sucrose in 0.2 M KPi buffer (pH 4.5) containing 2 M NaCl. The reaction mixture was incubated for 1 h with invertase alone to complete sucrose hydrolysis.

Following hydrolysis, the pH of the 2 mL reaction mixture was adjusted to pH 7.0 by adding 50 μL of 6 M NaOH, preparing it for the second step. Reaction solution 512.5 μL aliquot of the pH-adjusted solution was transferred to a 1.5 mL tube, and the total volume was adjusted to 1 mL by adding 100 μL each of 10 U/mL GDH, 60 U/mL MDH, and 10 mM NAD^+, followed by 0.2 M KPi buffer (pH 7.0) containing 2 M NaCl.

After the reaction, enzymes were removed using a Vivaspin 500 centrifugal concentrator with a 10 kDa molecular weight cutoff. The concentrations of substrates and products were then analyzed by HPLC before and after the reaction.

2.8. One-pot D-mannitol production

One-pot D-mannitol production was carried out in a 1.5 mL microcentrifuge tube with a total reaction volume of 1 mL. The reaction mixture consisted of 100 mM sucrose, 1 U/mL invertase (equivalent to 1.35 mg/mL at pH 7.0), 1 U/mL GDH, 6 U/mL MDH, and 1 mM NAD^+, all prepared in 0.2 M KPi buffer (pH 7.0) containing 2 M NaCl.

Following the reaction, enzymes were removed using a Vivaspin 500 centrifugal concentrator with a 10 kDa molecular weight cutoff. The concentrations of substrates and products were analyzed by HPLC before and after the reaction, following the same procedure used in the two-step D-mannitol production.

3. Results and discussion

3.1. Preparation of enzymes

Strep-tagged MDH and GDH were heterologously expressed in *E. coli* and purified using strep-tag, as described in the Materials and Methods section. SDS-PAGE analysis of the purified GDH showed a band between 25 and 37 kDa, consistent with its predicted molecular weight of 30,537 Da. Similarly, MDH migrated between 50 and 75 kDa (Fig. S1), corresponding to its theoretical molecular weight of 55,538 Da (Cha et al., 2023).

The experimentally determined molecular weights of GDH and MDH were 30,413 *m/z* and 55,556 *m/z*, respectively, which closely matched their theoretical values of 30,538 *m/z* and 55,539 *m/z*, with deviations of less than 0.5 % (Fig. S2). These results confirm the successful expression and purification of the Strep-tagged MDH and GDH enzymes.

3.2. Characterization of enzymes

The kinetic parameters of the purified GDH and MDH were subsequently determined. For GDH, k_{cat} and K_m values were measured for its two substrates, NAD^+ and D-glucose (Fig. S3a, c), and are presented in Table. S2. Similarly, for MDH, kinetic constants for NADH and D-fructose were determined (Fig. S3b, d) and summarized in Table. S1. These results demonstrate that both enzymes retained their expected catalytic activities following purification.

Enzyme catalytic efficiency of enzymes is significantly influenced by pH and temperature (Kabir and Ju, 2023; Zaretckii et al., 2024). To establish the optimal reaction conditions for the enzymatic conversion of sucrose to D-mannitol, we evaluated the activities of invertase, GDH, and MDH under various pH and temperature conditions. The kinetic values and pH stability obtained in this study are comparable to those previously reported for GDH and MDH, confirming the reliability and reproducibility of our enzyme preparations (Mitamura et al., 1989; Slatner et al., 1999).

Relative enzyme activities were analyzed across a pH range of 1–10. Invertase exhibited maximum activity at pH 4.0–5.0 (Fig. S4a), which is consistent with previously reported optimal conditions (Mohandesi et al., 2016; Zhou et al., 2016). Moreover, despite its peak activity within this range, invertase retained catalytic function over a broader pH spectrum (pH 3.0–8.0), aggregation at both highly acidic and alkaline conditions, likely contributing to their instability suggesting its potential applicability under various pH conditions. In contrast, GDH and MDH showed optimal activity at neutral pH (Fig. S4a) and exhibited significantly reduced activity under acidic conditions. Additionally, both enzymes displayed loss of function.

