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RESEARCH

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Production, purification, characterization, and safety evaluation of constructed recombinant D-psicose 3-epimerase

Nisit Watthanasakphuban[1], Pimsiriya Srila[1], Phitsanu Pinmanee[2], Charatda Punvittayagul[3], Nopphon Petchyam[4] and Boontiwa Ninchan[1,5*]

Abstract

Background D-psicose 3-epimerase (DPEase) is a potential catalytic enzyme for D-psicose production. D-psicose, also known as D-allulose, is a low-calorie sweetener that has gained considerable attention as a healthy alternative sweetener due to its notable physicochemical properties. This research focused on an in-depth investigation of the expression of the constructed *DPEase* gene from *Agrobacterium tumefaciens* in *Escherichia coli* for D-psicose synthesis. Experimentally, this research created the recombinant enzyme, explored the optimization of gene expression systems and protein purification strategies, investigated the enzymatic characterization, and then optimized the D-psicose production. Finally, the produced D-psicose syrup underwent acute toxicity evaluation to provide scientific evidence supporting its safety.

Results The optimization of DPEase expression involved the utilization of Mn^{2+} as a cofactor, fine-tuning isopropyl β-D-1-thiogalactopyranoside induction, and controlling the induction temperature. The purification process was strategically designed by a nickel column and an elution buffer containing 200 mM imidazole, resulting in purified DPEase with a notable 21.03-fold increase in specific activity compared to the crude extract. The optimum D-psicose conversion conditions were at pH 7.5 and 55 °C with a final concentration of 10 mM Mn^{2+} addition using purified DPEase to achieve the highest D-psicose concentration of 5.60% (w/v) using 25% (w/v) of fructose concentration with a conversion rate of 22.42%. Kinetic parameters of the purified DPEase were V_{max} and K_m values of 28.01 mM/min and 110 mM, respectively, which demonstrated the high substrate affinity and efficiency of DPEase conversion by the binding site of the fructose-DPEase-Mn^{2+} structure. Strategies for maintaining stability of DPEase activity were glycerol addition and storage at -20 °C. Based on the results from the acute toxicity study, there was no toxicity to rats, supporting the safety of the mixed D-fructose–D-psicose syrup produced using recombinant DPEase.

Conclusions These findings have direct and practical implications for the industrial-scale production of D-psicose, a valuable rare sugar with a broad range of applications in the food and pharmaceutical industries. This research should advance the understanding of DPEase biocatalysis and offers a roadmap for the successful scale-up production of rare sugars, opening new avenues for their utilization in various industrial processes.

Keywords Acute toxicity, Allulose, D-psicose 3-epimerase, D-psicose, Epimerization, Rare sugars

*Correspondence:
Boontiwa Ninchan
boontiwa.ni@ku.th
Full list of author information is available at the end of the article

Background

The global demand for low-calorie sweeteners has been steadily increasing as consumers seek healthier alternatives to traditional sugar [28]. Among the various options available, D-psicose, also known as D-allulose, has gained considerable attention due to its natural origin and notable physicochemical properties [8]. D-psicose has approximately 70% of the sweetness of sucrose but provides negligible calories, making it an appealing sugar substitute for individuals pursuing weight management or sugar-controlled diets [18–20]. Despite its potential as a low-calorie sweetener, the commercial availability of D-psicose has been hindered by limited natural sources and the complexity and cost of its chemical synthesis [27]. Consequently, there has been a growing interest in developing sustainable and cost-effective production methods for this valuable sugar, with a particular emphasis on scalability [29]. One approach to achieving efficient D-psicose synthesis is through biocatalysis, utilizing enzymes with D-psicose 3-epimerase activity [9, 11, 13, 21, 24, 36, 40].

D-psicose 3-epimerase (DPEase) is an enzyme that catalyzes the conversion of D-fructose to D-psicose, enabling the direct production of D-psicose from more readily available sugars [11, 13, 21, 36]. Among the microorganisms investigated for their DPEase activity, *Agrobacterium tumefaciens* has shown promising results [11, 13]. *A. tumefaciens* is a soil bacterium known for its ability to cause crown gall disease that is also used as a transient gene expression system in plants [37, 38]. This bacterium produces a DPEase enzyme that efficiently converts D-fructose to D-psicose [6, 7, 11, 13]. The utilization of this enzyme for industrial-scale production is appealing due to its specificity and catalytic efficiency [8]. However, the low enzyme yield from natural *A. tumefaciens* and the limitations of its safety evaluations pose challenges for large-scale enzyme production.

To overcome these limitations and address the scalability issue, heterologous expression systems have been used as an alternative approach for the efficient production of DPEase [29]. In particular, *Escherichia coli* offers numerous advantages for scalable production, including well-established genetic tools, fast growth rates, high biomass yields, and ease of cultivation [14]. By introducing the *DPEase* gene from *A. tumefaciens* into *E. coli*, it becomes possible to achieve high-level expression of the enzyme and to facilitate large-scale production of D-psicose. Therefore, the current research focused on an in-depth investigation of the expression of the *DPEase* gene from *A. tumefaciens* in *E. coli* for D-psicose synthesis, with a strong emphasis on scalability. Experimentally, this research created the recombinant DPEase and explored the optimization of gene expression systems and protein purification strategies, followed by investigating the enzymatic characterization to establish a robust and scalable process for D-psicose production. Additionally, the optimization was studied of D-psicose production using recombinant *A. tumefaciens* DPEase. Finally, the produced D-psicose syrup was evaluated based on oral acute toxicity to provide scientific data supporting its safe use.

This research can be a guideline for future studies for scaling up both DPEase and D-psicose production as the aim was to improve and ascertain the efficiency and safety of the recombinant DPEase-produced D-psicose. In addition, the analysis of D-psicose as a low-calorie sweetener was performed for its application in the food and pharmaceutical industries.

Materials and methods

Optimization of recombinant D-psicose 3-epimerase in *Escherichia coli*

Bacterial strains, plasmids, culture conditions, and chemicals

The relevant features of bacterial strains and plasmids used in this experiment are listed in Table 1. All restriction enzymes and DNA ligase were purchased from New England Biolabs, MA. All chemicals were analytical grade. D-fructose and D-psicose were supplied as standard sugars by Loba Chemie Pvt. Ltd (India) and SimSon Pharma Limited (India), respectively.

E. coli Neb5α was used as an intermediate cloning host, while *E. coli* BL21 (DE3) was used as the expression host. Both *E. coli* strains were grown in Luria–Bertani (LB) medium at 37 °C with 200 rpm agitation. Solid media contained 1.5% (w/v) agar. The kanamycin (kan) antibiotic was used at a final concentration of 50 μg/mL.

Plasmid construction and molecular cloning of recombinant DPEase

D-psicose 3-epimerase (DPEase) encoding gene sequences were codon optimized and artificially synthesized (GenScript, USA) from the *A. tumefaciens* C58

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant features	Source
Strains		
Escherichia coli		
Neb5α	Host strain for cloning	Lab stock
BL21 (DE3)	Host strain for expression	Lab stock
Psicose	*E. coli* BL21 harboring pET28a + *DPEase*	This study
Plasmids		
pET28a	*kan*^{r}; T7 *lac*	GenScript
pET28a + DPEase	*kan*^{r}; T7 *lac* derivative; D-psicose–3-epimerase biosynthesis gene	This study

sequence for expression in *E. coli* strains. The 6xHis-tag sequences were designed in front of stop codons at the 3' end for recombinant protein purification.

The construction of the DPEase expression vector (pET28a + DPEase) was carried out using restriction enzyme and standard T4 DNA ligase cloning procedures [37, 38]. The synthesized *DPEase* gene was ligated into the *Nco*I and *Xho*I cloning sites of the pET28a expression vector and the ligation mixture was transformed into *E. coli* Neb5α using electroporation. The transformant was selected on LB agar containing 50 μg/mL kanamycin and verified using colony polymerase chain reaction (PCR). The positive clones were confirmed by sequencing with specific primers.

Transformation and expression of DPEase

The DPEase expression vector was transformed into *E. coli* BL21 (DE3) competent cells using heat-shock and spread on to LB agar plates containing 50 μg/mL kanamycin. Then, after overnight incubation at 37 °C, the kanamycin-resistant colonies were picked and the positive clones with pET28a + DPEase plasmid were verified using colony PCR.

The *E. coli* psicose strain (*E. coli* BL21 (DE3) harboring pET28a + DPEase) was cultured in 10 mL LB broth (10 g/L of peptone, 5 g/L of yeast extract, and 10 g/L of NaCl) containing 50 μg/mL kanamycin and cultivated at 37 °C with 200 rpm agitation for 16 h. The overnight culture (0.5% (v/v) inoculum) was transferred into 50 mL LB broth with 50 μg/mL kanamycin and cultivated at 37 °C with 200 rpm agitation. The culture was grown until the optical density at a wavelength of 600 nm (OD_{600}) reached 0.4–0.6 [26], then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultivated at 25 °C with 200 rpm agitation for 16 h. The non-induced treatment was cultured under the aforementioned conditions, without the addition of IPTG supplementation, and served as a negative control.

The cells were separated by centrifugation at 7,000 × *g* for 10 min, then washed twice with sodium phosphate buffer (50 mM, pH 7.5), and re-suspended in 5 mL of cold lysis buffer (50 mM sodium phosphate buffer, pH 7.5, and 0.1% (v/v) Triton X-100). Cell lysis was carried out using sonication (VC 505, Sonics & Materials, Inc., USA) at 60% amplitude and a 10 s pulse (for 6 rounds) on ice. The cell lysate fraction (crude extract) and cell debris (cell fraction) were separated and collected using centrifugation at 15,000 × *g* and 4 °C for 30 min.

The protein concentration of the crude extract was measured using Bradford assay [2]. The crude extract and cell fraction of DPEase were diluted 30-fold with buffer, and then mixed with Laemmli buffer (2X), and heated at 95 °C for 15 min before loading the samples in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) to determine the size of protein. While, the enzyme activity was analyzed using high performance liquid chromatography (HPLC). The protein concentration and enzyme activity of the induced and non-induced treatments were further compared.

