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Characterization of a novel D-arabinose isomerase from *Thermanaeromonas toyohensis* and its application for the production of D-ribulose and L-fuculose

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ABSTRACT

D-Ribulose and L-fuculose are potentially valuable rare sugars useful for anticancer and antiviral drugs in the agriculture and medicine industries. These rare sugars are usually produced by chemical methods, which are generally expensive, complicated and do not meet the increasing demands. Furthermore, the isomerization of D-arabinose and L-fucose byDD-arabinose and L-fucose by D-arabinose isomerase from bacterial sources for the production of D-ribulose and L-fuculose have not yet become industrial due to the shortage of biocatalysts, resulting in poor yield and high cost of production. In this study, a thermostable D-ribulose- and L-fuculose producing D-arabinose isomerase from the bacterium *Thermanaeromonas toyohensis* was characterized. The recombinant D-arabinose isomerase from *T. toyohensis* (Thto-DaIase) was purified with a single band at 66 kDa using His-trap affinity chromatography. The native enzyme existed as a homotetramer with a molecular weight of 310 kDa, and the specific activities for both D-arabinose and L-fucose were observed to be 98.08 and 85.52 U mg^{-1}, respectively. The thermostable recombinant Thto-DaIase was activated when 1 mM Mn^{2+} was added to the reactions at an optimum pH of 9.0 at 75 °C and showed approximately 50% activity for both D-arabinose and L-fucose at 75 °C after 10 h. The Michaelis-Menten constant (K_m), the turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) for D-arabinose/L-fucose were 111/81.24 mM, 18,466/10,688 min^{-1}, and 166/132 mM^{-1} min^{-1}, respectively. When the reaction reached to equilibrium, the conversion rates of D-ribulose from D-arabinose and L-fuculose from L-fucose were almost 27% (21 g L^{-1}) and 24.88% (19.92 g L^{-1}) from 80 g L^{-1} of D-arabinose and L-fucose, respectively.

1. Introduction

Recently, rare sugars have fascinated the attention of researchers in terms of their uses and numerous purposes. The enzyme D-arabinose isomerase (D-AIase; EC 5.3.1.3), known as D-arabinose aldo-ketose isomerase, is very useful for producing D-ribulose from D-arabinose and L-fuculose from L-fucose (Fig. 1). D-Ribulose and L-fuculose are applicable in a variety of applications in different fields, but the production of these rare sugars requires a chemical method that is time-consuming and laborious. D-Ribulose is a rare aldopentose that plays a crucial role in antineoplastic, antiviral and antitumoral activities [6], while L-fuculose is one of the most important rare sugars and has been applied for a variety of purposes in pharmaceutical industrial molecules and materials. This rare sugar has been used to combat different kinds of diseases such as hepatitis B, HIV, cardioprotective, antiviral and anticancer drugs. Hence, our focus was to extract rare sugars such as D-ribulose and L-fuculose by using a simple method from economical and easily available sources [7].

According to the BRENDA enzyme database, D-arabinose isomerase (D-AIase) is usually produced by *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*) and recent studies also showed that D-arabinose could be metabolized by *Salmonella typhimurium* (*S. typhimurium*). The pH range for *K. pneumoniae* is 7.0–10, and manganese (Mn^{2+}) metal is applied to enhance the enzyme activity [8]. *Thermanaeromonas toyohensis* (Thto) is a strictly anaerobic, gram-positive, thermophilic and thiosulfate-reducing bacterium that was screened from a geothermal aquifer in the Toyoha Mines in Japan at a depth of 550 m, which was used for this research to produce D-AIase [9]. D-Xylose isomerase and L-arabinose isomerase have been studied in different kinds of microorganisms, such as numerous thermophiles, mesophiles

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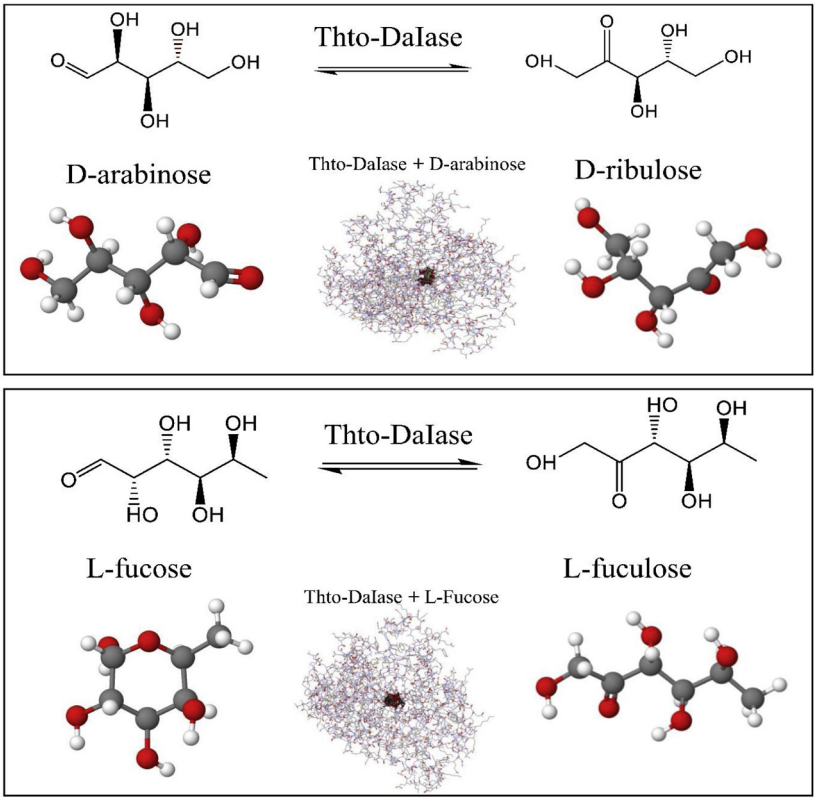

Fig. 1. Structural illustration for enzymatic production of D-ribulose and L-fuculose from D-arabinose and L-fucose, respectively, with the help of recombinant Thto-DaIase.

and hyperthermophiles [10,11]. Only three kinds of bacterial strains for D-AIase have been examined until now, such as *Aerobacter aerogenes* (*A. aerogenes* also known as *K. aerogenes*), *K. pneumoniae* and Bacillus pallidus (*B. pallidus*) [6]. Although there have only been a few bacterial strains used to characterize D-arabinose isomerase [12], *T. toyohensis* was reported for the first time as a strictly thermostable bacterial strain encoding a D-arabinose isomerase named Thto-DaIase.

The thermostable enzyme has a number of benefits due to its ability to withstand high temperatures, resistant to chemical and biochemical denaturation, increased reaction velocities, increased substrate solubility at high temperature, reduced risk of contamination and reduced number of undesirable byproducts during reactions [13]. The Thto-DaIase used in the catalytic reaction to isomerize aldose to ketose also promotes the formation of D-allulose from D-altrose (rare sugars), which is naturally present in very small amounts, *via* opening the ring of the substrate. Rare sugars have many advantages in the pharmaceutical, food, and agriculture industries and have various physiological functions. D-AIase, used against fucosidosis (human lysosomal disease), is affected by glycolipid and glycoprotein degradation. There are 36 kinds of hexoses and pentoses; among the 36 (24 aldoses and 12 ketoses), only seven are present in nature, while all others are called rare sugars [14]. Currently, new strategies have been completely developed for the bioproduction of rare sugars; so, D-AIase is considered to have much potential for industrial application. Earlier reports showed that D-psicose/D-allulose, D-ribulose, L-fuculose, L-mannose, D-talitol, L-ribose are rare sugars produced by enzymatic methods [15,16].

