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Efficient biotransformation of D-fructose to D-mannose by a thermostable D-lyxose isomerase from *Thermosediminibacter oceani*

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ABSTRACT

D-Mannose has prebiotic effect and potential medical application. Besides, it can be used as substrate to produce mannitol, a functional polyol widely used in food industry. As this result, it has attracted many researchers' attention. In this work, a thermostable D-mannose-producing D-lyxose isomerase (D-LI) was characterized from a hyperthermophile, *Thermosediminibacter oceani*. The recombinant D-LI could be remarkably activated by Mn^{2+}. It displayed maximal activity in presence of 1 mM Mn^{2+} at pH 6.5 and 65 °C, and was determined to be highly thermostable at 80 °C. The half-life was calculated to be 5.64, 2.82, 0.77, and 0.2 h at 70, 75, 80, and 85 °C, respectively. The enzyme showed the optimum activity using D-lyxose as substrate and could also effectively catalyze the isomerization between D-fructose and D-mannose. Under optimum conditions, 101.6 g/L D-mannose was produced from 400 g/L D-fructose after reaction for 9 h, giving a conversion yield of 25.4%.

1. Introduction

D-Mannose is the C-2 epimer of D-glucose and the aldose isomer of D-fructose. It is a naturally occurring monosaccharide, existing as the monomer unit in the polysaccharide mannan and an important component of many glycoproteins, therefore, it theoretically exists in a wide range of foods. D-Mannose has prebiotic effect and induces expression of pro- and anti-inflammatory cytokines [1]. As a supplement, D-mannose could reduce *Salmonella typhimurium* contamination to prevent urinary tract infections [2] and could treat the phosphomannose isomerase deficiency disease [3]. Furthermore, D-mannose is widely used as a cheap starting/basic material for synthesizing vitamins [4], immunostimulatory [5] and antitumor agents [6]. In addition, D-mannose is an important material to produce mannitol, which is a high-value sugar alcohol widely used in the food, pharmaceutical, medical, and chemical industries [7].

Nowadays, mannitol is mainly produced by chemical hydrogenation catalysis from D-fructose or invert sugar (D-fructose and D-glucose mixture). But due to the selectivity of hydrogenation, this reaction theoretically results in 50% (W/W) and 25% (W/W) mannitol from pure D-fructose [8] and invert sugar (50:50 D-fructose/D-glucose mixture) [9], respectively. However, if D-fructose is previously converted to D-manose by isomerase, then D-mannose can be further hydrogenated into 100% mannitol [10,11].

Biological production of D-mannose has been studied using several kinds of enzymes. D-Mannose isomerase (D-MI, EC 5.3.1.7) catalyzes the reversible isomerization reaction between D-fructose and D-mannose [12]. The conversion of D-fructose to D-mannose has previously been performed using D-MIs from *Escherichia coli* K12 [13], *Pseudomonas cepacia* [14], and *Agrobacterium radiobacter* M-1 [15]. A cellobiose 2-epimerase (2-CE, EC 5.1.3.11) from *Caldicellulosiruptor saccharolyticus* DSM 8903 is able to catalyze the epimerization of D-glucose to D-mannose, but the reaction also catalyzes the isomerization of D-glucose to D-fructose as a byproduct [16]. The production of D-mannose has also been performed by isomerization of D-fructose using D-lyxose isomerases (D-LI, EC 5.3.1.15) from *Serratia proteamaculans* KCTC 2936 [17] and *Providencia stuartii* KCTC 2568 [18].

D-LI is an aldo-keto isomerase catalyzing the isomerization reaction between D-lyxose and D-xylulose. So far, D-LIs have been characterized from *Aerobacter aerogenes* PRL-R3 [19], *Bacillus licheniformis* DSM13 [20], *Cohnella laevoribosii* RI-39 [21], *Dictyoglomus turgidum* DSM 6724 [22], *Escherichia coli* O157:H7 [23], *P.*

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stuartii KCTC 2568 [24], and *S. proteamaculans* KCTC 2936 [17]. D-LI exhibits broad substrate specificity with highest activity toward D-lyxose and may also catalyze the isomerization between D-fructose and D-mannose. However, only the D-LIs from *S. proteamaculans* [17] and *P. stuartii* [18] have been used for biological production of D-mannose from D-fructose [24]. Except the thermophilic *D. turgidum* DSM 6724 and *C. laevoribosii* RI-39, all previously reported D-LIs are from mesophilic microorganisms. For industrial application of aldose isomerases generally thermostable enzymes, such as D-xylose isomerase [25] and L-arabinose isomerase [26], are required.

Thermosediminibacter oceani DSM 16646 is an anaerobic hyperthermophilic bacterium isolated from deep sea sediments of Peru Margin and the optimal growth temperature is 65 °C [27]. The complete genome sequence of the strain has recently been determined and deposited in GenBank with accession No. NC_014377 [28]. The genome sequence reveals the presence of a putative D-LI gene (locus_tag: CP002131.1; protein ID: ADL08607.1). In this study, the putative D-LI gene was cloned and expressed in *Escherichia coli* BL21(DE3). This recombinant D-LI was then purified by metal affinity chromatography, characterized and the D-mannose production from D-fructose was studied.