Effect of manganese ions (Mn^{2+}) on DPEase activity

The requirement of 4 h incubation of DPEase with Mn^{2+} was investigated. The DPEase crude extracted from induced and non-induced recombinant psicose strain were analyzed for enzyme activity with and without 4 h incubation with the Mn^{2+} ion as the cofactor [11, 13]. In addition, the effect was studied of Mn^{2+} supplementation in culture medium during cultivation (LB + Mn^{2+}) and/or in the lysis buffer (lysis buffer + Mn^{2+}).

The *E. coli* psicose strain was cultured following the expression procedure above with and without 1 mM $MnCl_2$ supplementation. The cells were harvested using centrifugation at 7,000 × *g* for 10 min and re-suspended in 5 mL of two buffer solutions: sodium phosphate buffer (50 mM, pH 7.5) with 0.1% (v/v) Triton X-100 (lysis buffer) and sodium phosphate buffer (50 mM, pH 7.5) with 0.1% (v/v) Triton X-100 containing 1 mM $MnCl_2$ (lysis buffer + Mn^{2+}). The cell lysis was performed using sonication and the cell lysate was collected and compared to protein concentration and enzyme activity.

Effect of induction temperature on DPEase expression

The *E. coli* psicose strain was grown at 37 °C until the OD_{600} reached 0.4–0.6; then, it was induced with 1 mM IPTG and incubated at different temperatures (20, 25, 30, and 37 °C) for 16 h. The cells were harvested and the crude extract was collected after sonication. The protein concentration and enzyme activity of the crude extracts were analyzed using SDS-PAGE and HPLC, respectively.

Determination of optimal inducer concentration

To identify the optimal inducer concentration, the *E. coli* psicose strain was grown until OD_{600} reached 0.4–0.6, followed by induction using different IPTG concentrations (0, 0.5, 1.0, 1.5, and 2.0 mM). The DPEase production was monitored after 16 h induction at 25 °C. The enzyme activity and protein concentration of the crude extracts were analyzed, as mentioned above.

Scale-up production and purification of recombinant DPEase

Scale-up for recombinant DPEase production

The scale-up production of recombinant DPEase was carried out in a 5 L fermenter. A single colony of the *E. coli* psicose strain was inoculated in LB broth and the overnight culture (0.5% (v/v) inoculum) was transferred

into 200 mL LB broth containing 50 µg/mL kanamycin and cultivated at 37 °C with 200 rpm agitation for 16 h to prepare the seed culture. Then 10% of the seed culture was inoculated in 1.8 L LB medium containing 50 µg/mL kanamycin and cultivated at 37 °C with 200 rpm agitation at an aeration rate of 2 volume of air per volume of liquid per minute (vvm). At an OD_{600} of 0.4–0.6, 1 mM IPTG was added and the induction was performed at 25 °C for 16 h.

Purification of recombinant DPEase enzyme

Preparation of crude extract

The cells were harvested using centrifugation at 7,000×*g* for 10 min and washed twice with sodium phosphate buffer (50 mM, pH 7.5) before re-suspending in 80 mL cold binding buffer (20 mM sodium phosphate buffer containing 500 mM NaCl, and 20 mM imidazole) at pH 7.5. Cell lysis was carried out using a high-pressure homogenizer (M110EH, Microfluidics, massachusetts, USA) with a pressure of 12,000 psi for 2 passages.

The cell lysate fraction was collected using centrifugation at 15,000×*g* and 4 °C for 30 min. The crude extract was measured for protein concentration and enzyme activity. The purification of DPEase was performed using an affinity column.

Determination of optimal imidazole concentration for purification

A sample of 3 mL of crude extract (16.33 mg total protein content) was injected into a HisTrap™HP column (17,524,701, Cytiva), and the flowthrough fraction was collected. In the washing step, 5 mL of binding buffer was applied to the column and the washing solution was collected. The protein was eluted stepwise with 3 mL of elution buffer (20 mM sodium phosphate buffer 500 mM NaCl, pH 7.5 containing 100 mM (E1), 200 mM (E2), 300 mM (E3), and 400 mM (E4) imidazole). The eluted samples were collected using elution buffers E1–E4 and desalted using Amicon® Ultra Centrifugal Filters with a molecular weight cut-off at 10 kDa (Millipore, USA). The purified samples were measured for protein concentration and analyzed using SDS-PAGE.

Fast protein liquid chromatography (FPLC)

Enzyme purification was performed at 4 °C using fast protein liquid chromatography (FPLC) (ÄKTA purifier UPC-900) with a 20 mL HisPrep™ FF 16/10 column (28,936,551, Cytiva). The crude extract (80 mL, 436.37 mg total protein content) was injected into the column with a flow rate of 1 mL/min. The column was washed with 300 mL binding buffer with a flow rate of 2 mL/min. The elution step was performed using 100 mL elution buffer containing 200 mM imidazole with a flow rate of 2 mL/min. The eluted samples were desalted and the purified DPEase was checked for protein concentration and enzyme activity and the purification table was calculated.

Characterization and structure of constructed recombinant DPEase enzyme

Effects of metal ions, pH, temperature, and time reaction on purified DPEase activity

The characterization and optimization of purified DPEase were investigated using a one-factor-at-a-time experiment. The different metal ions (as LiCl, NaCl, KCl, $CoCl_2$, $MnCl_2$, $NiCl_2$, $MgCl_2$, $BaCl_2$, $ZnCl_2$, $CuCl_2$, and $FeCl_3$) were supplemented to final concentrations of 1 mM and 10 mM to study their influence on DPEase activity. A range of pH values (5.5–10.5) and various temperatures in the range 20–70 °C were studied to determine the optimum DPEase activity. In addition, the substrate-enzyme reaction time was explored at 30, 60, 120, and 180 min. The effects were analyzed of these parameters on the activity of DPEase.

DPEase stability

The crude and purified DPEase were investigated for their stability under storage conditions at different temperatures (4 °C and -20 °C) and storage times (15, 30, and 45 days). At -20 °C, glycerol was added to the final concentration of 50% (v/v) and then mixed. The enzyme activity of all DPEase samples was analyzed and compared.

Determination of kinetic parameters of purified DPEase

The kinetic parameters were analyzed based on the maximum velocity (V_{max}) and the Michaelis–Menten constant (K_m) of purified DPEase. The amount of psicose was determined using HPLC analysis based on the product from the fructose-DPEase reaction. D-psicose was used as a standard curve to calculate the Lineweaver–Burk plot.

Mn^{2+} and Co^{2+} binding site predictions

Metal docking was performed due to the lack of *A. tumefaciens* DPEase in metal binding form. The online software MIB2: Metal Ion-Binding site prediction and modelling server (http://bioinfo.cmu.edu. tw/MIB2/) was used to predict the metal binding site. The DPEase structure 2HK0 was used as the input structure and the ions (Mn^{2+} and Co^{2+}) were selected as the input metals. The predicted site with the highest score for each metal was chosen and superposed with 2HK0. The illustration was performed using Pymol [30].

Efficiency of DPEase enzyme on D-fructose conversion to D-psicose

Purified DPEase was determined for the efficiency of fructose conversion to the target product as psicose. Fructose concentrations at 5, 10, 15, 20, and 25% (w/v) were studied under the optimum conditions of DPEase activity at pH 7.5 and 50 °C for 60 min of substrate-enzyme reaction, supplemented with a final concentration to 10 mM of $MnCl_2$ as the cofactor enzyme in the reaction. The increase in the psicose content and the decrease in the fructose content were analyzed using HPLC to calculate the percentage of D-psicose concentration and the conversion rate.

Analysis

Analysis of DPEase activity

First, the DPEase enzyme was incubated separately for 4 h at 4 °C in 100 mM Tris–HCl buffer pH 8.0, supplemented with Mn^{2+} as $MnCl_2$, to a final concentration of 10 mM. In addition, substrate was prepared using 1% (w/v) fructose concentration in 100 mM Tris–HCl buffer solution (pH 8.0) and also added to a final concentration of 10 mM $MnCl_2$. Then, 1 mL of the substrate was incubated at 50 °C for 10 min; subsequently, 1 mL of DPEase enzyme was added and incubated for 10 min, after which the enzyme reaction was stopped by boiling for 10 min. The quantity of psicose released from the reaction of DPEase and fructose was determined using HPLC based on standard sugar as D-psicose to calculate the enzyme activity.

One unit (U) of enzyme was defined as the amount of enzyme that produced 1 µmol/mL free D-psicose per minute at pH 8.0 and 50 °C.

Analysis of D-fructose and D-psicose content using HPLC

The analysis was based on modified methods following Showa Denko [32], Ninchan and Noidee [22], and Noidee et al. [25]. After the substrate-enzyme reaction had been stopped, the samples were filtered through a cellulose acetate membrane with a pore size of 0.45 µm. Thereafter, analyses of the fructose and D-psicose content were performed using HPLC (Shimadzu Corporation, Japan), with a refractive index detector (RID-10A, Shimadzu Corporation, Japan) and a Shodex Asahipak NH2P-50-4E column (4.6 mm I.D. × 250 mm; Showa Denko America, Inc., Japan) at 40 °C, using a ratio of acetonitrile-to-deionized water (70:30) as the mobile phase at a flow rate of 0.4 mL/min and 20 µL of injection volume with standard sugars (D-fructose and D-psicose).

Analysis of cell growth

A sample (1 mL) of the cell culture was transferred to a cuvette for measurement. Cell growth was assessed based on measuring OD_{600} to analyze turbidity. A sample of the sterile culture medium was used as the blank for the analysis.

Oral acute toxicity study

Animals

The 10 female Sprague Dawley rats were purchased from Nomura Siam International Co., Ltd., Thailand. They were kept in the laboratory of the Animal Center, Chiang Mai University, under constant conditions (12 h light/dark cycle, 50 ± 10% humidity, 21 ± 1 °C) with a standard diet and RO water ad libitum. They were treated according to experimental protocols approved by The Animal Care and Use Committee, Chiang Mai University (Protocol No. 2567/ RT-0002). All experimental rats were acclimatized for one week before the experiment.