In this study, a highly thermostable D-arabinose isomerase from the novel thermophilic bacterium *T. toyohensis* (Thto-DaIase) was cloned and overexpressed in *E. coli*. The biochemical properties (molecular mass, pH, optimum temperature, metal ions and thermostability) were examined. Kinetic parameter analysis, molecular modeling and sequence similarity were identified by amino acid sequence alignment.

2. Materials and methods

2.1. Materials and chemicals

The resin column used to purify overexpressed *T. toyohensis* D-arabinose isomerase from recombinant *E. coli*, a fast flow chelating sepharose column, was obtained from GE Healthcare (Uppsala, Sweden). The isopropyl-β-D-1-thiogalactopyranoside (IPTG), D-arabinose, L-fucose and all other chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) and Bio-Rad (Hercules, CA, USA). The plasmid was synthesized and constructed by Sangon Biological Engineering Technology and Services (Shanghai, China). The *E. coli* strain of DH5α (Invitrogen, Carlsbad, CA, USA) was used in this study for cloning and propagating the plasmid.

2.2. Gene cloning and expression

The putative enzyme was obtained from the NCBI-BLAST tool (public database), and the protein sequence was acquired from an earlier deposited protein in GenBank (WP_084665866.1). ESPript software was used to generate the protein sequence alignment. According to NCBI, the complete genome of *T. toyohensis* was discovered, sequenced, and then published in GenBank (NCBI) with the reference sequence or accession No. WP_084665866.1. The sequence has different site interfaces: trimer interfaces for polypeptide binding, substrate binding sites for chemical binding and Mn^{2+} binding sites for ion binding. The DNA of the target gene and encoded protein of *T. toyohensis* (protein ID No. WP_084665866.1) was constructed by a commercial synthesis company (Generay Biotech Co., Ltd, Shanghai, China) and cloned into vector pET-22b (+) (Novagen, Darmstadt, Germany) with *Nde*I and *Xho*I sites to construct an in-frame fusion with a 6 × His-tag sequence at the C-terminus [17,18]. The resulting plasmid was Thto-DaIase in pET-22b (+), and D-AIase was transformed and

overexpressed in *E. coli* BL21. The cells harboring Thto-DaIase were cultivated in Luria–Brentani (LB) medium with ampicillin at a final concentration of 100 μg mL^{-1} and incubated at 37 °C until the optical density (OD_{600}) reached 0.6-0.8. IPTG (1 mM L^{-1}) was added for induction, and after 6–10 h, D-AIase expressing cells were grown at 28 °C with shaking at 200 rpm.

2.3. Protein purification and molecular mass determination of recombinant Thto-DaIase

The purification steps of D-arabinose isomerase from *T. toyohensis* (Thto-DaIase) were executed at 4 °C. The recombinant protein-expressing cells were collected from the broth culture by centrifugation at 10,000 × g for 15 min at 4 °C, and then these cells were resuspended in lysis buffer (50 mM Tris − HCl buffer, 100 mM NaCl) at pH 7.0. After mixing, the cell disruption was performed at 4 °C by using an ultrasonication Vibra-Cell 72,405 Sonicator (Bio-Block Scientific, Illkirch, France). The denatured and unlysed cells were discarded by centrifugation (10,000 × g, 20 min, 4 °C), and the supernatant was used as a crude extract of Thto-DaIase. The crude enzyme was applied to a resin column (chelating sepharose fast flow 1 cm × 10 cm) charged with Ni^{2+} followed by binding buffer (50 mM Tris − HCl buffer, 500 mM NaCl, pH 7.0), a process called nickel-affinity chromatography (Novagen). The bound nondenatured protein was detached using a washing buffer (50 mM Tris − HCl buffer, 50 mM imidazole, 500 mM NaCl, pH 7.0). The enzyme was eluted with elution buffer (50 mM Tris − HCl buffer, 500 mM imidazole, 500 mM NaCl, pH 7.0), and the solution containing purified enzyme was dialyzed overnight at 4 °C against dialysis buffers A (50 mM Tris − HCl buffer, 10 mM EDTA, pH 7.0) and B (50 mM Tris buffer, pH 7.0).

The instinctive molecular mass for Thto-DaIase was determined by analyzing the purified enzyme with high-performance liquid chromatography (HPLC, TSK G3000SW) column (Tosoh. Co., Ltd, Tokyo, Japan). By using differential refractometer (Optilab T-rEX, CA, USA) with a light-scattering detector (Dawn Heleos II, CA, USA) and a UV detector (Waters 2489, USA), the purified enzyme was eluted in 50 mM Tris − HCl buffer (pH 7.0) containing 300 mM NaCl with a flow rate of 1 mL min^{-1}. The subunit molecular mass of purified Thto-DaIase was calculated by SDS-PAGE (5% stacking gels and 12% resolving gels) under denaturing conditions using the prestained ladder as a reference protein (MBI Ferments, Glen Burnie, MD, USA). For the staining of protein bands, Coomassie Brilliant Blue 250 was used, and the destaining solution was used for visualization.

2.4. Determination of protein concentration and enzyme assay

The protein concentrations of Thto-DaIase were analysed by the Bradford method using bovine serum albumin (BSA) as a standard protein [19]. The activity of Thto-DaIase was determined by calculating the amount of D-ribulose from D-arabinose and L-fuculose from L-fucose. Experiments were performed on a high-performance liquid chromatography (HPLC) system (1200 Series; Agilent Technologies, Santa Clara, CA, USA) linked with an infrared (IR) detector (Shodex, RI-101), and the column was a SUGAR-PAK (6.5 × 300 mm) at 85 °C; the mobile phase was double distilled water at a 0.4 mL min^{-1} flow rate. D-Arabinose and L-fucose (substrates) were used to examine the activity of Thto-DaIase, and the substrates were transformed into D-ribulose and L-fuculose, respectively. The reaction was continued for 10 min at 75 °C in 50 mM glycine buffer, pH 9.0, with D-arabinose and L-fucose and purified Thto-DaIase. "The one unit of enzyme activity (U) was defined as the amount of enzyme required to increase 1 μ mol of D-ribulose/L-fuculose min^{-1} at 75 °C and pH 9.0".

2.5. Effects of pH and temperature on the activity of recombinant Thto-DaIase

The enzyme activity was determined by using a variety of buffer solutions with various pH ranges (5–10.5), and the optimal pH values were detected. Three different buffers with different pH were used: sodium phosphate buffer (50 mM, pH 5.0–7.0), Tris − HCl (50 mM, pH 7.0–9.0) and glycine-NaOH (50 mM, pH 9.0–10.5). The pH stability was tested by incubating the enzyme in various pH ranges (5.0–10.5) at 4 °C and 75 °C for 24 and 1 h, respectively. The optimal temperature for the enzyme activity with glycine-NaOH buffer at pH 9.0 was evaluated at 35–95 °C. The thermostability of Thto-DaIase was studied by incubating the enzyme in 50 mM glycine-NaOH buffer at pH 9.0 and various temperatures 45, 55, 65, 75, 85 and 95 °C within specific intervals of times. The effects of thermostability on Thto-DaIase were determined by log k (k: rate constant of thermal denaturation) and 1/T with an Arrhenius plot using the $k = 0.693/t_{1/2}$ equation.