The effect of temperature on enzyme activity and stability was evaluated for invertase, GDH, and MDH at room temperature (RT, 20–27℃), 37 °C, and 50 °C. Invertase exhibited relatively higher activity at 50 °C compared to RT; however, the impact of temperature was less pronounced than that of pH (Fig. S4). To assess thermal stability, GDH and MDH were incubated for 1 h at each temperature. GDH retained approximately 70 % of its activity at both 37 °C and 50 °C relative to its

activity at RT. In contrast, MDH retained only 16 % of its activity at 50 °C, indicating notable thermal sensitivity (Fig. S4b).

Based on these findings, it was confirmed that invertase and the GDH-MDH system exhibit substantial differences in their optimal reaction conditions. To minimize enzyme consumption and maximize efficiency, a straightforward strategy is to divide the process into two distinct stages: (i) sucrose hydrolysis by invertase, and (ii) D-mannitol production via the GDH-MDH cascade (Fig. 1a). However, given that invertase retains significant activity even under non-optimal conditions, we also consider an alternative approach by increasing the invertase concentration to enable a one-pot reaction (Fig. 1b). The latter strategy could significantly simplify the process, improving its practicality and scalability for industrial applications. Accordingly, this study evaluates both reaction strategies: (i) a two-step enzymatic process designed to optimize enzyme-specific conditions and conversion efficiency, and (ii) a one-pot system aimed at simplifying the reactions for industrial-scale implementation.

3.3. Sugar content analysis

The overall composition of sugarcane molasses is generally known to include not only sugars but also proteins, pectin, salts, and other minor components. However, these proportions can vary significantly depending on the production region, processing method, and batch (Vasconcelos, 2015). To quantify changes in sugar content resulting from enzymatic reactions and to characterize the composition of molasses, HPLC retention times were analyzed, and calibration curves were constructed, as described in Materials and Methods. The retention times for sucrose, D-glucose, D-fructose, and D-mannitol were determined to be 7.7, 9.4, 10.9, and 12.3 min, respectively (Fig. S5). Calibration curves were appropriately established to ensure accurate quantification.

Molasses is a highly complex matrix containing melanin pigments and crude proteins. Frequent injections of raw molasses into the HPLC system can potentially interfere with analysis by damaging the column resin and other system components (Palmonari et al., 2020). Therefore, appropriate pretreatment is necessary to ensure reliable analysis in this experiment. In this study, molasses samples were pretreated using Carrez reagents, followed by syringe filtration to remove interfering substances (Fig. S6).

Previous studies on Carrez clarification have shown that this step has a minimal impact on sugar recovery and is primarily used to improve analytical reliability during sugar quantification. Reported sugar recovery yields after Carrez treatment generally range from 95 % to 100 % (Hoehnel et al., 2022; Palmonari et al., 2021). Consistent with these findings, our results (Fig. S7) showed that the proportions of sugars before and after pretreatment remained nearly unchanged, and approximately 97 % of total sugars recovered. It should be noted, however, that the addition of Carrez solutions and associated processing steps resulted in dilution, reducing the final sugar concentration to approximately one-fortieth of the original (~1/2.5).

To assess the impact of the pretreatment process on molasses composition, a comparative analysis between raw and pretreated samples was conducted. For this purpose, raw molasses was analyzed by HPLC. Although direct injection of raw molasses is generally discouraged due to the risk of damaging the HPLC column, the sample was diluted 20-fold (v/v) and passed through a 0.2 μm syringe filter prior to analysis to minimize the risk of column fouling.

Quantitative analysis of the pretreated molasses revealed a sugar composition of 13 % D-glucose, 15 % D-fructose, and 72 % sucrose (Fig. S7). The composition remained unchanged before and after pretreatment, indicating that the method effectively removed impurities without altering the major sugar components. Unless otherwise stated, all subsequent experiments were conducted using pretreated molasses to ensure consistency and reproducibility in analytical measurements.