Oral acute toxicity study

The mixed D-fructose-D-psicose syrup that was produced based on the optimized production conditions to achieve the highest D-psicose content was concentrated using evaporation to obtain the mixed syrup (67°Brix). The toxic effect of mixed syrup in rats was studied according to the treatment procedure of the Organisation for Economic Co-operation and Development (OECD) Guideline Test No. 420: Acute Oral Toxicity—Fixed Dose Procedure (2002)—Limit test (OECD 2002). The 10 female Sprague Dawley rats (aged 8–weeks, 220–230 g) were randomly divided into 2 groups. Each group was fasted overnight and provided with only water. After the overnight fast, Group 1 served as the control group, with oral access to distilled water, while Group 2 was given a single dose of 5000 mg/kg body weight of mixed fructose-psicose syrup. Signs of toxicity, behavior, and mortality were observed closely for the first 4 h. Subsequent observations were made at regular intervals for 24 h. Body weight, food consumption, and water intake were measured weekly. On day 15 of the experiment, all rats were euthanized with 4% isoflurane mixed with oxygen inhalation. Blood samples were collected from the abdominal vein for hematology and clinical biochemistry evaluation. The internal organs were excised for weighing and gross pathological observation.

Statistical analysis

Analytical values were analyzed and reported as the mean ± standard deviation. All reported values were based on the mean of three replicates. Data were subjected to analysis of variance (ANOVA) using the IBM

SPSS Statistics Version: 29.0.0.0(241) software. Significance was tested at the $p < 0.05$ level in the ANOVA. In the acute toxicity study, differences between groups of acute toxicity tests were determined based on an independent samples T-test. Differences were considered significant at $p < 0.05$.

Results

Optimization of recombinant D-psicose 3-epimerase in *E. coli*

Construction of DPEase recombinant strain

The DPEase expression vector was constructed by inserting the codon-optimized *DPEase* gene (AAK88700.1) into the pET28a expression vector. Positive clones were selected using colony PCR, which resulted in DNA fragments of 1070 bp. The sequencing results confirmed the successful construction of the *E. coli* DPEase expression strain, with two positive clones (Psicose 1 and Psicose 2) being chosen for further expression studies.

Recombinant DPEase expression of *E. coli* psicose

Recombinant DPEase expression was carried out using the *E. coli* psicose strains. The intracellular protein concentrations of crude extracts from Psicose 1 and Psicose 2 were measured using the Bradford assay, with non-induced samples serving as controls. Based on the results, the protein concentration in all induced samples was lower than that in the non-induced samples (Table S1).

SDS-PAGE analysis was performed on both cell fractions and crude extracts of Psicose 1 and Psicose 2 (Induced and Non-induced) to confirm the expression of recombinant DPEase (Fig. 1). Samples (10 µg) of protein were loaded per well in 10% (w/v) agarose gel. In this experiment, the cell fraction was analyzed to determine the quantity of the enzyme of interest remaining in the cell fraction, as well as the included body protein content.

The SDS-PAGE analysis revealed that Psicose 1 and Psicose 2, when induced with IPTG (Induced), exhibited intense protein bands at about 30 kDa, corresponding to the predicted size of DPEase calculated as 33 kDa (Fig. 1). More intense bands were observed in the cell debris of the induced samples. No bands corresponding to DPEase were detected in the non-induced samples.

Effect of Mn^{2+} ions on DPEase activity

The conversion of D-fructose to D-psicose highlighted the pivotal role of the Mn^{2+} ions as essential cofactor in DPEase biocatalysis [5, 10–13]. The current study explored the necessity of a specific procedural step involving incubation for 4 h with Mn^{2+} cofactors for accurate DPEase activity measurement. Based on the results, the highest recorded DPEase activity (1.730 U/

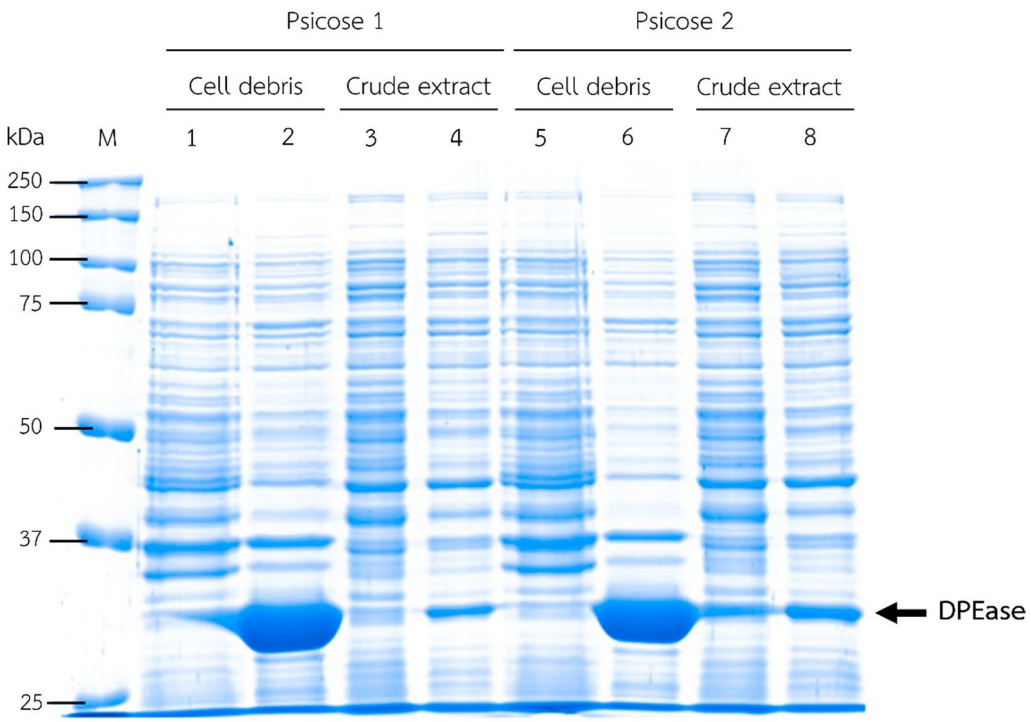


Fig. 1 SDS-PAGE analysis of recombinant DPEase in 2 DPEase recombinant *E. coli* strains; Psicose 1 (lanes 1–4) and Psicose 2 (lanes 5–8) at 10 µg protein of crude extract (lanes 3, 4, 7, 8) and 30-fold cell debris (lanes 1, 2, 5, 6). Lane M; protein marker

mL) was attained in the crude extract derived from induced recombinant psicose cells that had undergone incubation for 4 h with Mn^{2+} cofactors. In contrast, a discernible reduction in enzyme activity (18.5% decrease) was observed when the incubation step for 4 h was omitted (Table 2).

The impact of Mn^{2+} supplementation at various stages was evaluated by introducing 1 mM $MnCl_2$ into the culture medium (LB + Mn^{2+}) and/or the lysis buffer (lysis buffer + Mn^{2+}), with a focus on determining the highest DPEase activity attainable. Based on the results, the addition of 1 mM Mn^{2+} to the LB medium during cultivation and/or to the lysis buffer did not produce any discernible influence on the DPEase protein production or the protein content across all samples. Total protein levels remained consistent in the range 2.6–2.8 mg/mL (Table S2). These findings were corroborated by the protein profile data, which were in line with the results based on the SDS-PAGE analysis (Fig. 2). Furthermore, the DPEase activity of all samples was assessed in terms of D-psicose conversion using D-fructose as the substrate that was quantified using HPLC analysis. The supplementation of Mn^{2+} during cultivation or in the crude enzyme preparation produced no substantial variations in the protein profiles (Fig. 2). However, the presence of more Mn^{2+} in the crude enzyme resulted in lower DPEase activity (Table 3).

Table 2 Effect of Mn^{2+} incubation on D-psicose 3-epimerase activity

Incubation time	Sample	Enzyme activity (U/mL)
0 h	Non-induced	0.560 ± 0.016^{a}
	Induced	1.414 ± 0.066^{b}
4 h	Non-induced	0.545 ± 0.002^{a}
	Induced	1.730 ± 0.016^{c}

Values (mean ± standard deviation) in same column followed by different lowercase superscripts are significantly ($p < 0.05$) different

Effect of induction temperature on recombinant DPEase expression

Intense protein bands, corresponding to DPEase, were observed in the cell debris samples, indicating the presence of insoluble inclusion bodies in the recombinant DPEase expressed from the Psicose strain (Fig. 1 and Fig. 2). Different induction temperatures were tested during the induction process to mitigate the formation of inclusion bodies. It was observed that higher DPEase expression was achieved by using lower induction temperatures (20 °C and 25 °C). Based on the SDS-PAGE analysis, the highest DPEase protein expression occurred when induction was conducted at 20 °C, with expression gradually decreasing as the induction temperature

