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A novel L-arabinose isomerase from *Lactobacillus fermentum* CGMCC2921 for D-tagatose production: Gene cloning, purification and characterization

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ABSTRACT

The *araA* gene encoding L-arabinose isomerase (L-AI) from the acidophilus bacterium *Lactobacillus fermentum* CGMCC2921 was cloned and over-expressed in *Escherichia coli*. The open reading frame of the L-AI consisted of 1425 nucleotides encoding 474 amino acid residues. The molecular mass of the enzyme was estimated to be approximately 53 kDa on SDS–PAGE. The purified recombinant enzyme showed maximum activity at 65 °C and pH 6.5, which were extremely suitable for industrial applications. It required divalent metal ions, either Mn^{2+} or Co^{2+}, for enzymatic activity and thermostability improvement at higher temperatures. The enzyme was active and stable at acidic pH, it exhibited 83% of its maximal activity at pH 6.0 and retained 88% of the original activity after incubation at pH 6.0 for 24 h. Kinetic parameter study showed that the catalytic efficiency was relatively high, with a k_{cat}/K_m of 9.02 mM^{-1} min^{-1} for D-galactose. The purified *L. fermentum* CGMCC2921 L-AI converted D-galactose into D-tagatose with a high conversion rate of 55% with 1 mM Mn^{2+} after 12 h at 65 °C, suggesting its excellent potential in D-tagatose production.

1. Introduction

L-Arabinose isomerase (L-AI; EC 5.3.1.4) is an intracellular enzyme that catalyzes the reversible isomerization of L-arabinose to L-ribulose, a component of the pentose phosphate or the phosphoketolase pathway [1]. L-AIs are also referred to as D-galactose isomerases due to their ability, in vitro, to isomerase D-galactose into D-tagatose.

D-Tagatose is a hexoketose monosaccharide sweetener, which is an isomer of D-galactose and rarely found in nature. The sweetness of D-tagatose is equivalent to sucrose, but with only 38% of the calories when compared in 10% solutions [2]. Physiological studies demonstrated that D-tagatose consumption at recommended dose not promote tooth decay or elicit any increase in blood glucose level [3]. In addition, D-tagatose has been shown to have numerous health and medical benefits, including treatment of obesity [4], prevention of dental caries, regulation of intestinal flora [5], improvement of pregnancy and fetal development, and reduction of symptoms of type 2 diabetes [6]. Based on these properties, D-tagatose has attracted a great deal of attention in recent years as a low calorie sugar-substituting sweetener, which was also approved as a "generally recognized as safe (GRAS)" material under FDA regulations [7].

As a matter of fact, the use of D-tagatose was limited mostly due to its scarcity in nature and costly methods of production. Recently, there has been great interest in the biological manufacture of D-tagatose from D-galactose with L-AIs. A number of L-AIs have been identified from various microorganisms, such as *Geobacillus stearothermophilus*, *Geobacillus thermodenitrificans*, *Thermotoga maritima*, and *Thermus* sp. [8–11]. Nevertheless, L-AIs from those microbes were optimally active at an alkalescent pH (7.5–8.5), which would cause browning reaction and formation of undesirable sub-products in D-tagatose production process [12]. Other L-AIs, from *E. coli*, *Lactobacillus gayonii*, and *Mycobacterium smegmatis* [13–15] exhibited a lower optimum temperature (30–45 °C), which may be difficult to realize high conversion rate of D-tagatose from D-galactose [16]. Thus, L-AI with high activity and stability at a moderately low pH and higher temperatures would have the greatest potential for the production of the D-tagatose.

For the purpose of attaining L-AIs suitable for D-tagatose production, new organisms carrying the acidophilic and thermostable target enzyme need to be screened. Lactic acid bacteria are well known for their acid tolerance, and previously reported L-AI from *Lactobacillus* genera was extraordinarily thermostable [17]. Furthermore, L-AI has not yet been characterized from *Lactobacillus fermentum*. Thus, we chose *L. fermentum* CGMCC2921 as a candidate. Here we report the gene cloning, amino acid sequence inspection, over-expression and characterization of a novel L-arabinose isomerase (LFAI) from *L. fermentum* CGMCC2921.