3.4. Optimization of enzyme ratio

Given the substantial difference in the optimal reaction conditions between invertase and the GDH-MDH system, a two-step enzymatic approach was identified as the most effective strategy to achieve high conversion efficiency while minimizing enzyme usage and maximizing catalytic performance. All optimization experiments were conducted using 100 mM substrate concentrations.

To determine the most efficient balance between GDH and MDH, enzyme ratio optimization was performed. These enzymes function in a cascade system, with their activities coupled through the shared cofactor NAD^+/NADH. The key determinant for ratio optimization was the relative catalytic efficiency of each enzyme toward the cofactor. As shown in Table S2, kinetic parameters (k_{cat}/K_m) for NAD^+ and NADH reveal that MDH exhibits lower catalytic efficiency than GDH. Optimization was conducted under non-limiting substrate conditions where D-glucose and D-fructose were each supplied at 100 mM, ensuring that observed differences were solely due to enzyme kinetics rather than substrate depletion.

In the first step, sucrose hydrolysis was catalyzed by invertase at pH 4.5. To determine the minimum enzyme concentration required for complete conversion, various invertase concentrations were evaluated. The results indicated that 50 μg/mL of invertase was sufficient to fully hydrolyze 200 mM sucrose into equimolar amounts of D-glucose and D-fructose within 1 h (Fig. S8), Because molasses contains sucrose as the dominant sugar (~72 %), and this sucrose is quantitatively converted by invertase into glucose and fructose at an approximately 1:1 molar ratio, the actual substrate composition encountered by the GDH–MDH cascade is close to equimolar glucose and fructose. Thus, we used a 1:1 mixture of 100 mM glucose and 100 mM fructose in the optimization experiments to closely mimic the substrate environment after sucrose hydrolysis.

The second step focused on optimizing D-mannitol production by evaluating the effect of different GDH:MDH ratios on conversion efficiency, using 100 mM D-glucose and 100 mM D-fructose as substrates. At a ratio of 1:2, D-mannitol productivity increased significantly, indicating that MDH is the rate-limiting enzyme in the cascade (Fig. S9). Further optimization showed that D-mannitol production continued to increase with higher MDH loading up to a ratio of 1:6, whereas additional increases to 1:8 or 1:10 provided only marginal improvements (<1 % difference within the experimental error range). While the 24 h titers converge largely because of the system's thermodynamic equilibrium, time-limited productivity is also an important operational metric. We therefore examined the first-hour rate as a complementary readout. Under these conditions, increasing MDH up to 1:6 improved the early-stage rate, consistent with MDH contributing to rate control in the initial phase (Fig. S9a), whereas 24 h data reflect near-equilibrium performance (Fig. S9b). Taken together, both thermodynamic and kinetic considerations support the selection of 1:6 as a practical balance between performance and enzyme usage. On this basis, we identified 1:6 as minimal MDH loading required to achieve quantitative conversion (~99–100 mM within error) in 24 h, while also ensuring high early-stage productivity and avoiding unnecessary enzyme excess. Increasing the ratio beyond 1:6 led to only negligible changes in the initial rate and no significant improvement in the 24 h titer.

To optimize the one-pot D-mannitol production system, the activity ratio of invertase relative to GDH and MDH was evaluated at pH 7.0. At this pH, 1 U/mL of invertase corresponds to a concentration of 1.35 mg/mL. The activities of GDH and MDH were fixed at the previously optimized ratio of 1 U/mL: 6 U/mL, while the invertase activity was varied. Increasing invertase activity led to higher D-mannitol production within the first hour, with a pronounced increase observed at 1 U/mL (Fig. S10a). However, after 24 h, the final D-mannitol yields were similar between reactions containing 1 U/mL and 3 U/mL of invertase, indicating that higher invertase concentrations did not further improve overall yield (Fig. S10b). This result again highlights that enzyme ratios

primarily influence reaction kinetics and early-stage productivity, rather than the ultimate equilibrium yield. Based on these findings, the optimal enzyme ratio for the one-pot reaction was established as invertase: GDH: MDH = 1 U/mL: 1 U/mL: 6 U/mL.