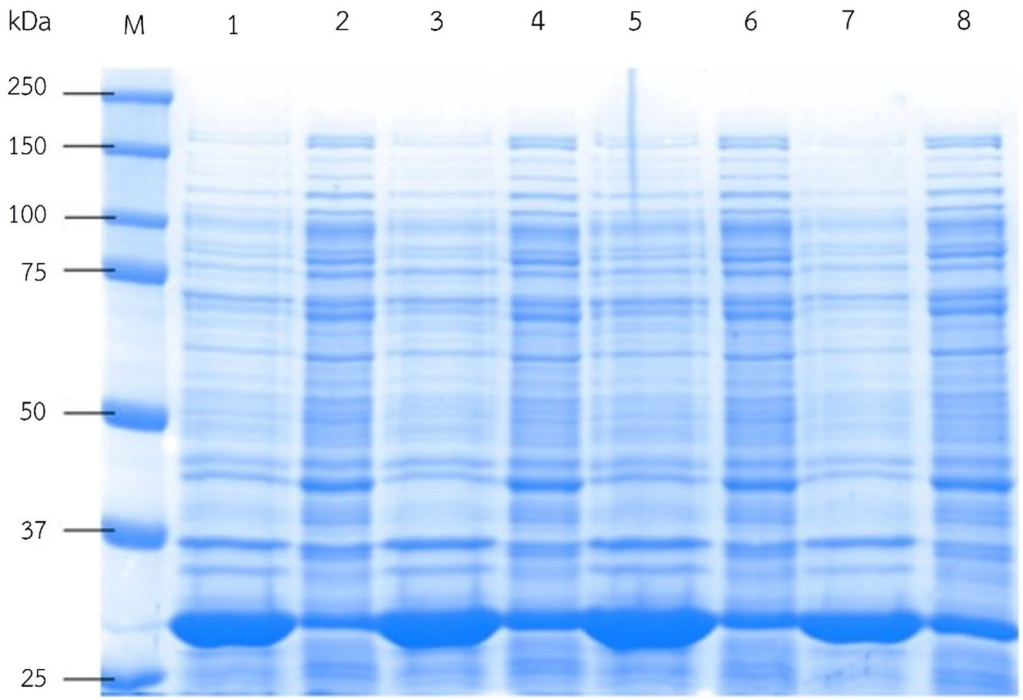


Fig. 2 SDS-PAGE analysis of Psicose strain cell debris (lanes 1, 3, 5, 7) and crude extract (lanes 2, 4, 6, 8). Lane M; protein marker, lanes 1–2; LB/lysis buffer, lanes 3–4; LB + Mn^{2+}/lysis buffer, lanes 5–6; LB/lysis buffer + Mn^{2+}, lanes 7–8; LB + Mn^{2+}/lysis buffer + Mn^{2+}

Table 3 Effect on DPEase activity of Mn^{2+}supplementation in various steps

Sample	Enzyme activity (U/mL)
LB / Lysis buffer / buffer	1.064 ± 0.063^{d}
LB + Mn^{2+} / Lysis buffer / buffer	1.935 ± 0.153^{a}
LB / Lysis buffer + Mn^{2+} / buffer	1.964 ± 0.170^{a}
LB / Lysis buffer / buffer + Mn^{2+}	1.705 ± 0.095^{b}
LB + Mn^{2+} / Lysis buffer / buffer + Mn^{2+}	1.663 ± 0.069^{b}
LB / Lysis buffer + Mn^{2+} / buffer + Mn^{2+}	1.672 ± 0.063^{b}
LB + Mn^{2+} / Lysis buffer + Mn^{2+} / buffer	1.664 ± 0.041^{b}
LB + Mn^{2+} / Lysis buffer + Mn^{2+} / buffer + Mn^{2+}	1.699 ± 0.119^{bc}

Values (mean ± standard deviation) in same column followed by different lowercase superscripts are significantly ($p < 0.05$) different;

LB: culture of recombinant bacteria in LB without Mn^{2+}

LB + Mn^{2+}: culture of recombinant bacteria in LB with Mn^{2+}

Lysis buffer: cell lysis using lysis buffer without Mn^{2+}

Lysis buffer + Mn^{2+}: cell lysis using lysis buffer with Mn^{2+}

Buffer: incubation of enzyme in buffer without Mn^{2+} for 4 h before the reaction

Buffer + Mn^{2+}: the incubation of the enzyme in buffer with Mn^{2+} for 4 h before the reaction

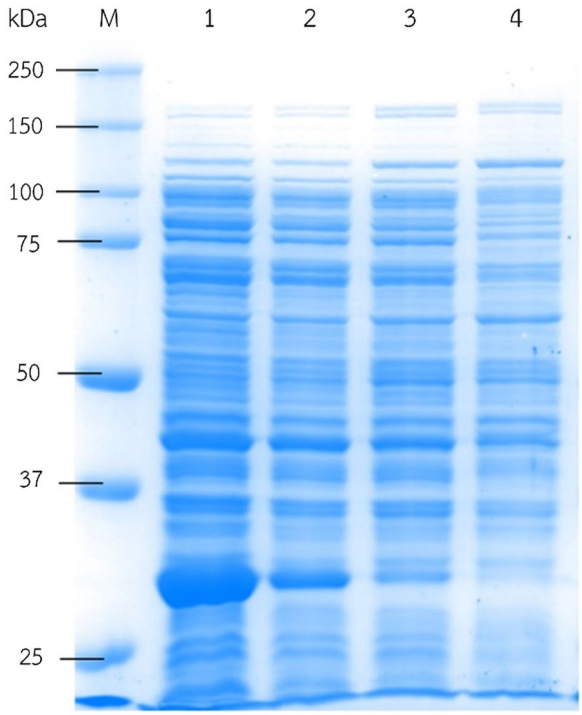


Fig. 3 SDS-PAGE analysis of recombinant DPEase in *E. coli* cultured in LB medium and induced at 20 °C (lane 1), at 25 °C (lane 2), at 30 °C (lane 3), and at 37 °C (lane 4). Lane M; protein marker

increased (Fig. 3). Notably, the highest DPEase specific activity (0.86 U/mg) occurred in the crude extract induced at 25 °C (Table 4). Notably, no DPEase protein bands or DPEase activity were detected in the crude extract when induction was performed at 37 °C. Consequently, an induction temperature of 25 °C was selected and applied as the optimal temperature for enzyme production in this experiment for *E. coli* Psicose expression.

Table 4 Effect of induction temperature on soluble D-psicose 3-epimerase production

Temperature (°C)	Protein (mg/mL)	Enzyme activity (U/mL)	Specific activity (U/mg)
20	3.971 ± 0.024	2.122 ± 0.035^{a}	0.53
25	2.427 ± 0.076	2.098 ± 0.13^{a}	0.86
30	1.610 ± 0.052	0.583 ± 0.067^{b}	0.36
37	1.092 ± 0.082	0.000 ± 0.000^{c}	0

Values (mean ± standard deviation) in same column followed by different lowercase superscripts are significantly ($p < 0.05$) different

Table 5 Effect of IPTG concentration on recombinant D-psicose 3-epimerase expression

IPTG concentration	Protein (mg/mL)	Enzyme activity (U/mL)	Specific activity (U/mg)
0 mM	3.509 ± 0.058	0.444 ± 0.12^{b}	0.13
0.5 mM	2.264 ± 0.037	1.983 ± 0.014^{a}	0.88
1.0 mM	1.772 ± 0.026	2.053 ± 0.018^{a}	1.16
1.5 mM	1.772 ± 0.036	2.084 ± 0.002^{a}	1.17
2.0 mM	1.899 ± 0.027	2.199 ± 0.131^{a}	1.16

Values (mean ± standard deviation) in same column followed by different lowercase superscripts are significantly ($p < 0.05$) different

Optimization of IPTG concentration on DPEase expression

The concentration of the IPTG as an inducer was systematically optimized (0, 0.5, 1.0, 1.5, and 2.0 mM) to identify the ideal concentration for achieving maximum DPEase expression. Based on the results, it was clear that in the absence of IPTG, no recombinant DPEase was produced. However, upon the addition of IPTG, the detection of DPEase activity became evident. Specifically, at an IPTG induction concentration of 0.5 mM, the specific activity reached 0.88 U/mg. Notably, higher specific activities (approximately 1.16 U/mg) were observed when using higher IPTG concentrations (1.0, 1.5, and 2.0 mM), as shown in Table 5 and Fig. 4.

Verification of imidazole concentration for DPEase purification

The purification of recombinant DPEase from the recombinant psicose strain, featuring a His-tag fused to the 3' end, was carried out utilizing a nickel column, which is an affinity column tailored for His-tagged proteins. An elution buffer containing imidazole was used, as it was

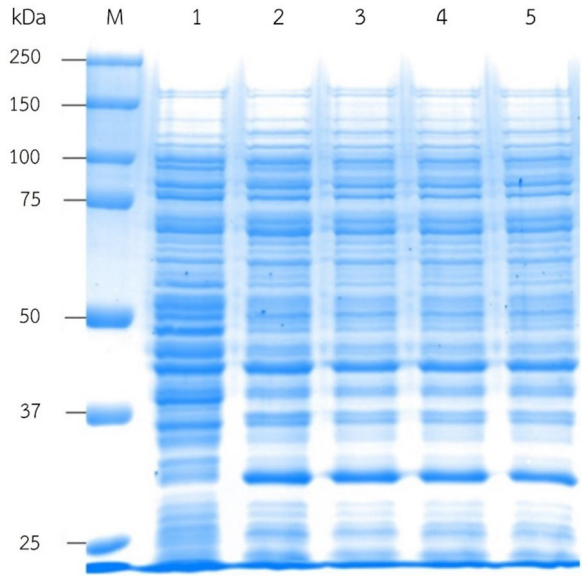


Fig. 4 SDS-PAGE analysis of effect of IPTG concentration on DPEase expression. Lane 1; 0 mM, lane 2; 0.5 mM, lane 3; 1.0 mM, lane 4; 1.5 mM, and lane 5; 2.0 mM. Lane M; protein marker

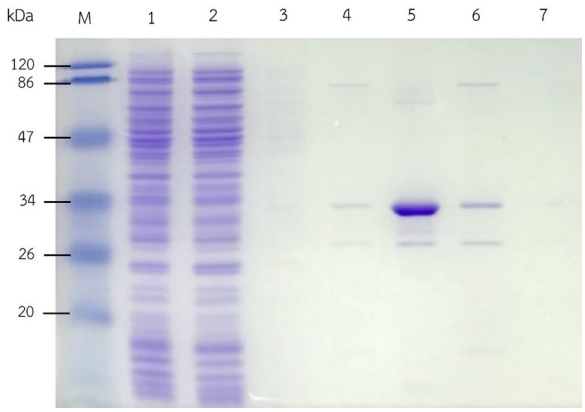


Fig. 5 SDS-PAGE analysis during purification steps using 1 mL HisTrap™HP column for recombinant DPEase. Lane M; protein marker, lane 1; crude extract, lane 2; flow-through fraction, lane 3; wash fraction, lane 4; eluted fraction 1 (elution buffer containing 100 mM imidazole), lane 5; eluted fraction 2 (elution buffer containing 200 mM imidazole), lane 6; eluted fraction 3 (elution buffer containing 300 mM imidazole), and lane 7; eluted fraction 4 (elution buffer containing 400 mM imidazole)

crucial to determine the appropriate imidazole concentration to achieve the highest yield and purity. Based on the results, the target DPEase was effectively eluted from the column by using an elution buffer containing 100–300 mM imidazole. The highest concentration of DPEase occurred in the fraction eluted with the E2 buffer, containing 200 mM imidazole (Fig. 5). This fraction had both high purity and a substantial quantity of DPEase protein, with an approximate molecular weight of 33 kDa, as confirmed from the SDS-PAGE analysis. Consequently, an elution buffer containing 200 mM imidazole was identified as the optimal concentration and was subsequently utilized for further purification of the target enzyme in large-scale production (Fig. 5).