2.6. Effects of metal ions on recombinant Thto-DaIase activity

As previously described, reactions were achieved in 50 mM glycine buffer (pH 9.0) comprising substrates (D-arabinose/L-fucose) and recombinant enzyme (Thto-DaIase) for 10 min at 75 °C. To inspect the influence of metal ions on the activity of Thto-DaIase, the recombinant enzyme was tested after treatment with EDTA and individual metal ions, including Ca^{2+}, Ni^{2+}, Mn^{2+}, Mg^{2+}, Ba^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+} at concentrations of 1 mM. The measured activities in the presence of metals were compared with the enzyme activity without the addition of a metal ion, which was used as a control. The effect of Mn^{2+} concentration for both D-arabinose and L-fucose was determined by incubating Thto-DaIase with different concentrations (1–5 mM) of Mn^{2+} at pH 9.0 at 75 °C for 10 min.

2.7. Homology modeling and amino acid sequence comparisons of recombinant Thto-DaIase

The homology structural and substrate docking of recombinant Thto-DaIase was designed by using the SWISS-MODEL server (https://swissmodel.expasy.org/), and the structure of Thto-DaIase was determined experimentally by using a template of *A. pallidus* (PDB ID: 3A9T). The secondary structure of PDB ID: 3A9T was designed by ESPript server (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) [4]. To verify and validate the structure, the verification server (http://services.mbi.ucla.edu/SAVES/) and structure analysis were used, while Discovery-studio were used to visualize and access the 3-dimensional structure.

To further analyze the amino acid sequence of Thto-DaIase, the sequence alignment tool ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to compare D-AIases from previously reported bacteria. A phylogenetic relationship of recombinant Thto-DaIase and previously identified bacteria with known accession numbers was created with MEGA-X using the neighbor-joining method. The scale of the tree indicates the branch length of the same unit, which is used to determine evolutionary distances based on phylogenetic trees formed by the Poisson correction method, and the branch length (optimum sum) of the tree was 0.85878910. The phylogenetic tree analysis involved 6 amino acid sequences [1–3].

The number of amino acids, isoelectric point, molecular weight, aliphatic index, instability index, estimated half-life and grand average of hydropathicity (GRAVY) were assessed by ExPASy-Compute pI/M_w tools at https://web.expasy.org/cgi-bin/protparam/protparam [20].

2.8. Specific activities, substrate specificities and kinetic parameters of recombinant Thto-DaIase

Different substrates were used to analyze the specific activities and

specificities of Thto-DaIase; D-arabinose, L-fucose, D-altrose, L-xylose, L-galactose, D-galactose and L-arabinose were used to a final concentration of 50 mM.

Different concentrations of D-arabinose, L-fucose, and L-xylose (10–300 mM) were applied to calculate the kinetics of recombinant Thto-DaIase. The reactions were executed in 50 mM glycine-NaOH (pH 9.0) at 75 °C for 10 min. The kinetic parameters, K_m (mM) and k_{cat} (min^{-1}), were calculated using the Michaelis-Menten equation by a nonlinear-regression method using GraphPad Prism software analysis.

2.9. Enzymatic production of D-ribulose and L-fuculose

The products D-ribulose and L-fuculose from D-arabinose and L-fucose, respectively, by recombinant Thto-DaIase, were examined by using HPLC with a sugar column (Waters SUGAR-PAK 1, Waters Corp., Milford, MA, USA). The mobile phase used to detect the sugars was deionized water with an 85 °C column temperature and a flow rate of 0.4 mL min^{-1} with the help of an attached refractive index detector. The reaction mixture was prepared for the biological synthesis of D-ribulose and L-fuculose in 50 mM glycine-NaOH, pH 9.0 at 75 °C with 1 mM Mn^{2+}, and three different concentrations were used: 30, 50, 80 g L^{-1} D-arabinose and 36, 50 and 80 g L^{-1} L-fucose.

3. Results and discussion

3.1. Homology and amino acid sequence comparison of putative Thto-DaIase among other D-AIases

The hypothetical protein from *T. toyohensis* has 600 amino acids and a predicted isoelectric point and molecular weight of 5.5 and 65,800.97 Da, respectively. The derived sequenced amino acids of the hypothetical protein from *T. toyohensis* (WP_084665866.1) showed 74.62, 71.67 and 69.32% identities with D-AIases from *A. pallidus* (WP_063389244.1), *Dictyoglomus turgidum* (*D. turgidum*) DSM6724 (YP_002352328.1) and *E. coli* W (ADT76410.1), respectively (Table 1). Thto-DaIase showed an aliphatic index of 84.08, which is higher than 82.82, 78.16 and 76.55 of *D. turgidum*, *E. coli* W and *A. pallidus*, respectively. The instability index computed by ExPASy-Compute pI/M_w tools was 33.30, which is less than 40, classifying the protein as stable, and was lower than other comparable bacterial strains, such as *D. turgidum* and *E. coli* W, which had instability indexes of 33.97 and 37.46, respectively, and higher than that of *A. pallidus* at 29.18 (Table 1). The estimated half-lives presented in Table 1 were 30 h, 20 h, and 10 h, which corresponded to *in vivo* for yeast, *in vitro* for mammalian reticulocytes, and *in vivo* for *E. coli*, respectively. The half-life values were similar to *D. turgidum*, *E. coli* W and *A. pallidus* strains. The GRAVY was −0.183, which was closer to positive than those of *D. turgidum* and *E. coli* W, which were -0.253 and -0.256, respectively (Table 1) [20].

The sequence alignment of Thto-DaIase with theD-AIases protein sequences from previously characterized D-AIases-producing thermostable bacteria is given in Fig. 2. The secondary structure was designed from the previously reported 3-dimensional structure of *A. pallidus* (PDB ID: 3A9T) by the ESPript server [4]. The multiple sequence alignment from *T. toyohensis* (GenBank: WP_084665866.1) compared with *A. pallidus* (GenBank: WP_063389244.1), *D. turgidum* DSM6724 (GenBank: YP_002352328.1), and *E. coli* W (GenBank: ADT76410.1), showed that all the sequences have more than 65% similarity. The red background showed the residues that were strictly conserved, the red type in blue boxes revealed highly conserved residues, and elements for the secondary structure were reported by comparing Thto-DaIase with the previously discovered structure of D-AIase from *A. pallidus* with the PDB ID: 3A9T, shown as star symbols on the residues (Fig. 2).

The phylogenetic relationship among different D-AIase-producing bacteria, which was previously characterized, showed that *T. toyohensis* producing D-arabinose isomerase (Thto-DaIase) has a closer relationship with *Caldicellulosiruptor saccharolyticus* (*C. saccharolyticus*) and *D.*

Table 1
Comparison between amino sequences of Thto-DaIase with other thermophilic microorganisms.