2. Materials and methods

2.1. Chemicals and reagents

The carbohydrate standards, including D-fructose, D-mannose, D-lyxose, and D-xylulose were obtained from Sigma (St. Louis, MO, USA). Tryptone and yeast extract for Luria-Bertani (LB) broth were purchased from Difco (Detroit, MI, USA). All other chemicals were at least of analytical grade obtained from Sinopharm Chemical Reagent (Shanghai, China) and Sigma (St. Louis, MO, USA).

2.2. Bacterial strain, plasmid, and culture conditions

The *E. coli* BL21(DE3) strain was used for heterologous expression and the plasmid pET-22b(+) was used as an expression vector. The *E. coli* cells were cultivated in Luria-Bertani (LB) broth (pH 7.5), composed of 10 g/L trypone, 5 g/L yeast extract, and 5 g/L of NaCl.

2.3. Gene cloning and expression

The putative D-LI gene (locus_tag: CP002131.1; protein ID: ADL08607.1) from *T. oceani* DSM 16646 has been published in the open GenBank database. In this work, the full length of the target gene was commercially synthesized by Shanghai Generay Biotech Co., Ltd (Shanghai, China), with an in-frame C-terminal 6 × histidine-tag sequence, and was cloned into the expression vector pET-22b(+) with *Nde*I and *Xho*I restriction sites at the 5′- and 3′-terminus.

The constructed plasmid harboring *T. oceani* D-LI genes was transformed into *E. coli* BL21(DE3) strain for overexpression. The recombinant strain was grown at 37 °C in LB medium containing 100 μg/mL of ampicillin. When the optical density at 600 nm reached 0.6, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the culture medium at 0.1 mM to induce the enzyme expression and the culture was further grown at 28 °C for 6 h.

2.4. Purification of recombinant D-LI

All purification steps were conducted at 4 °C. The grown cells of *E. coli* were harvested from 200 mL culture broth by centrifugation at 10,000 × *g* for 15 min, and then were resuspended in 15 mL cell lysis buffer (50 mM sodium phosphate buffer, 100 mM NaCl, pH 6.5). The harvested cells were disrupted by sonication for 12 min with a 2 s on: 3 s off cycle. The insoluble cell debris was removed by centrifugation at 10,000 × *g* for 30 min. The recombinant enzyme, expressed as 6 × histidine-tagged fusion protein, was purified by nickel-affinity chromatography using an ÄKTA purifier system (GE Healthcare, Sweden). The centrifuged supernatant (15 mL) as crude enzyme was filtrated by a 0.22-μm Millipore filter and then loaded onto a 5-mL HisTrap HP Ni-NTA column (GE Healthcare, Sweden), which was chelated with Ni^{2+} and pre-equilibrated with Binding Buffer (50 mM sodium phosphate buffer, 500 mM NaCl, pH 6.5). Then, the Binding buffer and Washing buffer (50 mM sodium phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH 6.5) were used in turn to remove the impurity protein. At last, the 6 × histidine-tagged target protein was eluted from the column using an Elution Buffer (50 mM sodium phosphate buffer, 500 mM NaCl, 500 mM imidazole, pH 6.5). The collected enzyme solution was dialyzed against dialysate A (50 mM sodium phosphate buffer, 10 mM ethylenediamine tetraacetic acid (EDTA), pH 6.5) for 12 h, and then against dialysate B (50 mM sodium phosphate buffer, pH 6.5) at 4 °C for another 12 h to remove EDTA.

2.5. Molecular mass determination

The subunit molecular mass was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. The separating and stacking gels were 12% and 5% (w/v) of acrylamide, respectively. Coomassie brilliant blue R250 was used to stain proteins for visualization.

The molecular mass of native enzyme was assessed by gel filtration using high-performance liquid chromatography (HPLC, Agilent 1200 LC Systems, Agilent technologies, Santa Clara, CA, USA) equipped with a Diode Array Detector and a TSKgel G2000SWxl column (125 Å, 5 μm, 7.8 mm id × 30 cm, Tosoh Bioscience LLC, Tokyo, Japan). The HPLC conditions were as follows: mobile phase, 0.1 M phosphate buffer (pH 6.5) containing 0.05% (W/V) NaN_3 and 0.1 M Na_2SO_4; column temperature, 25 °C; flow rate, 1 mL/min; detection wavelength, 260 nm.

2.6. Enzyme assay

The enzyme activity was determined by measuring the accumulation of D-fructose using D-mannose as a substrate. Unless otherwise stated, the reaction was carried out in 50 mM sodium phosphate buffer (pH 6.5) containing 10 mM D-mannose, 1 mM $MnCl_2$ and 0.2 U enzyme/mL at 65 °C for 10 min. After incubation, the reaction was stopped by the addition of trichloroacetic acid to final concentration of 150 mM. One unit of enzyme activity was defined as the amount of enzyme that produced 1 uM D-fructose from D-mannose per min at pH 6.5 and 65 °C.

2.7. Effects of metal ions, pH, and temperature

To investigate the effect of divalent metal ions on enzyme activity, the enzyme assay was carried out after adding 1 mM of each divalent metal ion such as $CoCl_2$, $MnCl_2$, $NiSO_4$, $MgCl_2$, $CaCl_2$, $ZnCl_2$, $CuSO_4$, $FeSO_4$ or $BaCl_2$ to the divalent metal-free enzyme. The reactions were performed in 50 mM sodium phosphate buffer (pH 6.5) at 65 °C. The activity of enzyme without adding any divalent metal ion was taken as 100%.