Scale-up production and purification of recombinant DPEase

The scale-up production of recombinant DPEase from psicose strain was performed in 5 L fermentation tanks at the laboratory scale. The selected production and purification conditions from the abovementioned results were applied. Cells were collected from the fermenter and the crude enzyme was extracted with total activity of 167.84 U (Table 6). After purification, the DPEase elution with the elution buffer containing 200 mM imidazole resulted in high purity DPEase, as seen clearly in the SDS-PAGE analysis (Fig. 6). Very few contaminated protein bands were observed, demonstrating the high efficiency of this purification method. The purity of the purified enzyme was confirmed based on its high specific activity (8.20 U/mg), with a purification factor of 21.03 compared to the crude enzyme (Table 6). The purified enzymes from this study were used to study the biochemical characterization of the DPEase, as well as the conversion of D-psicose.

Characteristic and structure of contracted recombinant DPEase enzyme

Effects of metal ions, pH, temperature, and reaction time on purified DPEase activity

Effects of metal ions on DPEase activity

The purified DPEase showed no activity without any metal ion addition. All ions (Li^+, Na^+, K^+, Co^{2+}, Mn^{2+},

Table 6 Purification table of purified recombinant DPEase using fast protein liquid chromatography

Sample	Total volume (mL)	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg)	Purification factor (-fold)
Crude extract	80	435.37 ± 1.14^a	167.84 ± 2.32^b	0.39	1.00
Purified DPEase	80	31.69 ± 0.23^b	259.84 ± 2.53^a	8.20	21.03

Values (mean ± standard deviation) in same column followed by different lowercase superscripts are significantly ($p < 0.05$) different

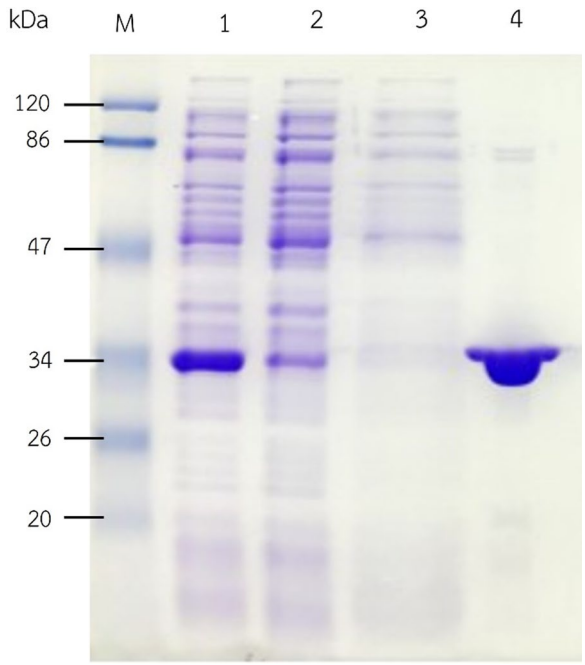


Fig. 6 SDS-PAGE analysis during purification steps using HisPrep™ FF 16/10 column of recombinant DPEase. Lane M; protein marker, lane 1; crude extract, lane 2; flow-through fraction, lane 3; wash fraction, lane 4; eluted fraction (elution buffer containing 200 mM imidazole)

Ni^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{3+}) were investigated for DPEase activity. Based on the results from the final concentration of ions (1 mM), there was DPEase activity with the addition of Co^{2+} and Mn^{2+} ions, as shown in Fig. 7A, with the DPEase having the highest activity of 1.25 U/mL. The other ions displayed no enzyme activity. Furthermore, increasing concentration of metal ions positively affected DPEase activity. The ion addition to final concentrations of 10 mM for Li^{+}, Na^{+}, K^{+}, Ni^{2+}, Mg^{2+}, Ba^{2+}, and Zn^{2+} produced higher DPEase activity; however, the activity was lower than from supplementing with 10 mM of Co^{2+} and Mn^{2+}. Neither Cu^{2+} nor Fe^{3+} activate DPEase. Some ions influenced DPEase activity by acting as a cofactor for achieving high activity, especially Mn^{2+} [10, 11, 13, 39], which in the current study had the maximum DPEase activity (3.16 U/mL) at 10 mM of final ion concentration.

Effects of pH on DPEase activity

Enzyme activity was conducted in the presence of 10 mM Mn^{2+} at 50 °C to investigate the effect of pH in the range 5.5–10.5 as shown in Fig. 7B. Purified DPEase had the highest activity at pH 7.5 (3.25 U/mL). However, within the pH range 7–9, DPEase had more than 80% activity. Notably, at other pH levels outside this range, there was more than 60% activity.

Effects of temperature on DPEase activity

Temperature is as important a factor influencing activity of enzyme as DPEase activity. Over the temperature ranges studied (20–70 °C) under the optimum conditions at pH 7.5 in the presence of 10 mM Mn^{2+} ion as the final concentration in the enzyme–substrate reaction, there were enzyme activity in the range 35–70 °C, with thermoactivated enzyme activity occurring at over 60 °C. Overall, DPEase had the highest activity at 55 °C (Fig. 7C).

Effects of reaction time on DPEase activity

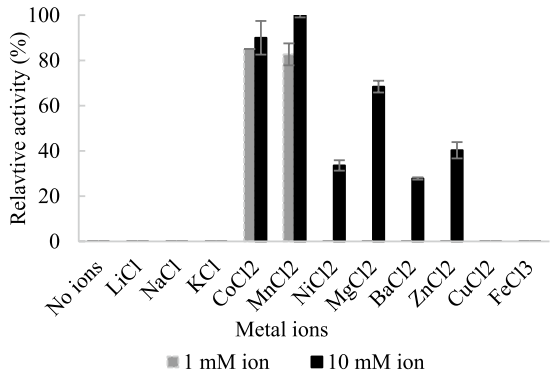
The reaction of fructose solution as substrate and DPEase under different reaction times was studied at pH 7.5 and 55 °C with 10 mM Mn^{2+} supplementation. The substrate-enzyme reaction was tested at 30, 60, 120, and 180 min. At times longer than 60 min, the DPEase produced stable enzyme activity, as shown in Fig. 7D. Thus, 60 min of reaction time could be the candidate timing for D-psicose production using purified DPEase.

DPEase stability

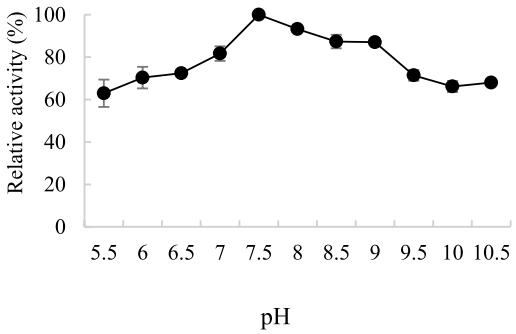
The results of crude and purified DPEase were analyzed for enzyme activity after storage at 4 °C and -20 °C for 15, 30, and 45 days as shown in Fig. 8. Notably, the crude DPEase activity clearly decreased compared to purified DPEase. At both storage temperatures, the purified DPEase had the relative activity of approximately 90%, 80%, and 60%, after storage for 15 days, 30 days, and 45 days, respectively. The DPEase enzymes kept at − 20 °C with glycerol addition had greater enzyme activity compared to keeping at 4 °C. Notably, the activity of crude DPEase kept at 4 °C decreased sharply, with no activity evident after 30 days of storage.

Determination of kinetic parameters of purified DPEase

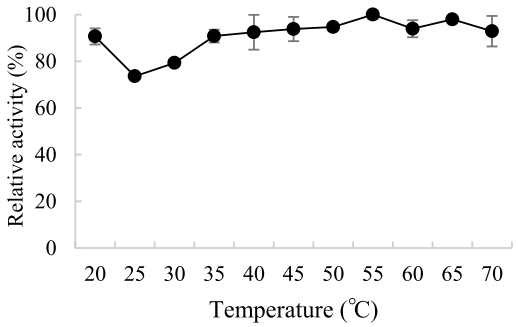
The kinetic parameters of the purified DPEase were investigated, based on the maximum velocity (V_{max}) and the Michaelis constant (K_m). V_{max} is the maximum rate of reaction when the DPEase enzyme is saturated with fructose (substrate), while K_m is the concentration of fructose at which the DPEase achieves half V_{max} and is referred to as the binding or affinity between the DPEase and fructose. The initial velocity (V_i) was studied at different fructose concentrations at pH 7.5 and 55 °C, with Mn^{2+} ion supplementation for 1 min of testing, which was the short time of enzyme–substrate reaction. The kinetic parameters were obtained from calculations based on the Michaelis–Menten equation and the Lineweaver–Burk plot, with the V_{max} and K_m values of DPEase being 28.01 mM/min and 110 mM, respectively. Other researchers have reported K_m values in the



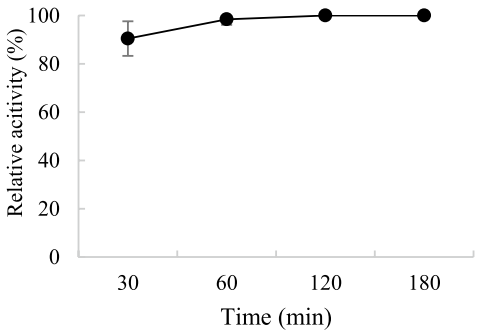
(A) Metal ions (conditions: 50°C, pH 8.0, and 60 min)