Source	Accession No.	No. of residues	Identity (%)				Theoretical pI/M_w	Estimated half-life		Instability index	Aliphatic index	(GRAVY)
			1	2	3	4		Mammalian reticulocyte *in-vitro* (h)	Yeast, *in vivo* (h)	*E. coli in vivo* (h)			
T. toyohensis	WP_084665866.1	600	100	71.67	69.32	74.62	5.50/65,800.97	30	20	10	33.30	84.08	−0.183
D. turgidum DSM6724	YP_002352328.1	606	71.67	100	62.03	72.27	5.69/68363.48	30	20	10	33.97	82.82	−0.253
E. coli W	ADT76410.1	591	69.32	62.03	100	63.67	5.59/64958.64	30	20	10	37.46	78.16	−0.256
A. pallidus	WP_063389244.1	595	74.62	72.27	63.67	100	5.23/66237.25	30	20	10	29.18	76.55	−0.349

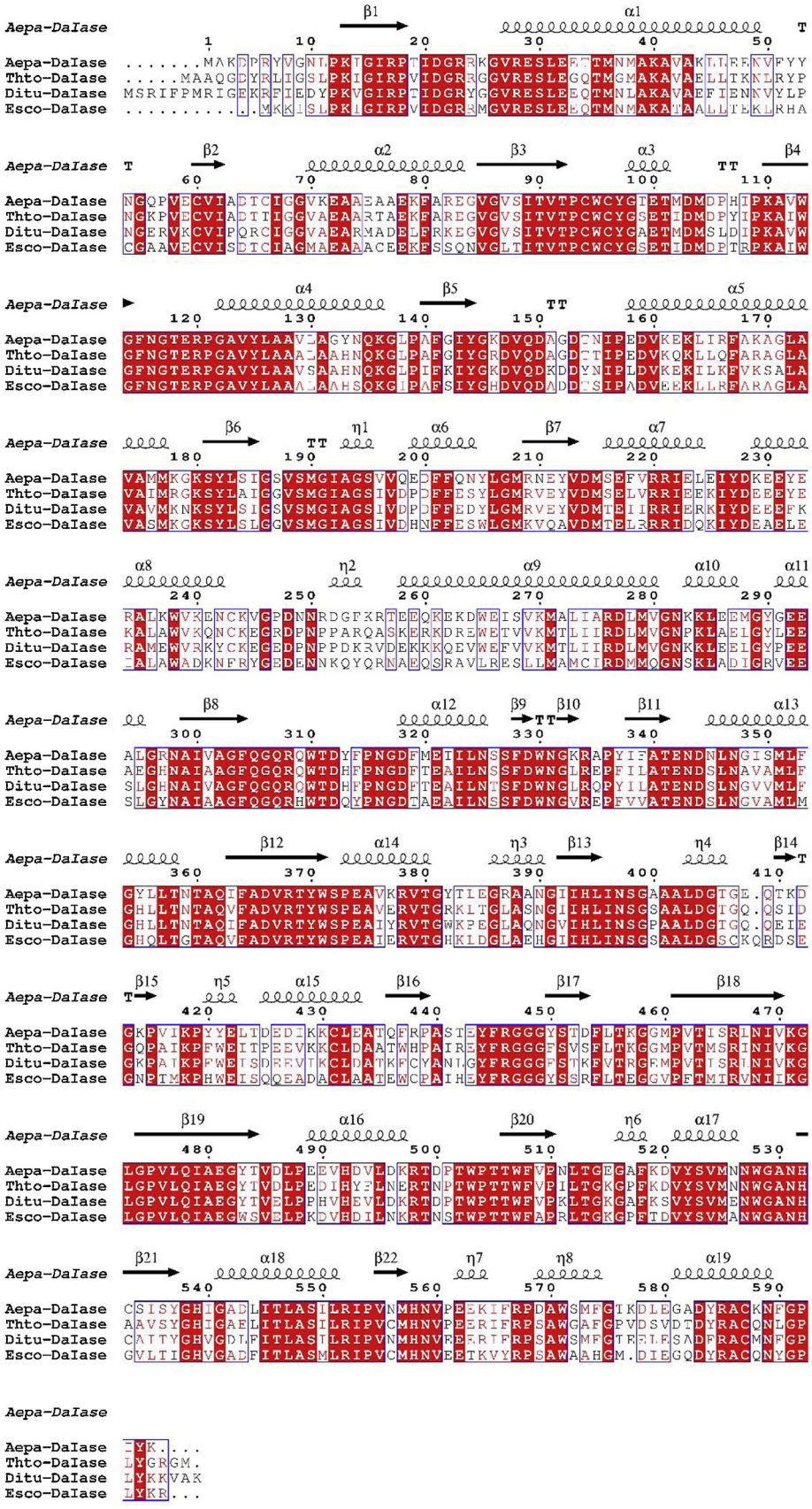

Fig. 2. Amino acid alignment of recombinant Thto-DaIase. The GenBank accession No. of the D-arabinose isomerases were Aepa-DaIase: A. pallidus (WP_063389244.1); Thto-DaIase: T. toyohensis (WP_084665866.1); Ditu-DaIase: D. turgidum DSM 6724 (YP_002352328.1); Esco-DaIase: E. coli W (ADT76410.1). The red background represents strictly conserved residues and the red type in blue boxes represent highly conserved residues. The star symbols above residues indicate secondary structure elements by the template A. pallidus with PDB ID: 3A9T [4]. ESPript was used to perform the alignment [5]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

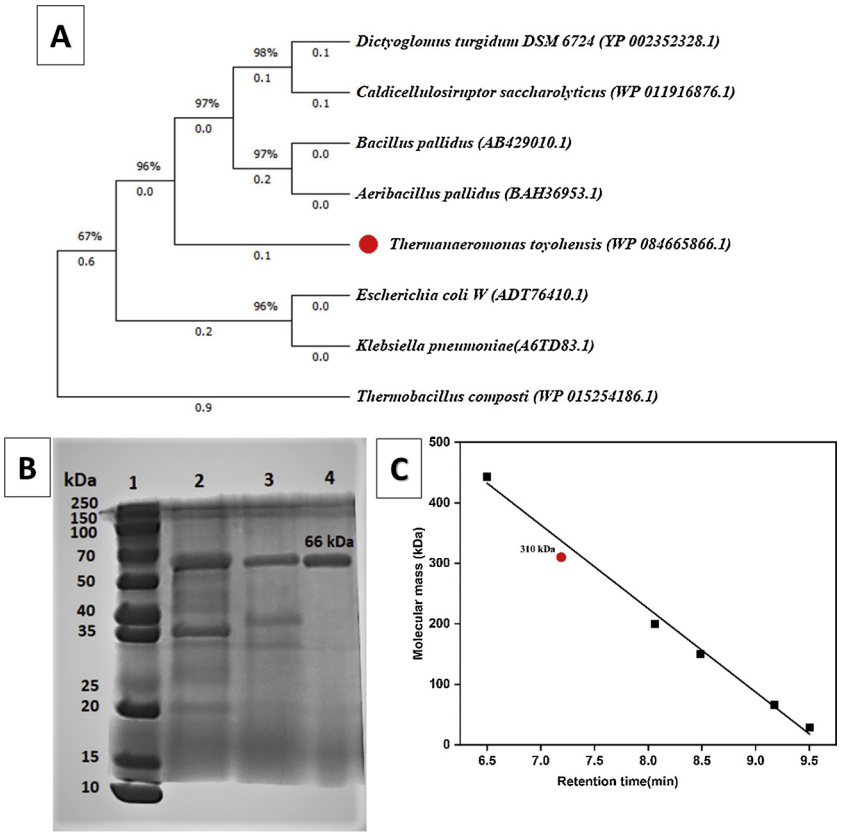

Fig. 3. (A) The phylogenetic relationship of recombinant Thto-DaIase with previously identified bacteria with known accession numbers. The tree was generated with MEGA-X by the neighbour-joining method. The branching point numbers show bootstrap values with genetic distance values on the substitution position [1–3]. (B) SDS-PAGE examination of purified Thto-DaIase overexpressed in E. coli BL21; lane 1 is protein marker, lane 2 indicates crude extract, lane 3 is a heat-treated enzyme and lane 4 is the purified His-Trap column product. (C) The native molecular mass of recombinant Thto-DaIase determined by HPLC using the reference standards apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa). The red circle at 310 kDa shows the native molecular mass of recombinant Thto-DaIase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

turgidum (approximately 98%), therefore *T. toyohensis* was used to characterize L-fucose isomerase (L-FIase) and D-AIase. The sequence similarity between *C. saccharolyticus* and *D. turgidum* was more than 70%, which provided evidence that their ancestors had a close relationship among themselves. The phylogenetic tree revealed that *T. toyohensis* also has similar phylogeny to *B. pallidus*, *E. coli* W, *K. pneumoniae* and *A. pallidus* (Fig. 3A).