To study the effect of pH on the recombinant D-LI, pH was varied from 4.5 to 9.0 using 50 mM acetate buffer (pH 4.0–5.5), 50 mM phosphate buffer (pH 6.0 – 7.0), and 50 mM Tris-HCl buffer (pH 7.5–9.0). The effect of temperature on the enzyme activity was evaluated in 50 mM phosphate buffer (pH 6.5) at various temperatures, from 50 to 85 °C. The activities at each pH or temperature were relative to the highest activity value (100%).

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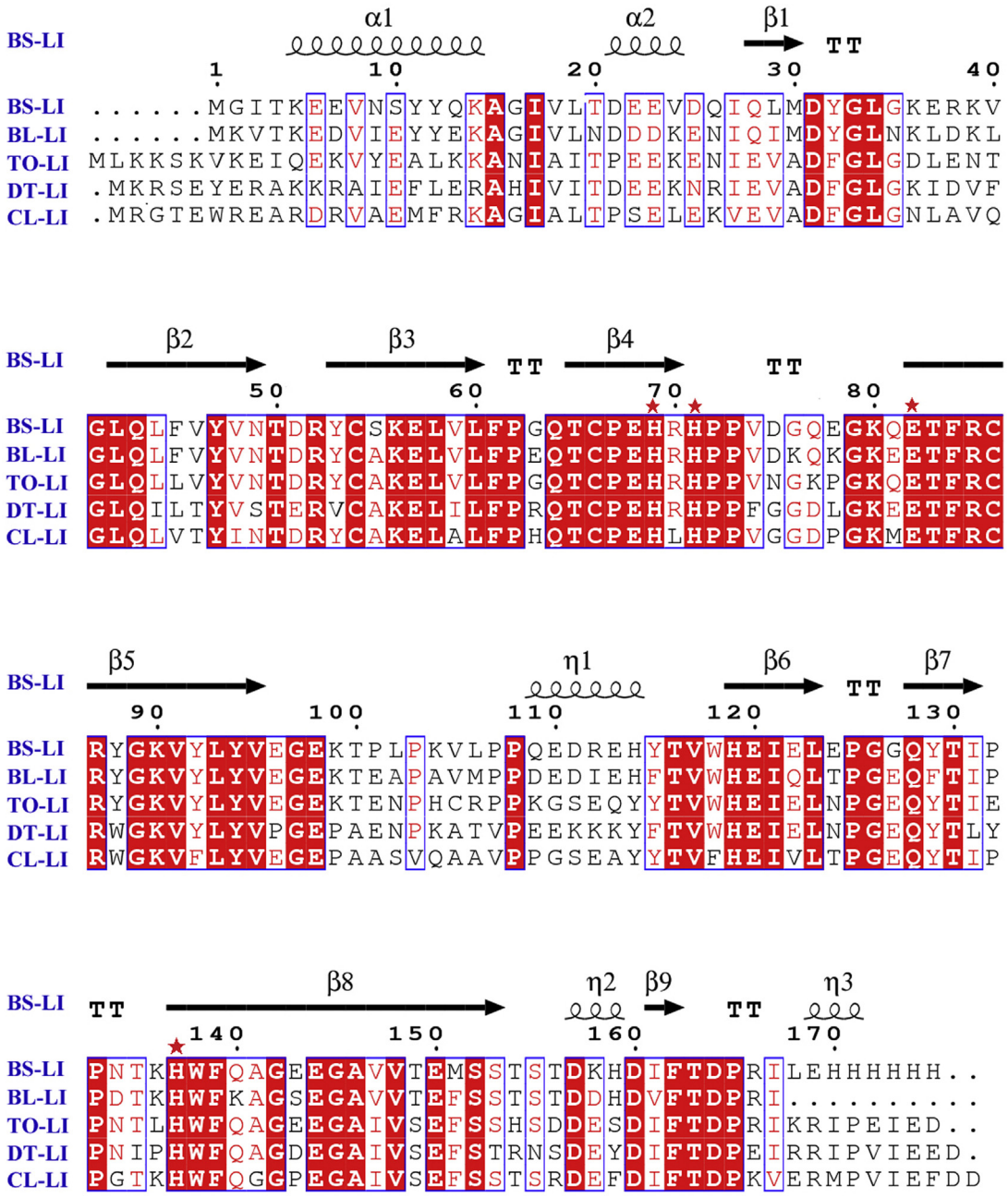

Fig. 1. Multiple sequence alignment of D-LIs and their homologs. Amino acid sequence for D-LI from *T. oceani* DSM 16646 (TO-LI; GeneBank accession No: ADL08607.1) was aligned with *B. subtilis* D-LI (BS-LI; AIY91703), *B. licheniformis* D-LI (BL-LI; AAU22106.1), *D. turgidum* D-LI (DT-LI; YP_002352606.1), and *C. laeviribosi* D-LI (CL-LI; ABI93960.1). The strictly conserved residues are shown on a red background, and the highly conserved residues are shown in red type and boxed in blue. The secondary structure elements and the residues involved in the metal coordinating sites (the symbol star above the residues) are symbolized based on the previously reported structure of *B. subtilis* D-LI (PDB No. 2Y0O_A) [29]. The alignment was performed using ESPript [42]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The thermal stability of the recombinant D-LI was studied by pre-incubating the enzyme in 50 mM sodium phosphate buffer (pH 6.5) at temperatures ranging from 70 to 85 °C. The residual activities withdrawn at different time intervals were determined after the isomerization reaction from D-mannose to D-fructose. The initial activity of enzyme without incubation was taken as 100%.