3.5. D-mannitol production from pure sucrose

We selected an appropriate buffer to facilitate the two-step reaction. In the first step, invertase hydrolyzes sucrose without causing significant pH fluctuations. However, in the second step, GDH produces 100 mM gluconolactone, which has the potential to alter the pH. Therefore, we considered buffers that include pH 7.0 within their buffering range and are capable of accommodating the presence of 100 mM gluconolactone. Additionally, since the pH needs to be adjusted from 4.5 (the reaction condition for step 1) to 7.0 using a small amount of alkali, we excluded buffers with buffering ranges below pH 5. As a result, the overall reaction was carried out in 0.2 M KPi buffer.

Sucrose hydrolysis was performed using 50 μg/mL invertase in 0.2 M KPi buffer (pH 4.5) for 1 h. The reaction successfully converted 200 mM sucrose into 194.5 ± 1.6 mM D-glucose and 193.7 ± 3.5 mM D-fructose, corresponding to a conversion efficiency exceeding 96 %.

Before proceeding to the second step, the reaction solution pH was adjusted to 7.0 by adding a small volume of 6 M NaOH. Since the effective buffering range of KPi buffer at RT is pH 5.8–8.0, pH 4.5 lies outside its effective range. In the second step, a 1:2 dilution was applied to compensate for the dilution effect caused by the addition of NaOH, resulting in final D-glucose and D-fructose concentrations of approximately 100 mM each. The reaction was then carried out for 24 h under optimized conditions, with the addition of 1 U/mL GDH, 6 U/mL MDH, and 1 mM NAD^+.

Initially, the reaction exhibited a rapid conversion rate, but after 6 h, the rate sharply declined. This is likely due to the high K_m value of MDH for D-fructose (Table. S1), leading to a gradual depletion of D-fructose as it was converted into D-mannitol, thereby significantly reducing reaction efficiency. In this second-step reaction, 96.8 ± 5.2 mM D-mannitol was produced, with no detectable sucrose, D-glucose, or D-fructose remaining, indicating a conversion efficiency exceeding 96 % (Fig. 2).

The one-pot reaction was performed at pH 7.0 using 1 U/mL (1.35 mg/mL) invertase, 1 U/mL GDH, 6 U/mL MDH, 1 mM NAD^+, and 100 mM sucrose. After 24 h, the reaction yielded 100.3 ± 4.3 mM D-mannitol, corresponding to a conversion efficiency of approximately 99 % (Fig. 3).

These findings confirm that the enzymatic cascade system efficiently converts sucrose into D-mannitol with high conversion efficiency. Furthermore, both reaction strategies demonstrated excellent performance: the two-step reaction minimized enzyme consumption, while the one-pot process provided a simplified and streamlined approach, enhancing the feasibility of industrial application.

3.6. Enzyme activity changes in molasses

Prior to implementing the enzymatic cascade system for molasses conversion, the activity change of each enzyme within the molasses matrix was evaluated. A synthetic sugar mixture containing sucrose, D-glucose, and D-fructose at concentrations equivalent to those found in molasses was prepared, and enzyme activity in this mixture was compared to that observed when using molasses as the substrate. Invertase, GDH, and MDH retained 93 %, 98 %, and 78 % of their activity, respectively, relative to the synthetic sugar mixture (Fig. 4). These results confirm that the enzymatic cascade system remains catalytically active in the presence of molasses.

To evaluate the impact of molasses pretreatment on enzymatic reactions, raw molasses was diluted in buffer, and the cascade reaction was conducted using both pretreated and untreated molasses. The results showed that the reaction proceeded identically under both conditions, indicating that pretreatment does not affect enzymatic activity (Fig. S11). Moreover, these findings suggest that D-mannitol can be directly produced from molasses without pretreatment, thereby simplifying the overall process and enhancing its industrial applicability.