3.2. Overexpression and purification of the recombinant Thto-DaIase

D-Arabinose isomerase from *T. toyohensis,* a putative gene with the identical sequence as that from GenBank accession No. WP_084665866.1 was emulated and expressed in *E. coli.* The enzyme showed activity for both substrates D-arabinose andL-fucose. The uncharacterized Thto-DaIase was purified by solubilizing the protein with heat treatment and affinity chromatography, obtaining a final purification of 32-fold, 98.08 U mg^{-1} specific activity and 44% yield for D-arabinose, and 35-fold, 85.52 U mg^{-1} specific activity and 48% yield for L-fucose (Table 2). The yield of active recombinant Thto-DaIase in *E. coli* from cell culture was 2960 U L^{-1} for D-arabinose and 2360 U L^{-1} for L-fucose (Table 4), which had not been previously described. The specific activity of Thto-DaIase was higher from the previously reported specific activities of 93 and 76 U mg^{-1} for *D. turgidum* and *C. saccharolyticus*, respectively [13,21]. The protein obtained on each purification step was used to evaluate its subunit molecular mass by SDS-PAGE, which displayed a single band near 66 kDa (Fig. 3B). This value was larger than the theoretical calculated molecular weight (65,800.97 Da) of recombinant Thto-DaIase with 600 amino acid residues and a His-tag on its C-terminus that was obtained by using ExPASy-Compute pI/M_w tools. The molecular mass of recombinant Thto-DaIase was measured by HPLC with a single peak at approximately 7.19 min in the elusion profile. Fig. 3C shows that the molecular mass of Thto-DaIase measured under nondenaturing circumstances was approximately 310 kDa by comparing Thto-DaIase with standard curves of different reference proteins, such as carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa) and apoferritin (443 kDa), and native enzyme corresponding to 310 kDa. While the theoretical molecular mass of one unit of recombinant Thto-DaIase is approximately 66 kDa, the native molecular mass of the enzyme was calculated to be 310 kDa, corresponding to four subunits. These results specify that recombinant Thto-DaIase is a homotetramer that is similar to the previously characterized D-arabinose from *B. pallidus* [22].

Table 2
Purification of Thto-DaIase.

Substrates	Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U mg^{-1})	Yield (%)	Purification Folds
D-Arabinose	Crude Extract	290	887	3.06	100	1
	Heat-Treatment	21.7	563	25.98	63	8
	His-Trap	4.0	392	98.08	44	32
L-fucose	Crude Extract	290	707	2.44	100	1
	Heat-Treatment	21.7	490	22.60	69	9
	His-Trap	4.00	342	85.52	48	35

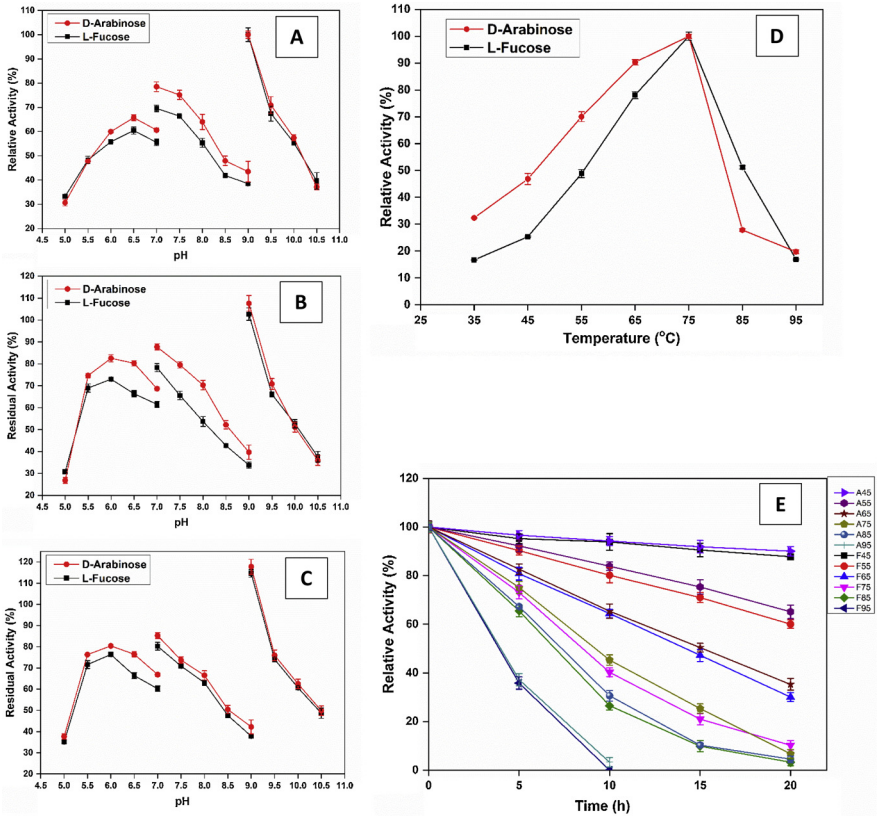

Fig. 4. Effect of pH, temperature and thermostability on the activity of recombinant Thto-DaIase. (A) Effect of pH; three different types of buffers at 50 mM [phosphate (pH 5.0–7.0), Tris − HCl (pH 7.0–9.0) and glycine (pH 9.0–10.5)] were used to determine the optimum pH of recombinant Thto-DaIase for D-arabinose and L-fucose. (B) pH stability of recombinant Thto-DaIase for D-arabinose and L-fucose; enzyme was incubated at different pH values (5.0–10.5) at 4 °C for 24 h to evaluate the residual activities. (C) Residual activities of recombinant Thto-DaIase for D-arabinose and L-fucose incubated at different pH values (5.0–10.5) at 75 °C for 1 h. (D) Effect of temperature on the activity of recombinant Thto-DaIase for D-arabinose and L-fucose; 50 mM glycine-NaOH with pH 9.0 was used. (E) Effect of the thermostability of recombinant Thto-DaIase for D-arabinose and L-fucose; different time intervals 0–20 h and temperatures 45, 55, 65, 75, 85 and 95 °C were used to incubate the enzyme. Data represent the mean of three replicates.