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2. Materials and methods

2.1. Strains and materials

L. fermentum CGMCC2921, which was used as a source of genomic DNA for *araA* gene cloning, was isolated from traditional Chinese pickles and grown at 37 °C in MRS medium. The host strains *Escherichia coli* JM109 and *E. coli* BL21 (DE3) were obtained from Novagen and grown in Luria–Bertani (LB) medium. Ex-taq DNA polymerase, T4 DNA ligase, restriction endonucleases and pMD-18T Vector were purchased from Takara Biotechnology (Takara, China). All the other chemicals were of the highest reagent grade and commercially available.

2.2. DNA amplification and subcloning of the L-AI gene

Genomic DNA was isolated from *L. fermentum* CGMCC2921 using a Takara Bacterial Genomic DNA Extraction Kit (Takara, China). Oligonucleotide primers specific for the full-length *araA* gene were derived from the putative *araA* gene of *L. fermentum* IFO3956 (Gene bank accession no. YP_001844370). The forward primer was *araA*s, 5′-AGAGAATTCATGCGTAAGATGCAAGATTAC-3′ (EcoRI site is underlined). The reverse primer was *araA*r, 5′-AAGCTCGAGCTACTTGATGTTGATAAAGT-3′ (XhoI site is underlined). The amplified 1.4 kb DNA fragment was cloned into the pMD18-T Vector and transformed into *E. coli* JM109 competent cells. Transformants containing the pMD18-T Vector harbouring the *araA* gene were selected, plasmid DNA (pMD18-T-*araA*) was isolated from the transformants and sequenced. To over-produce LFAI in *E. coli*, an expression plasmid pET-*araA* was constructed by ligation of gene *araA*, digested by EcoRI and XhoI from pMD-18T-*araA*, into the corresponding restriction sites of the pET-28a plasmid (Novagen) and transformed into *E. coli* BL21 (DE3).

2.3. Over-expression and purification of the recombinant L-AI

E. coli BL21 (DE3) cells harbouring the pET-28a plasmid carrying the *araA* gene were grown at 37 °C in LB medium containing kanamycin (25 μg/mL) until the $OD_{600\,nm}$ reached 0.5. Then, IPTG was added at 1 mM and growth was carried out at 20 °C for extra 12 h. Cells were harvested by centrifugation at 8000 × *g* for 10 min, washed with 50 mM phosphate buffer (pH 6.5). After sonication, the lysates were centrifuged to remove the cell debris and the supernatant was filtered through a 0.2 μm filter. The filtrate was loaded on a Ni-NTA resin column equilibrated with equilibration buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0). The column was then washed with the same buffer containing 10 mM imidazole, and a gradient of imidazole (from 50 mM to 250 mM) was applied to elute the recombinant protein. The fractions containing enzyme activity were pooled and dialyzed against phosphate buffer, and the dialyzed enzyme preparation was stored at 4 °C. Protein purity was determined by SDS–PAGE analysis.

2.4. Analytical methods

Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard protein [18]. L-AI activity was measured by determining the amount of formed D-tagatose (L-ribulose). Under standard conditions, the reaction mixture of 1 mL contained 50 mM D-galactose (L-arabinose), 1 mM $MnCl_2$, 2 mM $CoCl_2$, 100 μL of enzyme preparation at a suitable dilution and 50 mM phosphate buffer (pH 6.5). The reaction mixture was incubated at 65 °C for 10 min, followed by cooling samples on ice to stop the reaction. The generated D-tagatose (L-ribulose) was determined by cysteine–carbazole method [19], and the absorbance was measured at 560 nm. D-Tagatose production was also confirmed by high-performance liquid chromatography (HPLC) using Rezex RCM-Monosaccharide column (300 mm × 7.8 mm). The products were separated by isocratic elution with water at a flow rate of 0.5 mL/min and detected with a refractive index detector (SHODEX RI-101). Solutions of D-galactose and D-tagatose at 10 g/L each were used as standards. One unit of L-AI activity was defined as the amount of enzyme catalyzing the formation of 1 μmol keto-sugar per minute.

2.5. Effect of temperature and pH on enzyme activity and stability

The temperature optimum of LFAI activity was measured by assaying the enzyme samples over the range of 30–90 °C, at pH 6.5. Three buffer systems (sodium acetate/phosphate/Tris–HCl) were used for measuring the pH optimum of enzyme activity at 65 °C. The thermal stability of LFAI was studied by incubating the enzyme in phosphate buffer (pH 6.5) at 75 °C in the presence of 1 mM Mn^{2+}, 2 mM Co^{2+}, 1 mM Mn^{2+} plus 2 mM Co^{2+} and without adding ions, respectively. Samples were withdrawn at certain time intervals and residual activity was estimated under standard assay conditions. To determine the pH stability, the enzyme was incubated at various pH values (5.0, 5.5 and 6.0) at 4 °C for up to 24 h, the residual activity was also estimated.