3.7. D-mannitol production from molasses

D-mannitol production from molasses using the enzymatic cascade system composed of invertase, GDH, and MDH was conducted using both two-step and one-pot methods, under the same reaction conditions as those applied for the conversion of pure sucrose, which served as the reference substrate.

In the two-step process, the first step converted the initial sugar concentrations of 200 mM sucrose, 36.7 mM D-glucose, and 43.6 mM D-

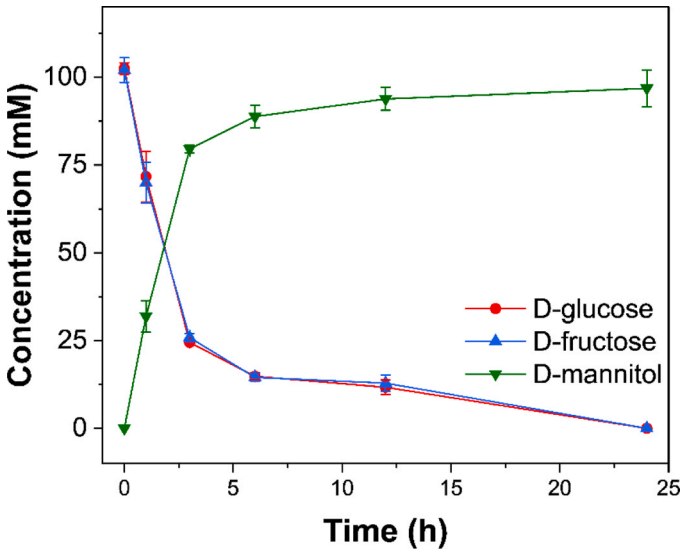

Fig. 2. The second step in two-step enzymatic production of D-mannitol from pure sucrose. Step 2: Time-dependent D-mannitol production following the addition of 1 U/mL GDH, 6 U/mL MDH, and 1 mM NAD^+ to the diluted reaction mixture.

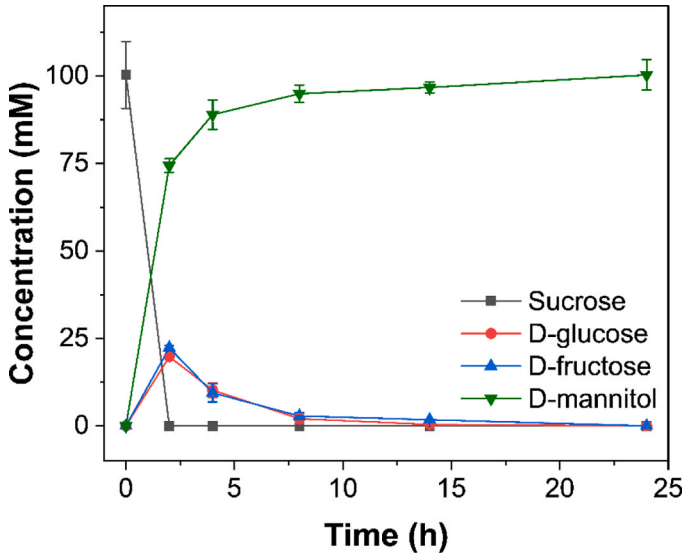

Fig. 3. One-pot enzymatic synthesis of D-mannitol from pure sucrose. A time-course analysis was performed to monitor substrate consumption and product formation using 100 mM sucrose at pH 7.0. The reaction was carried out with 1 U/mL invertase, 1 U/mL GDH, 6 U/mL MDH, and 1 mM NAD^+.