3.3. Influences of pH, temperature, and thermostability on the activity and stability of recombinant Thto-DaIase

The influence of pH, temperature, and thermostability were measured and are demonstrated in Fig. 4. The activity of recombinant Thto-DaIase was measured by using three different kinds of buffers: sodium phosphate (pH 5.0–7.0), Tris − HCl (pH 7.0–9.0) and glycine-NaOH (pH 9.0–10.5). The variation in pH values in the reaction mixture had significant effects on the dissociation state of the substrate and enzyme complex that changed the enzyme activity. Thto-DaIase exhibited optimum activity at pH 9.0 in the glycine-NaOH buffer for D-arabinose and L-fucose (Fig. 4A), which was similar to D-AIase from *K. pneumoniae* and D-AIase from *B. pallidus* [6,12]. The enzyme exhibited minimum activity at pH 5.0 in sodium phosphate for both D-arabinose and L-fucose, while at pH 7.0, both substrates retained more than 50% activity, which was increased by increasing the pH values. In Tris − HCl buffer (pH 7.0), the enzyme retained about 70% activity for D-arabinose and L-fucose substrates, which decreased by increasing the pH up to pH 9.0 (Fig. 4A). As shown in Fig. 4A, Thto-DaIase displayed optimum catalytic activity under alkaline conditions in a glycine-NaOH buffer (pH 9.0) for both substrates D-arabinose and L-fucose, which was decreased by increasing the pH from 9.0-10.5. The enzyme stability at different pH values was assessed by incubating purified enzyme at different pH values (5.0–10.5) at 4 °C for 24 h (Fig. 4B). The enzyme fully retained its activity from pH 5.0–10.5 in sodium phosphate buffer, Tris − HCl and glycine-NaOH buffers, which revealed no significant effect on activity, while at pH 9.0, the residual activity was increased for both substrates. The enzyme was also incubated at 75 °C in different pH values for 1 h to measure the thermal effects of pH on Thto-DaIase stability. The enzyme fully retained its activity at all pH values and showed the highest activities of at pH 9.0 for D-arabinose and L-fucose (Fig. 4C). The effects of temperature on Thto-DaIase activity were assessed from 35 to 95 °C for D-arabinose and L-fucose (Fig. 4D). The optimum activity was observed at 75 °C for both substrates, although in the case of D-arabinose, Thto-DaIase exhibited 90% activity at 65 °C and 70% activity at 55 °C, which demonstrated that Thto-DaIase is highly thermostable. The effect of temperature on Thto-DaIase for the L-fucose substrate was measured to be 50% activity at 55 °C and 80% at 65 °C (Fig. 4D). The results were closely related to the findings of Ju and Oh (2010), as they found that the optimum temperature for *C. saccharolyticus* D-AIase, which isomerizes D-arabinose and L-fucose, was 75 °C.

Thto-DaIase was further tested at different temperatures (45, 55, 65, 75, 85 and 95 °C), and the activity was observed from 0, 5, 10, 15 and 20 h (Fig. 4E). The results showed that Thto-DaIase was more than 60% active and stable with D-arabinose and L-fucose at 45, 55, 65, 75 and 85 °C for 5 h, while at 95 °C, it showed sudden inactivation and decreased the activity. After 10 h, the enzyme showed more than 60% activity at 45, 55 and 65 °C, which is a property of thermostable enzymes. Moreover, the activity reduced when the temperature was increased to 75, 85, and 95 °C. By increasing time and temperature, the activity of Thto-DaIase with D-arabinose and L-fucose decreased; after 20 h, it retained approximately 40% activity at 65 °C (Fig. 4E). The half-life ($t_{1/2}$) of Thto-DaIase for D-arabinose, based on first-order kinetics, was measured as 133, 32, 13, 5, 4 and 2 h at 45, 55, 65, 75, 85 and 95 °C, respectively. The present results revealed that recombinant Thto-DaIase was more stable than the enzyme from *D. turgidum*, which exhibited half-lives of 20, 12, 7, 5 and 2 h at 65, 70, 75, 80 and 85 °C, respectively [13]. In the case of the L-fucose substrate, Thto-DaIase exhibited 111, 27, 11, 5, 4 and 3 h half-lives at 45, 55, 65, 75, 85 and 95 °C, respectively. Ju and Oh (2010) reported that the half-life values of L-fucose isomerase (L-FIase) from *C. saccharolyticus* at 60, 65, 75 and 80 °C were 62, 13, 6, 2 and 1 h, respectively, which were lower than those of the present findings; therefore, the present findings are good for industrial applications.

3.4. Effects of metal ions on recombinant Thto-DaIase activity

It was necessary to remove metal ions from the purified enzyme

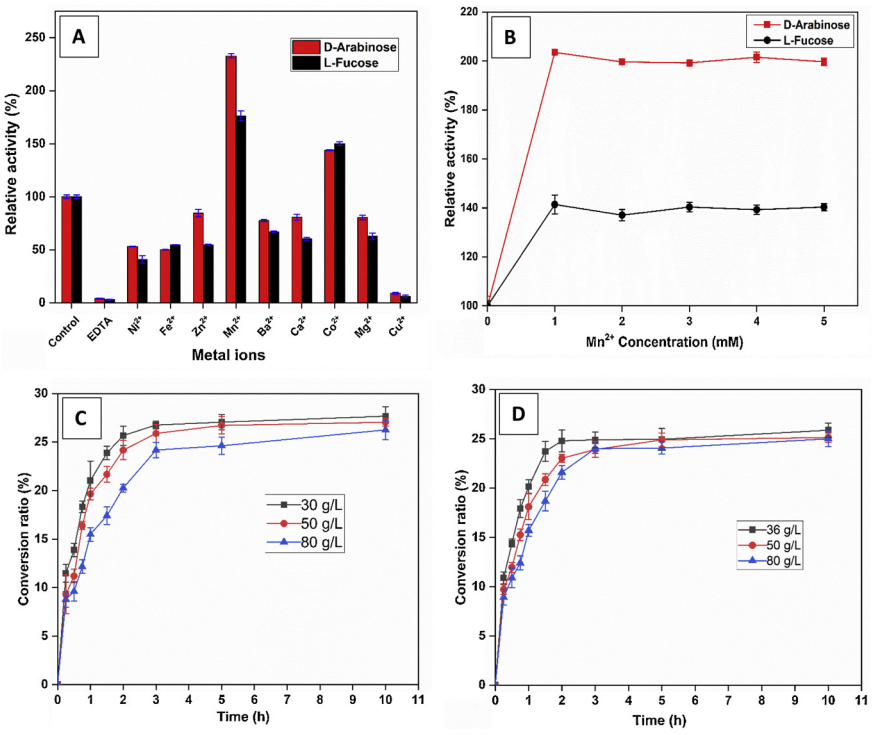

Fig. 5. Effects of metal ions, the concentration of metal ions, and production of D-ribulose/L-fuculose from recombinant Thto-DaIase (A) Influence of metal ions on the activity of recombinant Thto-DaIase for D-arabinose and L-fucose; reactions were performed in 50 mM of glycine buffer at 75 °C for 10 min. The control showed a purified enzyme without metal. (B) Effect of Mn^{2+} concentration on the activity of recombinant Thto-DaIase for D-arabinose and L-fucose. (C) D-ribulose production from D-arabinose by recombinant Thto-DaIase at different concentrations (30, 50 and 80 g L^{-1}) of D-arabinose. (D) L-Fuculose production from L-fucose by recombinant Thto-DaIase at different concentrations (36, 50 and 80 g L^{-1}) of L-fucose.