2.8. Determination of kinetic parameters

Various concentrations (from 10 mM to 200 mM) of monosaccharide substrates (D-lyxose, D-mannose, and D-fructose) were used to measure the kinetic parameters. The reactions were performed in 50 mM phosphate buffer (pH 6.5) at 65 °C containing 1 mM Mn^{2+}. The enzyme kinetic parameters, including Michaelis–Menten constant (K_m), turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_m), were determined using Michaelis–Menten equation and Lineweaver-Burk plots.

2.9. Enzymatic production of D-mannose from D-fructose

Biological production of D-mannose was performed in 50 mM phosphate buffer (pH 6.5) containing 1 mM Mn^{2+} and 4 U/mL

enzyme at 60 °C. 100, 200, 300 and 400 g/L of D-fructose were used as initial concentrations of substrate.

2.10. Analysis of monosaccharides

The concentrations of monosaccharides were determined using a Bio-LC system (Dionex ICS-5000, Sunnyvale, CA, USA) with a pulse ampere detector and a CarboPac PA20 column (6.5 μm, 3 × 150 mm, Thermo Fisher Scientific, Massachusetts, USA). NaOH (20 mM) was used as the mobile phase with the flow rate of 0.5 mL/min. The column temperature was 30 °C.

3. Results and discussion

3.1. Heterologous expression and protein purification

So far, D-LIs from *C. laevoribosii* (GenBank accession No. ABI93960.1, 182 aa) [21], *B. licheniformis* DSM13 (AAU22106.1, 167 aa) [20], *D. turgidum* DSM 6724 (YP_002352606.1, 181 aa) [22], *Bacillus sutbilis* strain 168 (AIY91703, 167 aa) [29], *S. proteamaculans* (BAJ07463.1, 228 aa) [17], *E. coli* (Q8 × 5Q7, 227 aa) [23] and *P. stuartii* KCTC2568 [24] have been identified in recombinant forms. To find a D-LI with good thermostability, the protein blast search using the known D-LIs as controls was performed and the thermophilic bacterium sources were selected for further analysis. Since the amino acid sequence of the D-LI from *P. stuartii* KCTC2568 is not available in GenBank, and the *S. proteamaculans* D-LI and *E. coli* D-LI have much higher molecular weight and show no significant amino acid sequence similarity with other characterized D-LIs, those three are not used as controls. The putative D-LI from a thermophilic strain, *T. oceani* DSM 16646 (ADL08607.1, 181 aa), exhibited 69%, 65%, 65%, and 64% amino acid sequence identity to the D-LIs from *B. sutbilis, C. laevoribosii*, *B. licheniformis* DSM13, and *D. turgidum* DSM 6724, respectively (Fig. 1). The crystal structures of D-LI from *B. sutbilis* and *E. coli* have recently been determined and released with PDB No. 2Y0O and 3MPB, respectively [29]. The structure of *B. sutbilis* D-LI forms a dimer with a molecule related by the crystallographic twofold axis. It contains two divalent metal coordinating sites, which are composed of three histidines (H69, H71, and H137) and one glutamic acid (E82). What's more, these four residues are strictly conserved across all D-LIs including *T. oceani* DSM 16646 (Fig. 1). Although the *E. coli* D-LI shares a low sequence identity (24%) with *B. sutbilis* D-LI, it is also a dimer in the asymmetric unit. The overall fold of is a cupin-type β-barrel which forms a deep hydrophobic pocket. Interestingly, the metal-binding site in the hydrophobic pocket is also conserved. Similar to other D-LIs, the metal is coordinated by H103, H105, E110, H171 and two water molecules [23].

In this work, the putative D-LI from the thermophilic strain *T. oceani* DSM 16646 was selected for study in order to obtain a thermostable D-mannose-producing D-LI. The putative D-LI-encoding gene (GenBank accession No. CP002131.1) was heterologously expressed in *E. coli* BL21(DE3) with C-terminal 6 × histidine-tag. The theoretical molecular weight of the recombinant protein was calculated to be 21,664 Da based on the 181 amino acid residues and six histidine residues, as measured using the ExPASy Computer pI/Mw tool. As shown in Fig. 2, the target protein didn't appear without adding inducer. As the induction time increased, the band of target protein became more and more obvious. After expression by IPTG induction for 6 h at a low temperature (28 °C), a strong protein band of approximately 22 kDa appeared on the electrophoresis gel of SDS-PAGE, compared to the electrophoresis result of the control *E. coli* BL21(DE3) (data not shown), indicating that the target recombinant protein with the predicted molecular weight had been overexpressed. The recombinant enzyme was purified to homogeneity, exhibiting a single band of 22 kDa in SDS-PAGE (Fig. 2) and the native purified enzyme existed as a homodimer with a molecular weight of 44 kDa as measured by gel filtration chromatography, using TSK G2000SWxl column (data not shown). D-LIs from *C. laevoribosii* [21], *D. turgidum* DSM 6724 [22], and *P. stuartii* KCTC2568 [24] were also identified as homodimers with subunit molecular weight of 21 or 22 kDa *S. proteamaculans* D-LI was identified as a homodimer as well [17], but the subunit molecular weight (27 kDa) was much higher than other D-LIs and it displayed very low amino acid sequence identity with other D-LIs as mentioned above.