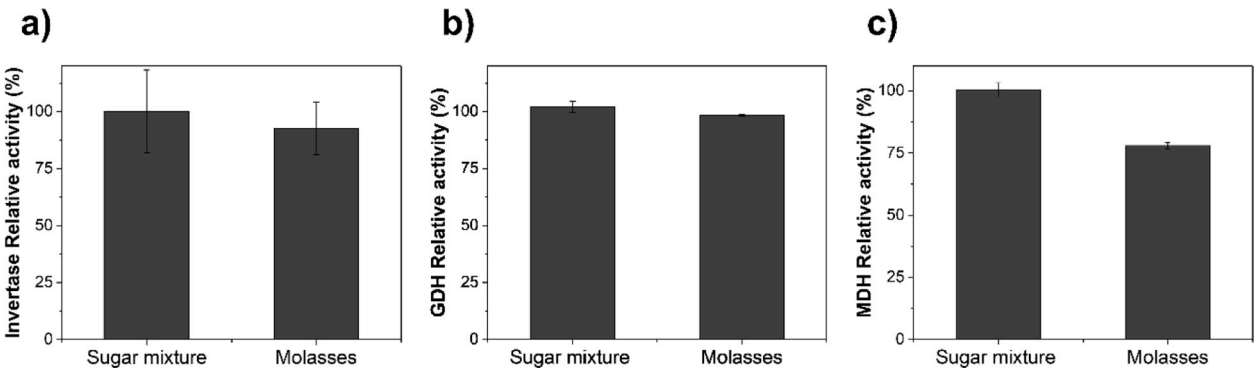

Fig. 4. Stability of a) invertase, b) GDH, and c) MDH in the presence of molasses. Enzymatic activities were evaluated in molasses and compared to those in a defined sugar mixture containing equivalent concentrations of sucrose, D-glucose, and D-fructose. Invertase activity was determined using the PAHBAH assay, while GDH and MDH activities were assessed spectrophotometrically by monitoring NADH production and consumption at 340 nm, respectively.

fructose into 223.6 ± 0.3 mM D-glucose and 232.3 ± 0.03 mM D-fructose (Fig. 5). The reaction mixture was then diluted 1:2 to allow for pH adjustment and enzyme addition. In the second step, 137 ± 13 mM D-mannitol was produced in a single-tube reaction (Fig. 5).

In the one-pot process, the reaction yielded 123.1 ± 1.3 mM D-mannitol from a substrate mixture containing 99.6 ± 1.6 mM sucrose, 22.6 ± 0.2 mM D-glucose, and 31.7 ± 0.8 mM D-fructose (Fig. 6).

These results demonstrate that the enzymatic cascade system effectively converts the sugar components in molasses into D-mannitol, achieving over 90 % productivity in both approaches. However, residual D-fructose was detected in both methods, with concentrations of 12.1 ± 3.2 mM and 6.8 ± 2.8 mM remaining in the two-step and one-pot reactions, respectively (Figs. 5, 6). To address this, additional D-glucose was supplemented to the molasses prior to the reaction, which resulted in increased D-mannitol yield and complete consumption of D-fructose (Fig. S12). This outcome suggests that the inherent imbalance between D-glucose and D-fructose in molasses leads to the premature depletion of D-glucose, thereby limiting NADH regeneration by GDH and causing incomplete conversion of D-fructose to D-mannitol. From a process perspective, another important consideration is the choice of expression host for the enzymes used in this study. Although *E. coli* was employed to rapidly and efficiently produce recombinant enzymes for proof-of-concept validation, it is not classified as a GRAS organism. For commercial application of D-mannitol as a food-grade ingredient, enzymes expressed in GRAS-certified hosts such as *Bacillus* or *Corynebacterium* would be more appropriate. Transferring the system to such hosts