(B) pH (conditions: 50°C, 10 mM $MnCl_2$, and 60 min)



(C) Temperature (conditions: 10 mM $MnCl_2$, pH 7.5, and 60 min)



(D) Time (conditions: 10 mM $MnCl_2$, pH 7.5, and 55 °C)

Fig. 7 Effects of metal ions (**A**), pH (**B**), temperature (**C**), and time reaction (**D**) on purified DPEase activity

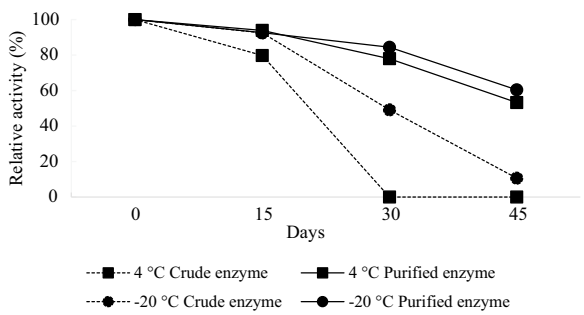


Fig. 8 Stability of DPEase activity (crude and purified enzymes) under storage conditions at different temperatures (4 °C and – 20 °C) and storage times (0–45 days)

range 0.279–549 mM, depending on the substrates and DPEase-producing microorganisms (BRENDA 2003; [35, 41]). Considering the fructose as the substrate, the K_m value of the recombinant *A. tumefaciens* DPEase used in the current research had a lower value than *Blautia produca* DPEase [35] and *Treponema primitia* ZAS-1 DPEase [41] that had values of 235.4 mM and 279 mM, respectively. In fact, a low K_m value indicates a high affinity for the enzyme and substrate; on the other hand, a high K_m correlates with low affinity [23, 34]. Consequently, the low K_m of the DPEase from the current result probably implied high affinity of DPEase and fructose for greater conversion to the target product of D-psicose. These results were confirmed by Fig. 9D, showing the binding of substrate (fructose) and enzyme (DPEase) under Mn^{2+} as the co-factor.

Structure–function relationships of *A. tumefaciens* DPEase: Mn^{2+} and Co^{2+} binding site predictions

To implement the biochemical characterization of the *A. tumefaciens* DPEase (AgtuDPEase) strain C58, we described the structural features of this enzyme. Crystal structures of DPEase *A. tumefaciens* strain C58 have been reported in two forms: apoform and D-fructose bound complex. AgtuDPEase are homotetramers that adopt the

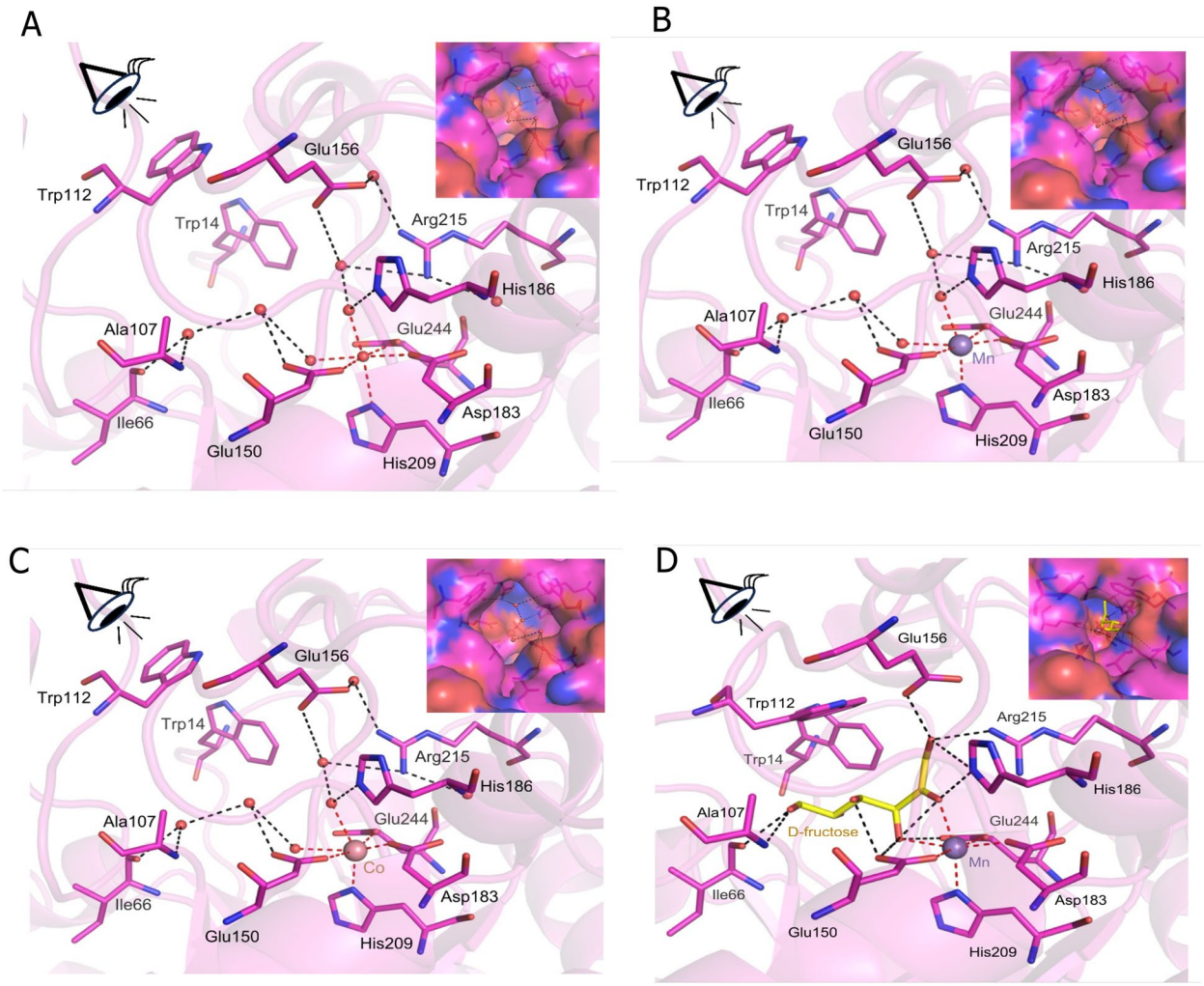
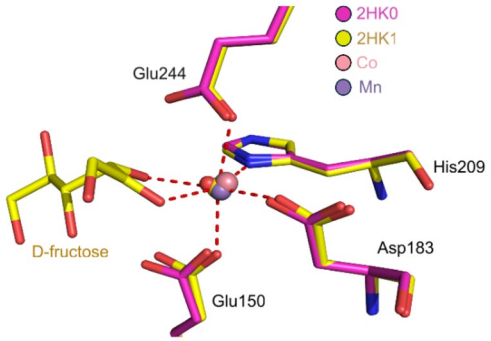


Fig. 9 Active sites of *A. tumefaciens* DPEase in apoform, metal bound complex, and D-fructose bound complex. DPEase structure illustrated using PDB code 2HK0 for apoform and metal bound complex, and 2HK1 for D-fructose bound complex. **A** Active site in DPEase apoform showing water network occupying the substrate binding site. **B**, and **C** Active site of DPEase (2HK0) in metal bound complex, in which Mn^{2+} and Co^{2+} are docked to metal binding site, respectively. **D** Active site of substrate bound complex shows binding of D-fructose replacing water network and interacting directly with catalytic residues (Glu150, and Glu244) and metal cofactor. Inset figures show active site pocket in different states along view indicated by eye icon. Dotted black lines represent hydrogen bonds, dotted red lines represent hydrogen bond involving in metal coordination or water in apoform, while D-fructose is shown as yellow sticks

highly conserved (α/β)8-TIM barrel domain. The active site of AgtuDPEase is located in the hydrophobic cavity in each subunit. The metal binding site is found in the center of the active site coordinated by Glu150, Asp183, His209, Glu244, and two water molecules (Fig. 9). In the AgtuDPEase apoform, the metal site is replaced by one water molecule that adopts similar geometry to the metal (Fig. 9A). In addition, the active site is occupied by the water network, which maintains the interactions of catalytic and metal binding residues [11, 13]. Due to the lack of AgtuDPEase in metal binding form (without substrate), the metal ions (Mn^{2+} and Co^{2+}) and positively active-AgtuDPEase were docked to the AgtuDPEase apoform. The docking site was in proximity to the coordinated water. Notably, the docking site of Co^{2+} was similar to that of Mn^{2+}; however, Co^{2+} had shifted toward the side chain of Asp183 and His209 (Figs. 9C and 10). Local changes occurred upon the binding of D-fructose [11, 13]. The catalytic, and metal binding residues adopted slight movement of their side chains to accommodate the incoming D-fructose, which displaced the ordered water network at the active site of the enzyme. In addition, hydrophobic Trp112, and Trp14, located at the gate of the cavity, adopted a close conformation to



Distance of metal cofactor to metal binding residues of AgtuDPEase

Metal ions	AgtuDPEase Structure (PDB code)	Distances to metal binding residues (Å)
Water	2HK0	2.3 to OE2 of Glu150 2.3 to OD2 of Asp183 2.5 to ND1 of His209 2.4 to OE1 of Glu244
Mn^{2+} (Docked)	2HK0	2.2 to OE2 of Glu150 2.0 to OD2 of Asp183 2.4 to ND1 of His209 2.5 to OE1 of Glu244
Co^{2+} (Docked)	2HK0	2.4 to OE2 of Glu150 1.9 to OD2 of Asp183 2.2 to ND1 of His209 2.3 to OE1 of Glu244
Mn^{2+}	2HK1	2.2 to OE2 of Glu150 2.3 to OD2 of Asp183 2.2 to ND1 of His209 2.4 to OE1 of Glu244

Fig. 10 Alignment of apoform, AgtuDPEase, metal bound complex, and D-fructose bound complex, and distances of metal cofactor to metal binding residues of AgtuDPEase. Prediction of metal docking into 2HK0 was performed using MIB2 [17]. Alignment was performed using Cα of 2HK0, and 2HK1. Metal ions are shown as spheres with indicated colors. Mn^{2+} of 2HK1 is shown as yellow sphere and coordinated water of 2HK0 is shown as red sphere, while red dotted line represents only hydrogen bond involved in Mn^{2+} coordination of D-fructose bound complex (2HK1). D-fructose is shown as yellow sticks

ensure the binding of D-fructose, narrowing the entrance to the hydrophobic cavity (Fig. 9D). Then, the epimerization was catalyzed by Glu150, and Glu244, generating D-psicose through the intermediate, cis-enediolate. Once the reaction has been completed, the involved residues return to open conformation priming for the next turnover. The structure is still unknown regarding how metal specificity affects the binding of sugar substrate, as only a limited number of metal ions have been co-crystalized with DPEase, so far including Mn^{2+} and Mg^{2+} [11, 13, 16].