with EDTA to attain the accurate effects of metals on enzyme activity and subsequently dialyze against Tris−HCl buffer (pH 7.5) to remove the effect of EDTA. Few metal ions Ca^{2+} and Mg^{2+} are already involved in enzyme purification after affinity chromatography, which forces Mn^{2+} to bind to the binding site of the enzyme molecule. D-Arabinose isomerase is a metalloenzyme in which divalent metal ions act as a cofactor for the isomerization of rare sugars [21]. The recombinant Thto-DaIase was incubated with various divalent metal ions (EDTA, Ni^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+}, Mg^{2+}, and Cu^{2+}) at concentrations of 1 mM (Fig. 5A). The results demonstrated that manganese (Mn^{2+}) increased the activity of Thto-DaIase by 133% and 76% for D-arabinose and L-fucose, respectively, with respect to the control (100%), while Ni^{2+}, Fe^{2+}, Zn^{2+}, Ba^{2+}, Ca^{2+}, Mg^{2+}, and Cu^{2+} decreased the activity of Thto-DaIase (Fig. 5A). Copper (Cu^{2+}) and EDTA strongly declined the activity of the enzyme for both substrates. However, Mn^{2+} enhanced the activity of Thto-DaIase significantly, which corroborated that this enzyme is more dependent on Mn^{2+} than the other metals tested to increase its activity. Co^{2+} also increased the enzyme activity by approximately 43% and 50% compared with the control for D-arabinose and L-fucose respectively, which was highly dependent after Mn^{2+} (Fig. 5A). A previous study showed that Mn^{2+} and Co^{2+} metal ions were highly effective for L-FIase from *D. turgidum*, which promotes the isomerizing reactions of L-fucose and D-arabinose and is highly thermostable [13]. D-Arabinose isomerase from *K. pneumoniae* and *B. pallidus* showed that this enzyme was highly dependent on Mn^{2+} to enhance the activity [12,22].

Among all other metal ions, Mn^{2+} significantly heightened the activity of Thto-DaIase; subsequently, different concentrations of Mn^{2+} (from 1 to 5 mM) were applied with D-arabinose and L-fucose (Fig. 5B). Thto-DaIase showed the highest activity at 1 mM Mn^{2+}, which was considered the optimal concentration for both substrates (Fig. 5B). Mn^{2+} is a cofactor for L-fucose and D-arabinose isomerization by L-FIase from *C. saccharolyticus* [21]. All reported D-AIases and L-FIases from *C. saccharolyticus, B. pallidus* and *E. coli* displayed Mn^{2+} metal ion was used to activate and characterize the enzyme [6,13,21].

3.5. Substrate specificity of recombinant Thto-DaIase and enzyme kinetics

Various reports are available on rare sugar production, such as D-arabinose from D-ribulose, L-galactose from D-tagatose and L-galactose from L-tagatose, that elaborate the catalytic reactions of several substrates [23–26]. The hydroxyl groups present in aldose substrates oriented in the left-handed configuration at the C2 position and in the right-handed configuration at the C3 and C4 positions, such as D-arabinose, L-fucose, D-altrose, and L-galactose. But the C5 has different configuration for all of these substrates which is inactive during the catalytic reaction. The open-chain structures of the aldose substrates responsible for the aldose-ketose isomerization reactions catalyzed by the D-arabinose isomerase. The specificity of recombinant Thto-DaIase for different monosaccharides (substrate) of D- and L- forms (pentoses

Table 3
Substrate specificity and Kinetic parameters of recombinant Thto-DaIase.

Substrate	Product	Specific Activity (U mg^{-1})	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m (mM^{-1} min^{-1})
D-Arabinose	D-ribulose	98.0 ± 0.97	111 ± 1.7	18,466 ± 27	166 ± 2.8
L-fucose	L-Fuculose	85.5 ± 1.81	81.2 ± 2.1	10,688 ± 30	132 ± 3.5
D-Altrose	D-Allulose	15.0 ± 1.50	69 ± 2.4	7328 ± 11	106 ± 2.0
L-Galactose	L-Tagatose	01 ± 0.32	ND	ND	ND
L-Xylose	L-Xylulose	01 ± 0.02	ND	ND	ND
D-Galactose	D-Tagatose	0.63 ± 0.26	ND	ND	ND
L-Arabinose	L-Ribulose	0.00 ± 0.00	ND	ND	ND

ND: not displayed.

Table 4
Comparison of biochemical properties and Kinetic parameters of recombinant Thto-DaIase with D-AIases/L-FIases.

Enzyme	Substrate	Microorganisms	Molecular weight (kDa)	Temperature (°C)	Optimum pH	Specific Activity (U mg^{-1})	Expression yield (U L^{-1} medium)	Metal ions	K_m (mM)	V_{max} (U mL^{-1})	Reference
D-arabinose isomerase	D-Arabinose	*T. toyohensis*	66	75	9.0	98	2960	Mn^{2+}	111	9.3	This study
	D-Arabinose	*K. pneumoniae*	65	40	9.3	38	NR	Mn^{2+}	51	NR	[12]
	D-Arabinose	*B. pallidus*	66	55	9.0	NR	NR	Mn^{2+}	NR	NR	[6]
	L-fucose	*T. toyohensis*	66	75	9.0	85.5	2360	Mn^{2+}	81.2	3.7	This study
L-fucose isomerase	L-fucose	*D. turgidum*	68	80	7.0	93	NR	Mn^{2+}	90	2.3	[13]
	L-fucose	*E. coli* B/r	65	37	8.0	64	NR	Co^{2+}	42	0.25	[27]
	L-fucose	*E. coli* K-2	65	37	8.0	63	NR	Co^{2+}	45	0.26	[27]
	L-fucose	*C. saccharolyticus*	68	75	7.0	76	NR	Mn^{2+}	141	0.25	[21]

NR: not reported.

and hexoses), such as D-galactose, D-altrose, L-fucose, L-galactose, D-arabinose, L-xylose, and L-arabinose, was investigated (Table 3).

The recombinant Thto-DaIase exhibited the highest activity for D-arabinose and L-fucose and moderate activity against D-altrose, whereas the lowest activity was towards L-galactose, L-xylose, and D-galactose, while no activity was observed against L-arabinose. The specific activities of recombinant Thto-DaIase for D-arabinose, L-fucose and D-altrose were 1.6-fold, 1.12-fold and 1-fold higher, respectively from *C. saccharolyticus*. The results revealed that recombinant Thto-DaIase is more efficient for the isomerization reaction of these substrates than D-AIase/L-FIase from *C. saccharolyticus* [21]. The specific activity of recombinant Thto-DaIase for D-arabinose and D-galactose was higher (3- and 1.25-fold) and lower (1.08- and 1.53-fold) for L-fucose and D-altrose compared to L-FIase/D-AIase from *D. turgidum*, respectively. Highly thermostable recombinant Thto-DaIase showed higher activity for D-arabinose, L-fucose and D-altrose, which were 2.64-, 2.25- and 15-fold, respectively, and lower activity for D-galactose, which was 1.9-fold that of D-AIase from *K. pneumoniae* [12].

The results shown in Table 3 prompted the researchers to further study the isomerization and transformation processes to produce rare sugars that are useable in the food and pharmaceutical industries. Thto-DaIase enzymatically has the ability to isomerize two kinds of substrates D-arabinose and L-fucose to produce D-ribulose and L-fuculose, the most expensive sugars, respectively. This enzyme also introduced activity towards D-altrose to D-allulose, which corroborates that Thto-DaIase has different substrate binding sites than D-AIase from *K. pneumoniae*. Although similar to other kinds of isomerases used for the transformation of rare sugars, Thto-DaIase has the ability to be used in the formation of many rare sugars, such as D-ribulose from D-arabinose, L-fuculose from L-fucose, D-allulose from D-altrose, D-tagatose from D-galactose, L-xylulose from L-xylose and L-tagatose from L-galactose by an isomerization process (Table 3). We revealed that Thto-DaIase is the most promising enzyme for producing D-altrose from D-allulose and needs further study.