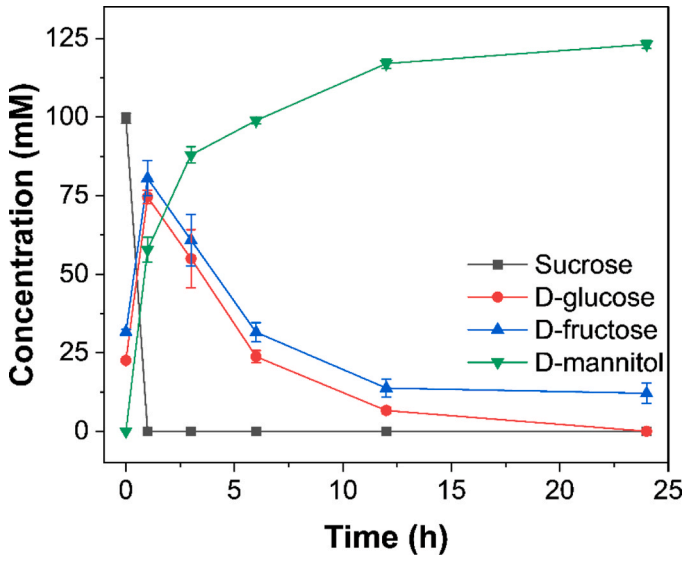

Fig. 6. One-pot enzymatic synthesis of D-mannitol from molasses. A time-course analysis was performed to monitor substrate consumption and product formation using 100 mM sucrose at pH 7.0. The reaction was carried out with 1 U/mL invertase, 1 U/mL GDH, 6 U/mL MDH, and 1 mM NAD^+.

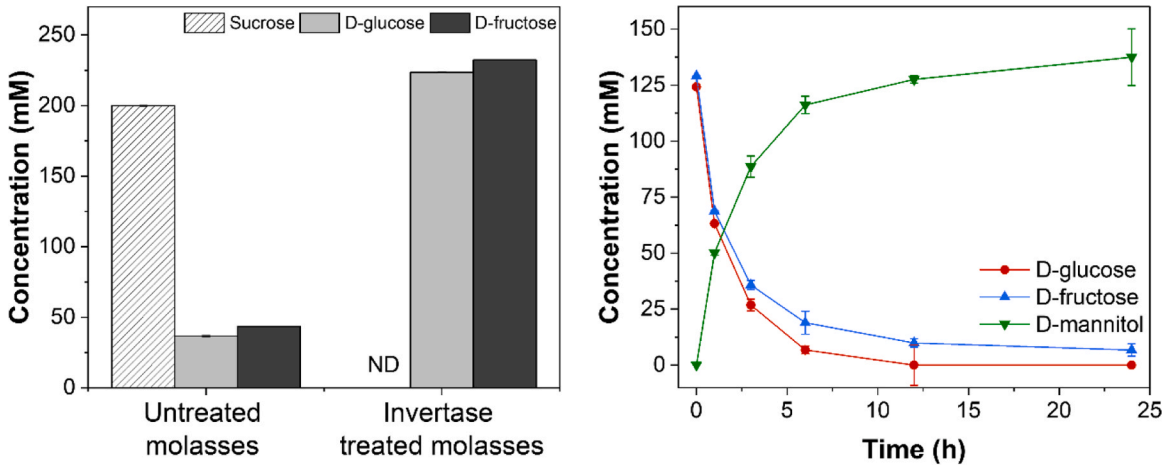

Fig. 5. Two-step enzymatic production of D-mannitol from molasses. Step 1 was initiated with 200 mM sucrose and followed by a 1:2 dilution before proceeding to Step 2. a) Step 1: Sucrose hydrolysis catalyzed by 50 μg/mL invertase at pH 4.5 for 1 h. b) Step 2: Time-dependent D-mannitol production following the addition of 1 U/mL GDH, 6 U/mL MDH, and 1 mM NAD^+ to the diluted reaction mixture. ND: Not detected.

would simplify regulatory approval and enhance process safety, and thus represents an important direction for future development.

4. Conclusion

In this study, we demonstrated a value-added enzymatic conversion strategy to produce D-mannitol from molasses, leveraging its high sugar content as a renewable and cost-effective feedstock. The use of invertase enabled the efficient hydrolysis of sucrose into D-glucose and D-fructose, thereby enhancing the reactivity of molasses and supplying sufficient substrates for downstream enzymatic reactions.