2.6. Effect of various metal ions on enzyme activity

Before studying the effects of metal ions on L-AI activity, the purified enzyme was dialyzed against phosphate buffer containing 10 mM EDTA overnight at 4 °C. Subsequently, the enzyme was dialyzed against phosphate buffer to remove EDTA. Then, the enzymatic activity was assessed in the presence of several metal ions ($MgCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $CaCl_2$, $CuCl_2$, $NiCl_2$, and $BaCl_2$) with a final concentration of 1 mM. For the purpose of determining the effect of Mn^{2+} and Co^{2+} concentration on enzyme activity, the reactions were performed using the EDTA-treated enzyme with the addition of Mn^{2+} and Co^{2+} at concentrations from 0.1 to 5 mM. Then samples were taken for activity assays.

2.7. Determination of substrate specificity and kinetic parameters

A substrate concentration of 50 mM was used to investigate the substrate specificity of the enzyme. Reactions were carried out under standard reaction conditions with different substrates (L-arabinose, L-xylose, L-ribose, D-galactose, D-glucose, D-xylose, and D-mannose). The values were compared to the enzyme activity in the D-galactose solution.

Kinetic parameters of LFAI were determined in 50 mM phosphate buffer (pH 6.5), 1 mM Mn^{2+}, 2 mM Co^{2+} and 1–600 mM substrate (D-galactose or L-arabinose). The samples were incubated at 65 °C for 10 min. The enzyme reaction was stopped by chilling on ice, and the amount of D-tagatose (L-ribulose) was determined. Kinetic parameters, such as K_m (mM) and V_{max} (U/mg protein) for substrates were obtained using the Lineweaver–Burk equation. All assays were performed in triplicate at least two separate times.

2.8. Analysis of the isomerization of D-galactose to D-tagatose with LFAI

The conversion media (1 ml) contained 50 mM of D-galactose, 1 mM Mn^{2+} and 1 mg of the purified enzyme (9.98 U) in 50 mM phosphate buffer (pH 6.5). The study of the kinetic conversion of D-galactose was investigated until 24 h at 60 °C, 65 °C and 70 °C. Samples were taken periodically, and the concentration of the generated D-tagatose was determined by the cystein–carbazol–sulfuric acid method and confirmed by HPLC as indicated in Section 2.4. The