Efficiency of D-fructose conversion to D-psicose

The efficiency was studied of D-psicose production using purified DPEase and the pure fructose solution was the substrate for the enzyme conversion. The different substrate concentrations of 5, 10, 15, and 25% (w/v) were compared based on the efficiency of conversion under the optimum conditions for the enzyme. The concentration of D-psicose and its associated conversion rate from D-fructose to D-psicose are shown in Table 7. Based on these results, the substrate concentration in terms of fructose solution was negatively correlated with the rate of conversion from D-fructose to D-psicose because of the substrate inhibition. While, the D-psicose concentration had a strongly positive correlation with the fructose concentration as the substate in the enzyme reaction.

Table 7 Efficient enzyme conversion of purified DPEase for D-psicose production using different fructose concentrations as substrate for reaction at pH 7.5 and 55 °C, with Mn^{2+} ion supplementation

Fructose concentration (%, w/v)	D-psicose concentration (%, w/v)	Conversion rate (%)
5	1.45 ± 0.00^{e}	29.02 ± 0.07^{a}
10	2.65 ± 0.07^{d}	26.51 ± 0.66^{b}
15	3.75 ± 0.14^{c}	25.02 ± 0.92^{b}
20	4.50 ± 0.14^{b}	23.02 ± 0.71^{c}
25	5.60 ± 0.03^{a}	22.42 ± 0.12^{c}

Values (mean ± standard deviation) in same column followed by different lowercase superscripts are significantly ($p < 0.05$) different

Based on the current results, the highest D-psicose concentration was clearly produced from the highest fructose concentration at 25% (w/v), as shown in Table 7. The final product from this reaction was obtained as a mixed syrup of D-fructose and D-psicose. The highest production of D-psicose was under 25% (w/v) of initial fructose concentration that was concentrated by evaporation to achieve a higher concentration, reaching 67°Brix. Then, the DPEase was denatured by boiling and was precipitated as a protein and filtered through an 11 µm membrane, followed by filtering

through a 0.45 µm membrane to remove the fine precipitate particles and to obtain the clear sugar solution that was subsequently passed through the evaporation process. Next, the mixed syrup was evaluated for its acute toxicity in rats to provide documentary evidence of its safe use.

Table 8 General parameter observations of rats in acute toxicity test

Parameter	Distilled water	Mixed syrup 5000 mg/kg bw
Initial body weight (g)	230 ± 18[a]	229 ± 13[a]
Final body weight (g)	279 ± 18[a]	277 ± 14[a]
Food intake (g/rat)	18.8 ± 0.8[a]	19.2 ± 2.1[a]
Water intake (ml/rat)	31.4 ± 2.2[a]	32.9 ± 1.8[a]

Values (mean ± standard deviation) followed by same lowercase superscripts are not significantly

($p < 0.05$) different between the two groups; bw: Body weight

Oral acute toxicity study

Oral administration of 5,000 mg/kg body weight of mixed D-fructose–D-psicose syrup (mixed syrup) showed no mortality or toxicity-related clinical signs in rats. The body weight, food consumption, and water intake of rats treated with mixed syrup were not significantly different to those of the control rats (Table 8). There were no significant differences in the absolute and relative organ weights between the control group and the mixed syrup-treated group (Table 9).

There were no significant differences among the hematological parameters between the control and the mixed syrup-treated groups. Additionally, there were no significant differences among the biochemical parameters between the control and the mixed syrup-treated groups. The results are summarized in Tables 10–11. Based on these findings, mixed syrup was not toxic to rats. The 50% lethal dose (LD50) of the mixed syrup was greater than 5,000 mg/kg body weight.

Table 9 Absolute and relative organ weights of rats in acute toxicity study

Parameter		Distilled water	Mixed syrup 5000 mg/kg bw
Final body weight (g)		279 ± 18[a]	277 ± 14[a]
Absolute organ weight (g)	Liver	12.03 ± 1.86[a]	12.85 ± 0.71[a]
	Spleen	0.52 ± 0.07[a]	0.56 ± 0.03[a]
	Kidney	2.05 ± 0.06[a]	2.36 ± 0.25[a]
	Lung	1.35 ± 0.07[a]	1.27 ± 0.08[a]
	Heart	0.81 ± 0.07[a]	0.78 ± 0.07[a]
	Pancreas	0.54 ± 0.08[a]	0.62 ± 0.06[a]
	Uterus & ovary duct	0.61 ± 0.07[a]	0.58 ± 0.12[a]
	Thymus	0.45 ± 0.07[a]	0.43 ± 0.05[a]
	Stomach	1.49 ± 0.06[a]	1.41 ± 0.20[a]
	Adrenal gland	0.075 ± 0.004[a]	0.080 ± 0.005[a]
	Ovary	0.158 ± 0.014[a]	0.152 ± 0.005[a]
Relative organ weight (%)	Liver	4.29 ± 0.42[a]	4.64 ± 0.22[a]
	Spleen	0.19 ± 0.02[a]	0.20 ± 0.02[a]
	Kidney	0.74 ± 0.05[a]	0.85 ± 0.06[a]
	Lung	0.48 ± 0.01[a]	0.46 ± 0.01[a]
	Heart	0.29 ± 0.04[a]	0.28 ± 0.02[a]
	Pancreas	0.19 ± 0.02[a]	0.22 ± 0.02[a]
	Uterus & ovary duct	0.22 ± 0.04[a]	0.21 ± 0.05[a]
	Thymus	0.16 ± 0.01[a]	0.15 ± 0.01[a]
	Stomach	0.54 ± 0.05[a]	0.51 ± 0.05[a]
	Adrenal gland	0.027 ± 0.002[a]	0.029 ± 0.001[a]
	Ovary	0.057 ± 0.007[a]	0.055 ± 0.003[a]

Values (mean ± standard deviation) followed by same lowercase superscripts are not significantly

($p < 0.05$) different between the two groups; bw: Body weight

Table 10 Hematological values of rats in acute toxicity test

Parameter	Distilled water	Mixed syrup 5000 mg/kg bw
RBC ($\times 10^6$ /µL)	7.70 ± 0.31[a]	8.03 ± 0.51[a]
Hb (g/dL)	14.74 ± 0.40[a]	15.26 ± 0.53[a]
HCT (%)	44.1 ± 2.05[a]	45.16 ± 1.60[a]
MCV (fL)	57.28 ± 1.34[a]	56.34 ± 1.81[a]
MCH (pg)	19.16 ± 0.36[a]	19.06 ± 0.71[a]
MCHC (%)	33.46 ± 0.66[a]	33.82 ± 0.31[a]
PLT ($\times 10^3$ cell/µL)	1,026.8 ± 70.46[a]	1,054.0 ± 28.13[a]
WBC ($\times 10^3$ cell/µL)	7.87 ± 2.61[a]	7.73 ± 1.74[a]
Neu (%)	12.2 ± 3.84[a]	9.76 ± 3.17[a]
Lym (%)	79.84 ± 4.01[a]	82.26 ± 4.64[a]
Mon (%)	6.74 ± 0.54[a]	6.76 ± 1.33[a]
Eos (%)	1.16 ± 0.34[a]	1.16 ± 0.46[a]
Bas (%)	0.06 ± 0.13[a]	0.06 ± 0.05[a]

Values (mean ± standard deviation) followed by same lowercase superscripts are not significantly

($p < 0.05$) different between the two groups; RBC: Red blood cell, Hb: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelet, WBC: White blood cell, Neu: Neutrophil, Lym: Lymphocyte, Mon: Monocyte, Eos: Eosinophil, Bas: Basophil, bw: Body weight

Table 11 Biochemical parameters of rats in acute toxicity test

Parameter	Distilled water	Mixed syrup 5000 mg/kg bw
BUN (mg/dL)	33.14 ± 5.90[a]	30.22 ± 4.41[a]
Creatinine (mg/dL)	0.66 ± 0.06[a]	0.66 ± 0.02[a]
Uric acid (mg/dL)	3.64 ± 1.33[a]	5.22 ± 2.16[a]
ALT (U/L)	31.60 ± 4.04[a]	32.80 ± 5.07[a]
ALP (U/L)	117.00 ± 29.11[a]	112.00 ± 17.66[a]
AST (U/L)	79.20 ± 8.81[a]	77.60 ± 8.91[a]
Total protein (mg/dL)	7.04 ± 0.43[a]	7.08 ± 0.45[a]
Albumin (g/dL)	4.28 ± 0.22[a]	4.24 ± 0.15[a]
Globulin (g/dL)	2.76 ± 0.29[a]	2.84 ± 0.31[a]
Albumin/ Globulin ratio	1.56 ± 0.15[a]	1.51 ± 0.14[a]
Total bilirubin (g/dL)	0.28 ± 0.08[a]	0.24 ± 0.05[a]
Direct bilirubin (g/dL)	0.04 ± 0.01[a]	0.03 ± 0.01[a]

Values (mean ± standard deviation) followed by same lowercase superscripts are not significantly

($p < 0.05$) different between the two groups; bw: Body weight

Discussion

The current study applied codon optimization to construct the DPEase recombinant strain, redesigning the DPEase gene from *A. tumefaciens* to enhance the expression of the target protein. Based on the results, the recombinant DPEase strain had a high and comparable specific activity of DPEase expression compared to other findings [11, 13]. The purified DPEase had a specific activity of 8.2 units/mL after single-step purification, in contrast to the three-step purification reported by Kim et al. [11, 13]. This underscored the success of codon optimization with culture condition optimization in enhancing DPEase expression and streamlining the purification process.