The MDH-GDH cascade system efficiently converted D-fructose to D-mannitol while simultaneously regenerating NADH in situ via the GDH-catalyzed oxidation of D-glucose. This cofactor recycling approach eliminated the need for external NADH supplementation, improving both the economic feasibility and practical applicability of the process. The cascade achieved high conversion efficiency, underscoring its potential for producing high-value D-mannitol from low-cost industrial waste.

To address the disparity in optimal pH conditions—pH 4–5 for invertase and pH 7 for GDH and MDH—two reaction formats were evaluated: (1) a two-step approach, designed to optimize enzyme-specific conditions and reduce enzyme usage, and (2) a one-pot system, which prioritizes operational simplicity.

Both strategies were first applied into pure sucrose. In the two-step method, 200 mM sucrose successfully was hydrolyzed, achieving 96 %. The reaction mixture was then diluted 1:2 for second step, which produced 96.8 ± 5.3 mM D-mannitol was produced, indicating conversion efficiency exceeding 96 %. In the one-pot method, 100 mM sucrose was converted into 100.3 ± 4.3 mM D-mannitol, corresponding to a conversion efficiency of approximately 99 %. Both methods prove to be effective, demonstrating high conversion efficiencies exceeding 95 %.

Enzymatic activity was well-retained in the molasses, with invertase, GDH, and MDH maintaining 93 %, 98 %, and 78 % activity, respectively—confirming compatibility with this complex substrate. The enzyme cascade system successfully produced D-mannitol directly from raw sugarcane molasses without any pretreatment. Therefore, the initial process design did not include a pretreatment step. Notably, similar D-mannitol yields were obtained from both untreated and pretreated molasses, confirming that pretreatment is unnecessary and allowing simplified the overall process workflow.

We implement this enzymatic conversion system into molasses. In the two-step method, 137 ± 13 mM D-mannitol was produced, achieving a conversion efficiency of ~92 %. In contrast, the one-pot method yielded 123.1 ± 1.3 mM D-mannitol, corresponding to a conversion efficiency of ~95 %. Both strategies demonstrated high-yield performance, validating their feasibility for industrial-scale implementation.

Overall, this work highlights the feasibility of directly converting molasses into high-value D-mannitol via an efficient, robust, and scalable enzymatic cascade, thereby contributing to the valorization of agro-industrial waste and aligning with the principles of the circular bioeconomy and sustainable bioprocessing.

The substrate concentration used in this study (100 mM) was appropriate for laboratory-scale proof-of-concept experiments; however, it does not meet the criteria for economic efficiency in the sugar industry. Molasses is inherently a highly concentrated and viscous mixture due to its high sugar content and various impurities. High viscosity can negatively impact enzymatic reaction rates by reducing V_{max} and slowing overall conversion (Sashi and Bhuyan, 2015; Uribe and Sampedro, 2003). Consequently, at higher substrate concentrations, pretreatment to reduce viscosity and remove impurities becomes increasingly essential—though this would likely increase overall process costs. Further investigation will be necessary to evaluate the economic feasibility of operating at higher substrate concentrations with appropriate pretreatment, and we consider this an important direction for future research.

In addition, in this study, we produced the necessary enzymes using *E. coli* as the expression host to enable rapid and efficient enzyme production during the experimental stage. However, since *E. coli* is not classified as a Generally Recognized As Safe (GRAS) organism, the use of enzymes produced in *E. coli* would require additional safety validation and regulatory assessment prior to application in food, pharmaceutical, or other industries where GRAS status is critical. Adopting GRAS-certified microbial hosts for enzyme production in future studies would offer significant advantages for commercial application, including regulatory simplification, enhanced product quality, and improved process safety.

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CRediT authorship contribution statement

Hyeonseon Bak: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Jimin Park:** Writing – original draft, Validation, Investigation. **Inchan Kwon:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.122016.

Data availability

Data will be made available on request.

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