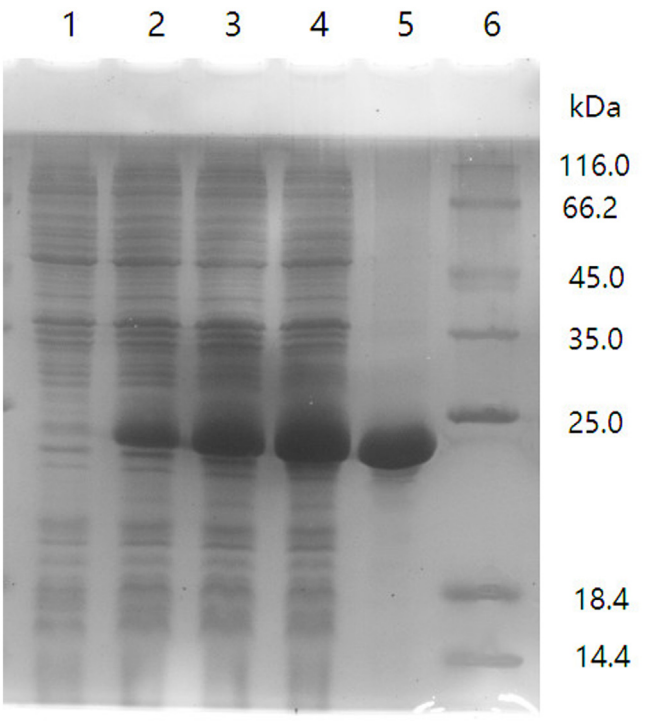

Fig. 2. SDS-PAGE analysis of proteins. lane 1, whole recombinant *E. coli* cells without IPTG induction; lane 2–4, the cells after IPTG induction for 2, 4, and 6 h, respectively; lane 5, purified recombinant *T. oceani* D-LI by metal affinity chromatography; Lane 6, protein markers. The proteins on gel were stained by Coomassie brilliant blue R250.

3.2. Effect of metal ions on the enzyme activity

To investigate the effect of metal ions, the purified recombinant *T. oceani* D-LI was firstly treated with EDTA to remove the divalent metal ions and dialyzed against divalent metal-free phosphate buffer (50 mM, pH 6.5) to remove EDTA. The relative activity was detected in presence of various divalent metal ions at the final concentration of 1 mM (Table 1). The enzyme displayed a maximal activity in the presence of Mn^{2+}. The Mn^{2+}-bound D-LI showed 1250% of the relative activity compared to the metal-free enzyme. The Ni^{2+}, Co^{2+} and Fe^{2+} increased significantly the catalytic activity to 1179%, 913%, and 214% of the initial relative activity, respectively. However, other tested metal ions, especially Cu^{2+} and Zn^{2+}, inhibited the enzyme activity up to 87% (Table 1).

Based on the *B. subtilis* D-LI structure data, the metal coordinating sites are present in the active center of the enzyme; therefore, the metal ion probably has important effect on the catalytic activity [29]. All the reported D-LIs have been identified to be metal-dependent enzymes, which especially are activated by Mn^{2+} and Co^{2+}. The D-LIs from *C. laevoribosii* [21], *B. licheniformis* DSM13 [20], *S. proteamaculans* [17], *E. coli* O157:H7 and *P. stuartii* KCTC2568 [24] also require Mn^{2+} as an optimal metal cofactor. Fur-

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Table 1
Effect of Metal Ions on the Recombinant *T. oceani* D-LI.

Metal ion (1 mM)	Relative activity (%)[a]
None	100 ± 4
Mn^{2+}	1250 ± 59
Ni^{2+}	1,179 ± 68
Co^{2+}	913 ± 42
Fe^{2+}	214 ± 18
Ca^{2+}	86 ± 4
Mg^{2+}	68 ± 5
Ba^{2+}	64 ± 2
Cu^{2+}	16 ± 1
Zn^{2+}	13 ± 1

[a] The activities were measured at pH 6.5 and 65 °C. Results were the mean values of three experiments ± standard deviation.

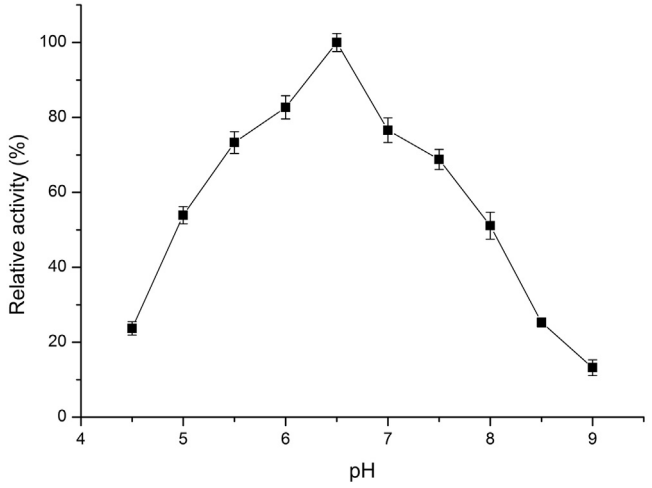

Fig. 3. Effect of pH on the activity of the recombinant *T. oceani* D-LI. The relative activity was investigated in presence of 1 mM Mn^{2+} at 65 °C and different pH values. Values are means of three replications and error bar represents standard deviation.

thermore, the Co^{2+} is the optimal cofactor of D-LI from *D. turgidum* DSM 6724 [22], but strongly inhibited the activity of *P. stuartii* D-LI [24]. The activity enhancement by Mn^{2+} or Co^{2+} was also found in a wide range of aldose isomerases and ketose epimerases, such as D-glucose isomerase [25], L-arabinose isomerase [26], and D-tagatose 3-epimerase family enzymes [30]. Moreover, some enzymes were identified to be strictly metal-dependent, which did not display any activity without divalent metal ion, such as L-rhamnose isomerase from *Thermoanaerobacterium saccharolyticum* NTOU1 [31], L-arabinose isomerase from *Geobacillus stearothermophilus* DSM22 [32], and D-psicose 3-epimerases from *Clostridium* species strains [33–35].