The systematically optimized expression conditions were also applied for DPEase production. The optimization included the use of Mn^{2+} as a cofactor, IPTG induction, and precise temperature control during induction. Mn^{2+} and Co^{2+} played a crucial role as a cofactor, enhancing DPEase activity, which was consistent with other research highlighting the importance of metal ions in DPEase catalysis [9]. Based on the results, Mn^{2+} could be recommended as the optimal cofactor for the recombinant DPEase. This choice could be justified by its superior enzyme activity and cost-effectiveness, which can potentially lead to reduced production costs for industrial applications. Furthermore, the addition of the cofactor during cell cultivation demonstrated the potential of ensuring the availability of the cofactor for DPEase. Furthermore, this approach had the advantage of reducing the incubation time after harvesting the crude enzyme, thereby benefiting the overall enzyme production process.

The effect of Mn^{2+} supplementation at various steps on DPEase activity indicated that higher Mn^{2+} supplementation led to reduced DPEase activity. This was attributed to the increased accumulation of NaCl in the enzyme solution. In this study, Mn^{2+} was supplemented as $MnCl_2$ dissolved in sodium phosphate buffer, which could contribute to NaCl accumulation through chemical reactions. Elevated NaCl concentrations in the solution potentially influenced enzyme activity and stability [3].

The higher protein concentrations in the non-induced samples (Table S1) suggested that induction of the recombinant strain not only required energy for plasmid maintenance and replication but also imposed a major metabolic burden for DPEase expression (Silva et al., 2012), especially in the *E. coli* expression system, with a strong T7 promoter capable of producing high concentrations of the target protein. This metabolic demand in the induced samples resulted in less energy being available for cell growth, resulting in reduced biomass production. Consequently, in the current study, intracellular protein was collected from biomass, revealing lower total protein concentrations in all induced samples compared to non-induced samples under the same culture conditions.

The expression of DPEase in *E. coli* resulted in the formation of inclusion bodies, particularly noticeable during cultivation at 37 °C. This occurrence was consistent

with established findings in the field of recombinant protein expression within the *E. coli* system, where the formation of inclusion bodies, consisting of misfolded and non-functional proteins, is a common occurrence [1, 29, 33]. The notable rate of protein expression, driven by the T7 promoter, in conjunction with the limited presence of chaperones, contributed to incomplete protein folding and the accumulation of misfolded proteins [1, 31]. Furthermore, this rapid protein expression led to elevated protein concentrations within the cytoplasm or periplasm, compromising protein solubility and initiating protein aggregation [29, 33]. This challenge can be addressed by using a successful strategy involving the reduction of the culturing temperature. This approach effectively retards cell growth, resulting in a decreased rate of transcription and translation, ultimately yielding reduced levels of misfolded proteins and mitigating protein aggregation [1, 29, 33]. These findings corroborated the established principle that temperature plays a major role in shaping protein folding and solubility, potentially enhancing DPEase yield during the enzyme production step. Using a nickel column and elution with 200 mM imidazole proved to be a highly efficient purification strategy, yielding purified DPEase with a notable 21-fold increase in specific activity compared to the crude extract. The purification of DPEase represents a critical phase in the commercial production of D-psicose, especially considering the regulatory framework surrounding the utilization of genetically modified microorganisms (GMOs) in food applications. Food safety regulations applicable worldwide place strict limitations on the presence of GMO contamination in final food products. These regulations are designed to ensure consumer safety and maintain the integrity of non-GMO food products. Enzyme purification serves a dual role: it eliminates cellular contaminants while enhancing enzyme activity to meet these rigorous standards.

The determination of kinetic parameters, specifically V_{max} and K_m, provided essential insights into the catalytic efficiency of DPEase and substrate affinity. Our calculated values of V_{max} (28.01 mM/min) and K_m (110 mM) indicated that DPEase had high substrate affinity and an efficient conversion rate of D-fructose to D-psicose. These results were consistent with other studies that emphasized the importance of low K_m values in achieving high enzyme–substrate affinity [23, 34]. Notably, a unique three-dimensional structure of *A. tumefaciens* DPEase contains the Mn^{2+} ion binding site [11–13] as does the structure of DPEase that was constructed from *A. tumefaciens* C58 in the current research (Fig. 9), resulting in high activity of DPEase in the presence of Mn^{2+} ions in the reaction. The Mn^{2+} ions play a pivotal role as a cofactor by anchoring the bound substrate of D-fructose and by providing coordination bonds and electrostatic interactions that lead to the epimerization reaction [5, 10–13]. Reasonable DPEase kinetic results are characterized by low K_m and a high affinity of DPEase and fructose leading to high efficiency of D-psicose conversion. Based on our experiments on D-psicose production using purified DPEase, the highest D-psicose concentration was achieved with the highest fructose concentration at 25% (w/v), underscoring the pivotal role of the substrate concentration in determining D-psicose production efficiency. The study of enzyme stability demonstrated that purified DPEase exhibited higher stability compared to the crude enzyme during storage at both 4 °C and -20 °C. The addition of glycerol and a lower temperature (-20 °C) further improved enzyme stability, highlighting the importance of proper storage conditions for preserving enzyme activity. Subsequently, the concentration of the mixed syrup containing D-psicose and D-fructose through evaporation resulted in a substantial increase in the Brix concentration to 67. This concentration step is vital for practical applications of D-psicose production in the food industry. Our research has important implications for D-psicose production, a valuable rare sugar with potential applications in the food and pharmaceutical industries. The high affinity of DPEase for D-fructose, as indicated by the low K_m value, suggests that DPEase is a promising candidate for efficient psicose production. In conclusion, our study has contributed to a comprehensive understanding of DPEase production, purification, and characterization, offering insights into its potential applications in various biocatalytic processes. These findings should contribute to the advancement of enzyme biotechnology and open new avenues for the production of valuable rare sugars such as D-psicose. Furthermore, the results of the oral acute toxicity study supported the safety of mixed D-fructose-D-psicose syrup produced using recombinant DPEase, making it suitable for food and functional food applications.

Conclusion

The production, purification, and characterization of recombinant DPEase from the psicose strain were systematically investigated. Based on the results, several important finding were produced: (1) successfully optimizing the expression conditions for DPEase, including the utilization of Mn^{2+} as a cofactor that was confirmed by the efficient binding site on structure of fructose-DPEase-Mn^{2+} binding; (2) fine-tuning the IPTG induction parameters; and (3) exploring the impact of induction temperature. These optimization processes led to increased DPEase production and activity. The formation of insoluble inclusion bodies was observed that were effectively addressed by lowering the

induction temperature, resulting in enhanced soluble DPEase expression. The purification strategy applied a column and elution with an imidazole-containing buffer (specifically 200 mM imidazole) demonstrated exceptional efficiency. This approach yielded purified DPEase with a notable 21.03-fold increase in specific activity compared to the crude extract. The purified enzyme was utilized for comprehensive biochemical characterization and its potential in D-psicose conversion. Purified DPEase performed well under a wide range of pH and temperature. However, the optimum conditions of the enzyme were at pH 7.5 and 55 °C, with Mn^{2+} addition as the enzyme cofactor. The kinetic parameters V_{max} and K_m had values of 28.01 mM/min and 110 mM, respectively. In fact, the purified DPEase was more stable than the crude enzyme at both tested temperature (4 °C and -20 °C) with a decrease of around 20% in enzyme activity after storage for 30 days, with additional reduced activity during longer storage. The efficient conversion rate of purified DPEase from fructose as the substrate into D-psicose was negatively correlated with the substrate concentration because of substrate inhibition. In contrast, the D-psicose concentration was positively correlated with the fructose concentration. Notably, the mixed syrup of D-fructose -D-psicose was nontoxic to rats. The LD50 value of the mixed syrup was greater than 5,000 mg/kg body weight. All the results could be useful in the development of guidelines for further research into both DPEase enzyme and D-psicose production, including purification on the pilot scale and the industrial scale.

In summary, this study has provided important insights into the production and purification of recombinant DPEase from *A. tumefaciens* C58, highlighting its potential for large-scale enzyme production and diverse biocatalytic applications, particularly in the synthesis of D-psicose, a low-calorie sweetener. These findings should not only advance understanding of low-calorie sweetener production but also should underscore the feasibility of scaling up DPEase production for industrial applications. Ongoing research is required on DPEase and D-psicose production through the development of enzyme immobilization technology or other advanced technology to achieve the highest efficiency of production and to underpin economic investment on an industrial scale.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02487-x.

Additional file1: Table S1 Protein concentration of recombinant DPEase expression of E. coli Psicose samples. Table S2 Protein concentration of recombinant Psicose samples after supplementation of Mn2+ at various stages.

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Author contributions
BN and NW designed the conceptualization and methodology. All authors carried out the experiments and draft the manuscript. BN and NW reviewed and revised the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article [and its additional files].

Declarations

Ethics approval and consent to participate
The certificate of approval for animal experiments was obtained from the Experimental Animal Ethics Committee of For Use of Laboratory Animal at Laboratory Animal Center, Chiang Mai University. The title of project was "Acute toxicity test of D-psicose syrup in laboratory animals" under protocol number: 2567/RT-0002.

Consent for publications
Approved by all named authors.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Chatuchak, Bangkok 10900, Thailand. [2]Enzyme Technology Research Team, National Center of Genetic Engineering and Biotechnology (BIOTEC), Pathum Thani 12120, Thailand. [3]Center of Veterinary Medical Diagnostic and Animal Health Innovation, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand. [4]Center for Advanced Therapeutics, Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom 73170, Thailand. [5]Sugars and Derivatives Analytical Laboratory, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.

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