The kinetic parameters of recombinant Thto-DaIase are given in Table 3 for D-arabinose, L-fucose, and D-altrose. The Michaelis-Menten constant (K_m) and K_{cat}/K_m were higher for D-arabinose than for L-fucose and D-altrose, which indicates that the enzyme is a D-arabinose isomerase. Although, Thto-DaIase has a higher affinity for D-altrose than D-arabinose and L-fucose but the isomerization rate of L-fucose and D-altrose was slower than that of D-arabinose. Table 4 demonstrates the comparison of the biochemical and kinetic parameters of recombinant Thto-DaIase with L-FIase/D-AIase from other microorganisms [12,13,21,22,27].

3.6. Bioproduction of D-ribulose and L-fuculose by recombinant Thto-DaIase

D-ribulose and L-fuculose were produced by recombinant Thto-DaIase in 0.5 mL of the reaction mixture at pH 9.0 and 75 °C. By applying 30, 50 and 80 g L^{-1} of D-arabinose substrate, after 3, 5 and 10 h, the enzymatic reaction reached approximate equilibrium by exhibiting conversion ratios of 27.66, 27.04 and 26.25%, respectively (Fig. 5C). After the reaction reached equilibrium, recombinant Thto-DaIase produced 8.29, 13.52 and 21 g L^{-1} of D-ribulose from 30, 50 and 80 g L^{-1} of D-arabinose, respectively, which was relatively high, while by applying 36, 50 and 80 g L^{-1} of L-fucose substrate, recombinant Thto-DaIase produced 9.31, 12.56 and 19.92 g L^{-1} of L-fuculose with 25.87, 24.88 and 24.9% conversion ratios, respectively (Fig. 5D). The results were compared with other bacteria producing the same isomerizing enzyme, such as L-FIase from *C. saccharolyticus* used to isomerize L-fucose and D-arabinose [21], which demonstrated a conversion ratio of 24% for both D-arabinose and L-fucose substrates at pH 7.0 and 70 °C after 3 h. The D-AIase from *K. pneumonia* showed a 10% conversion ratio for both substrates at pH 9.0 and 40 °C [12]. Reaction conditions such as pH and temperature affect the conversion ratio and productivity. Therefore, comparing the conversion yields of recombinant Thto-DaIase

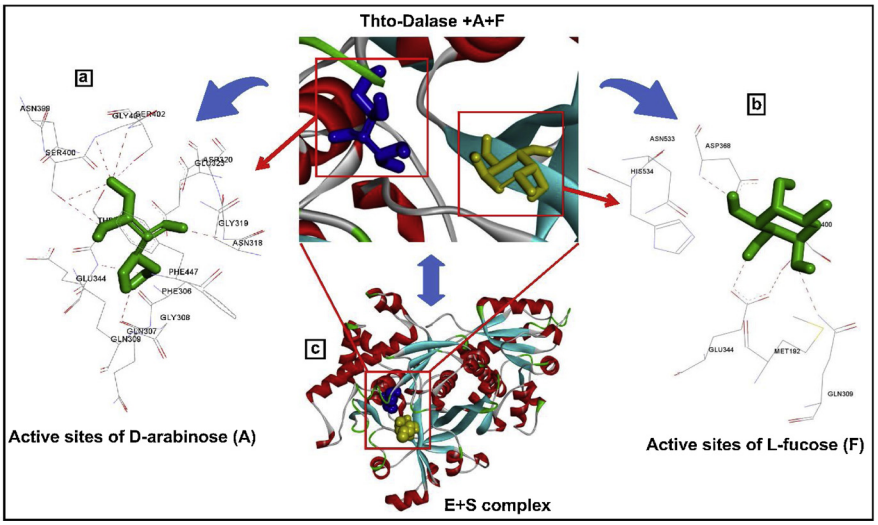

Fig. 6. 3-Dimensional structure of the active site of recombinant Thto-DaIase complexed with substrates D-arabinose (a) and L-fucose (b) and the enzyme-substrate complexes (c). The molecular model indicates the hypothetical active-site residues of D-arabinose and L-fucose that were closer to substrates and were used to enhance the activity of recombinant Thto-DaIase. The dashed lines show hydrogen bonds, while the green sticks show the substrates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with other D-ribulose- and L-fuculose-producing enzymes showed that the present enzyme was acceptable, and no byproducts were detected. Therefore, it tends to be a very important property for industrial application because it can lower the cost of the downstream industry by simplifying the purification process.

3.7. Docking and molecular modeling for active site residues of recombinant Thto-DaIase

The docked molecular model of Thto-DaIase for both substrates D-arabinose and L-fucose was created by a previously known template structure of *A. pallidus* (PDB ID: 3A9T) (Fig. 6) [4]. The recombinant Thto-DaIase has 74.62% similarity with the template organism *A. pallidus*; therefore, it was completely docked. Sometimes, environmental changes affected the amino acid arrangements and cause problems during mutation and docking [28,29].

The molecular model was used to identify specific residues with a ligand-docking tool using a linear form of D-arabinose and L-fucose. The molecular model of Thto-DaIase exhibiting the active site residues for D-arabinose are Phe306, Gln307, Gly308, Gln309, Asn318, Gly319, Asp320, Glu323, Thr343, Glu344, Asn399, Ser400, Gly401 and Ser402 (Fig. 6A), and for L-fucose, Met192, Gln309, Glu344, Asp368, Ser400, Asn533 and His534 (Fig. 6B), which showed a close relationship with the substrates. These residues may impart a decisive role to enhance the activity of the enzyme and could be designated as promising candidates of enzyme activity enhancer. The active site residues presented parallel geometry with the associated protein (Fig. 6C).

4. Conclusions

A recombinant thermostable D-AIase from a *T. toyohensis* strain was cloned, expressed, purified and characterized. The recombinant Thto-DaIase illustrated the highest activity at pH 9.0 and 75 °C, a suitable catalytic temperature for both D-arabinose and L-fucose substrates. The enzyme exhibited maximum catalytic activity with Mn^{2+} supplementation for D-arabinose and L-fucose from the initial activity. The thermostability of recombinant Thto-DaIase was more than 50% after 10 h, which is good for industrial applications. The specific activities for D-arabinose, L-fucose, and D-altrose were 98.08, 85.52 and 15 U mg^{-1}, respectively, which was greater than other D-AIase-producing bacteria. The active site residues were Phe306, Gln307, Gly308, Gln309, Asn318, Gly319, Asp320, Glu323, Thr343, Glu344, Asn399, Ser400, Gly401 and Ser402 for D-arabinose, while for L-fucose, Met192, Gln309, Glu344, Asp368, Ser400, Asn533, and His534 could be strong candidate for catalytic activity enhancer of Thto-DaIase. When 80 g L^{-1} of D-arabinose was applied, the conversion ratio of D-ribulose from D-arabinose was approximately 27.66% and produced 21 g L^{-1} of D-ribulose, while for L-fuculose from L-fucose, a 24.88% conversion ratio was observed, with 19.92 g L^{-1} of production yield and no byproducts were observed. The results showed that recombinant Thto-DaIase has great potential for D-ribulose and L-fuculose production in industries with a single enzyme by a simple purification method, which is good to lower the cost for the downstream industry.

5. Ethical statement

This study does not involve any human testing.

Declaration of Competing Interest

The authors declare that they do not have any conflict of interest.

Acknowledgments

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