3.3. Effects of pH on the enzyme activity

The enzyme assay was performed in presence of 1 mM Mn^{2+}. The purified recombinant enzyme showed an optimal activity at pH 6.5, and retained more than 50% of maximal activity at pHs ranged from 5.0 to 8.0 (Fig. 3). These results were in accordance with other studies (done by whom) which stated that D-LI from *C. laevoribosii* exhibited maximal activity at pH 6.5, and the optimal pH values of the D-LIs from *B. licheniformis* DSM13, *D. turgidum* DSM 6724, *S. proteamaculans*, *E. coli* O157:H7 and *P. stuartii* KCTC2568 were determined to be in the alkaline range of pH 7.5 – 8.0. Generally, a slightly acidic pH optimum is considered as an advantage for enzymatic reaction of reducing sugars in industry [36,37]. The acidic pH condition can remarkably reduce the non-enzymatic reactions which easily happen at alkaline pH and lead to unwanted by-products [38]. Therefore, many studies focused on the identification of aldose isomerases with slightly acidic pH optima [39] and the molecular modification to shift the pH optima toward acidic side [40].

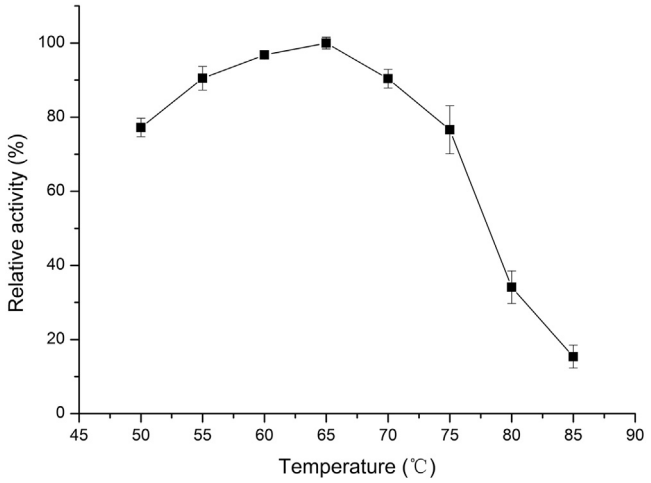

Fig. 4. Effect of temperature on the activity of the recombinant *T. oceani* D-LI. The relative activity was investigated in presence of 1 mM Mn^{2+} at pH 6.5 and various temperatures. Values are means of three replications and error bar represents standard deviation.

3.4. Effects of temperature on the enzyme activity and stability

The activity was examined over a temperature range of 50 – 85 °C at pH 6.5 in presence of 1 mM Mn^{2+}. The recombinant *T. oceani* D-LI showed the highest activity at 65 °C, and retained 77% of maximal activity at 75 °C, but the relative activity dropped significantly above 75 °C (Fig. 4). By comparison, the D-LIs from *D. turgidum* DSM 6724 [22] and *C. laevoribosii* [21] showed the highest relative activity at 75 and 70 °C, respectively, and the temperature optima of other reported D-LIs were measured to be less than 50 °C (Table 2).

The effect of temperature on the enzyme stability was studied at 70 – 85 °C (Fig. 5). The enzyme retained 65%, 51%, and 50% of its initial activity after incubation at 70 °C for 4 h, 75 °C for 2 h, and 80 °C for 1 h, respectively. For the determination of half-life, the first order deactivation kinetic model was selected. The stability decreased remarkably when incubated at 85 °C, and the residual activity dropped to less than 50% after 10 min. The decay constant k_d was calculated to be 0.123, 0.246, 0.904, and 3.473 at 70, 75, 80, and 85 °C, respectively. Therefore, based on the calculation formula of half-life ($t_{1/2}$) = ln2/k_d, the $t_{1/2}$ was determined to be 5.64, 2.82, 0.77, and 0.20 h at these temperatures, respectively. The thermostability was also studied at the optimal temperature (65 °C), and results showed that the enzyme almost did not lose any activity after incubation for 5 h and retained 80% of initial activity after incubation for 50 h (data not shown). This data indicated that the recombinant *T. oceani* D-LI showed a very good thermostability. By comparison, the half-lives of D-LIs from *S. proteamaculans* [17], *P. stuartii* KCTC2568, and *B. licheniformis* DSM13 were 0.09, 1.4, and 7 h at 50 °C, respectively. The thermostability of D-LI from the thermophilic *C. laevoribosii* RI-39 was not reported. The D-LI from the hyperthermophile, *D. turgidum* DSM 6724, has the optimum temperature at 75 °C and a half-life of 9.1 h at 60 °C [22]. Although the optimum temperature of *T. oceani* D-LI (65 °C) was relatively lower than that of *D. turgidum* D-LI, the *T. oceani* D-LI exhibited much higher thermostability.

In general, commercial applications of aldose isomerases, such as D-glucose isomerase [12] and L-arabinose isomerase [41], require the enzymes to have a high optimum temperature, a good ther-

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Table 2
Comparison of Biochemical Properties of Various Microbial D-LIs.

Microorganism	Subunit molecular mass (kDa)	Total molecular mass (kDa)	Optimum pH	Optimum Temperature (°C)	Optimum metal	Half-life (h)	Reference
T. oceani DSM 16646	22	44	6.5	65	Mn^{2+}	12.7 (70 °C) 6.8 (75 °C) 1.7 (80 °C) 0.5 (85 °C)	This study
C. laevoribosii RI-39	21	42	6.5	70	Mn^{2+}	NR[a]	[21]
B. licheniformis DSM13	19.5	NR	7.5–8.0	40–45	Mn^{2+}	140 (30 °C) 62 (35 °C) 30 (40 °C) 18 (45 °C) 7 (50 °C)	[20]
D. turgidum DSM 6724	22	44	7.5	75	Co^{2+}	9.1 (60 °C)	[22]
S. proteamaculans	27	54	7.5	40	Mn^{2+}	84 (30 °C) 17 (35 °C) 2.6 (40 °C) 0.3 (45 °C) 0.09 (50 °C)	[17]
E. coli O157:H7	NR	NR	7.5	50	Mn^{2+}	NR	[23]
P. stuartii KCTC2568	22	44	7.5	45	Mn^{2+}	36 (30 °C) 14 (35 °C) 8.9 (40 °C) 3.4 (45 °C) 1.4 (50 °C)	[24]

[a] NR, not reported.

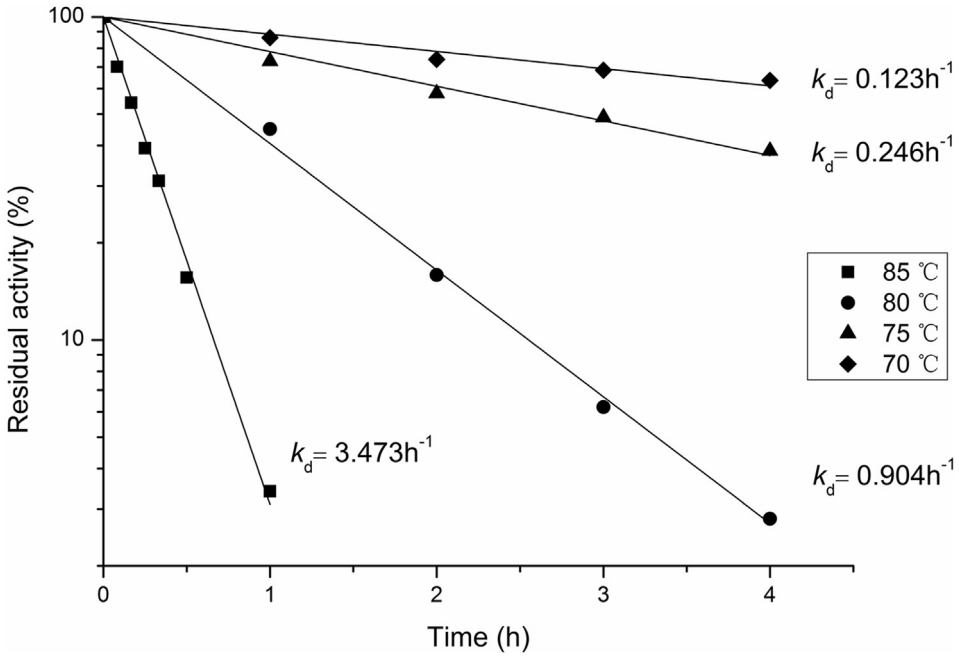

Fig. 5. Effect of temperature on the stability of the recombinant *T. oceani* D-LI. Thermal stability was studied by incubating the enzyme at various temperatures for different time intervals at pH 6.5. The parameter k_d represents decay constant.

mostability, and a slightly acidic pH optimum. Elevated reaction temperatures could increase the carbohydrate solubility, reduce viscosity of reaction mixture, and decrease the possibility of microbial contamination. High thermostability is helpful for the biocatalysts to maintain activity and extend the reaction period. Slightly acidic pH optimum reduces the non-enzymatic reactions between the proteins and carbohydrates forming brown-colored by-products. In this study, the recombinant *T. oceani* D-LI showed the optimum pH and temperature at pH 6.5 and 65 °C, and was the most thermostable of the D-LIs reported to date, indicating its good potential for industrial application.

3.5. Substrate specificity and kinetic parameters

The specific activity of the recombinant *T. oceani* D-LI was determined for D-lyxose, D-mannose, and D-fructose as substrates (Table 3). The isomerization reactions showed only one product from each substrate, and the optimum substrate was D-lyxose. The specific activities were measured to be 7.1 ± 0.2, 5.3 ± 0.1, and 1.2 ± 0.1 U/mg, for D-lyxose, D-mannose, and D-fructose, respectively.

The kinetic parameters for the three monosaccharides were calculated (Table 3). The K_m for D-lyxose was much lower than those for D-mannose and D-fructose, indicating that the recombinant

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Table 3
Specific Activities and Kinetic Parameters of the Recombinant *T. oceani* D-LI.[a]

Substrate	Product	Specific activity (U/mg)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
D-lyxose	D-xylulose	7.1 ± 0.2	15.5 ± 1.2	3,108 ± 32	201 ± 6
D-mannose	D-fructose	5.3 ± 0.1	32.8 ± 1.8	5,686 ± 39	173 ± 5
D-fructose	D-mannose	1.2 ± 0.1	27.3 ± 1.3	1,030 ± 26	38 ± 1

[a] Results were the mean values of three experiments ± standard deviation.

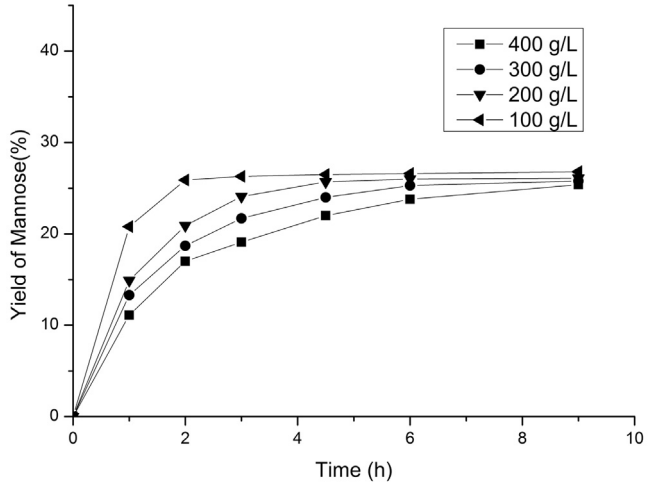

Fig. 6. Enzymatic production of D-mannose from D-fructose by the recombinant *T. oceani* D-LI. D-mannose was produced by 4 U/mL enzyme in 50 mM phosphate buffer (pH 6.5) containing 1 mM Mn^{2+} at 60 °C, from four concentrations of D-fructose, 100, 200, 300 and 400 g/L. Values are means of three replications and error bar represents standard deviation.

T. oceani D-LI showed the highest affinity toward D-lyxose. The k_{cat}/K_m values were 201 ± 6, 173 ± 5, and 38 ± 1 for D-lyxose, D-mannose, and D-fructose, respectively. All these results indicates that D-lyxose was the optimum substrate for *T. oceani* D-LI. The k_{cat} and k_{cat}/K_m values for D-mannose were much higher than those for D-fructose, the reaction from D-mannose to D-fructose was favorable. But D-mannose was much more expensive than D-fructose, and it was meaningful to carried out the reverse reaction from D-fructose to D-mannose. In addition, interestingly, the K_m values of *T. oceani* D-LI for D-lyxose, D-mannose, and D-fructose were slightly lower than most other known D-LIs., while the k_{cat} and k_{cat}/K_m values were not prominent (data not shown).

3.6. Production of D-mannose from D-fructose

D-Mannose production by the recombinant *T. oceani* D-LI was performed at pH 6.5 and 60 °C in 100 mL of reaction volume (Fig. 6). Upon adding 100, 200, 300 and 400 g/L D-fructose, the enzymatic reaction reached the equilibrium at 2, 4.5, 6 and 9 h, exhibiting conversion rates of 26.8%, 26.1%, 25.8% and 25.4% respectively. As the substrate concentration added, the amount of production increased, which led to the end-product inhibition and slight decrease of conversion rate. During high-level D-mannose production by *T. oceani* D-LI, 26.8, 52.2, 77.4 and 101.6 g/L of D-mannose was produced from 100, 200, 300 and 400 g/L D-fructose, respectively. By comparison, the *S. proteamaculans* D-LI produced 100 g/L D-mannose from 500 g/L D-fructose in 5 h, with conversion ratio of 20% [17]. D-Mannose production from D-fructose was also studied using free and immobilized *P. stuartii* D-LI. The free *P. stuartii* D-LI produced 150 g/L D-mannose from 600 g/L D-fructose in 2 h, with conversion ratio of 25%. The immobilization of D-LI displayed a good potential for the industrial application. The immobilized *P. stuartii* D-LI with Duolite A568 as carrier exhibited a 25% (W/W) conversion yield of D-fructose to D-mannose after 23 cycles and the D-mannose concentration could still retain 52% of its initial value after 35 cycles [17].

Compared with the conversion rate of other D-mannose producing enzymes (almost 20% – 25%), the conversion rate of *T. oceani* D-LI was acceptable. Besides, some other rare sugar isomerase also displayed the similar conversion rate, that D-psicose 3-epimerase could convert D-fructose to D-psicose with the rate of 20% – 30%. In the industrial production of rare sugars, high thermostability and slightly acidic pH optimum were required, because high thermostability could improve the utilization efficiency of the enzymes and slightly acidic pH optimum was beneficial for reducing non-enzymatic reactions and unwanted by-products. Interestingly, the thermostable *T. oceani* D-LI exhibited maximum activity at acidic pH (pH 6.5), which could meet the requirements of industrial production.

4. Conclusion

A thermostable D-mannose-producing D-LI was characterized from a thermophilic strain, *T. oceani* DSM 16646. The purified recombinant *T. oceani* D-LI showed a slightly acidic optimum pH at 6.5 and a relatively high optimum temperature at 65 °C. It exhibited a very high thermostability compared to other reported D-LIs and was stable up to 80 °C for 1 h of incubation. All these data indicated that the *T. oceani* D-LI had a great potential for industrial D-mannose production.

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