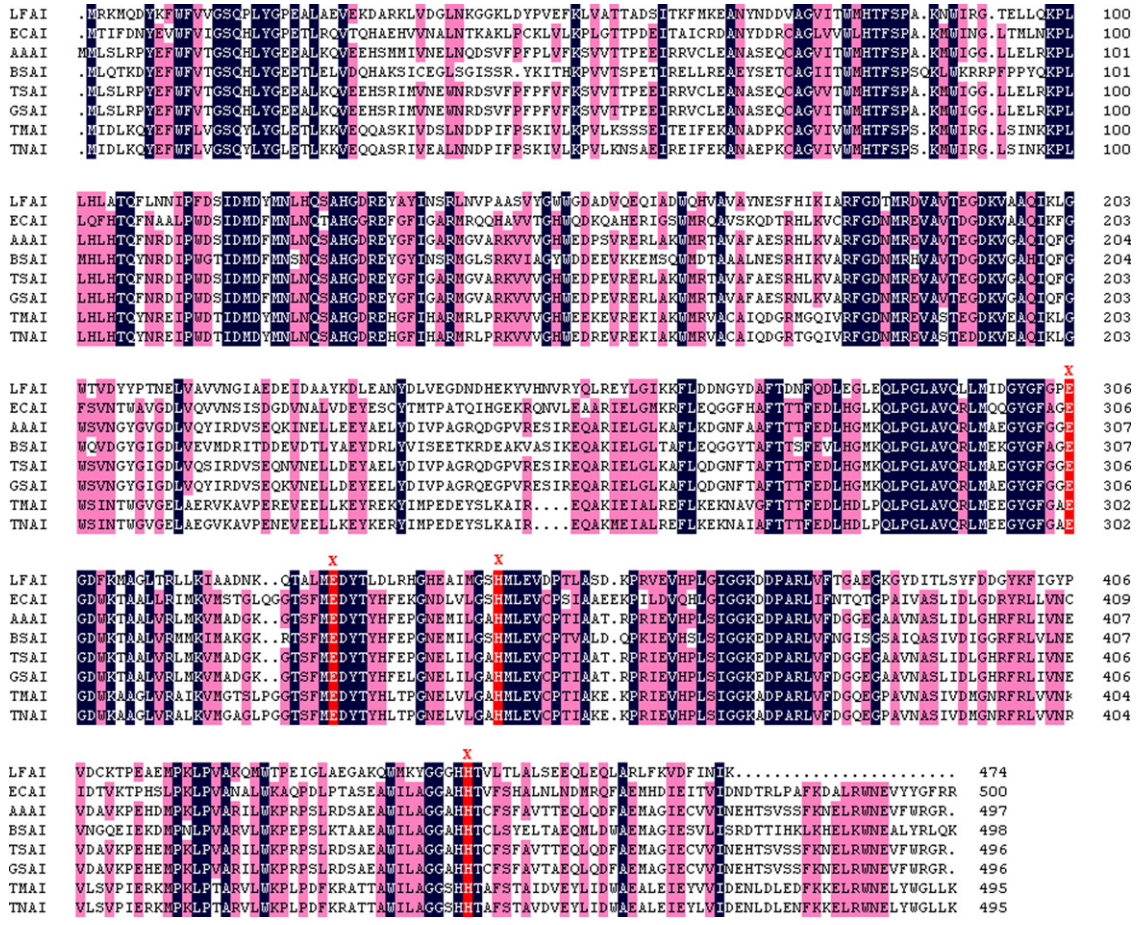

Fig. 1. Multiple sequence alignment of L-arabinose isomerases (L-AIs) from *L. fermentum* CGMCC2921 (LFAI), *E. coli* (ECAI), *A. acidocaldarius* (AAAI), *B. subtilis* (BSAI), *Thermus* sp. (TSAI), *G. stearothermophilus* (GSAI), *T. maritima* (TMAI), and *T. neapolitana* (TNAI). The alignment was performed using Clustal X program. Strongly conserved or weakly conserved residues are shaded dark blue or pink. The putative active residues (E306, E333, H350 and H450) are shaded red and marked as X at the top of the alignment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

bioconversion rate represents the ratio between the concentration of D-tagatose formed and the initial D-galactose concentration.

2.9. Separation of D-tagatose from the conversion mixture

In order to purify the formed D-tagatose by LFAI from the conversion mixture, an Amberite Ca^{2+} column (35 mm × 550 mm) was used in a water warm system (60 °C) to achieve chromatographic separation. Identification of D-tagatose was performed by ^1H NMR spectrometry (Bruker 400-MHz NMR spectrometer, 8% D-tagatose in DMSO, 25 °C).

2.10. Nucleotide sequence accession number

The nucleotide sequence of *araA* gene from *L. fermentum* CGMCC2921 was submitted to GenBank under the accession number HM150718.

3. Results and discussion

3.1. Sequence analysis of gene araA

The DNA sequence analysis of gene *araA* revealed an open reading frame of 1425 bp, encoding a polypeptide of 474 amino acids with a calculated isoelectric point of pH 4.82 and molecular mass of 53428 Da. It shows 99% identity with the putative *araA* gene from *L. fermentum* IFO 3956, suggesting a perfect conservation of the gene among *L. fermentum* strains. However, Its amino acid sequence exhibits low identity with other compared mesophilic, thermophilic and hyperthermophilic L-AIs (Fig. 1), demonstrating that LFAI is a special member of the L-arabinose isomerase superfamily. The protein sequence of LFAI shows 49% amino acid identity with L-AIs from *Alicyclobacillus acidolcaldarius* (AAY68209), *G. stearothermophilus* (AAD45718) and *Thermus* sp. (AY225311); 46% identity with L-AI from *Bacillus subtilis* (ACT82395); 44% identity with L-AIs from *T. maritima* (NP_228089), *Thermotoga neapolitana* (AY028379) and *E. coli* (AAA23463). As expected, essential catalytic amino acids E306, E333, E350 and H450 as well as residues Q16, L18, Y19, Q125, H128, M185, F279, Y335, M351, I373 and H449 that contribute to substrate recognition and isomerization reaction are perfectly conserved in LFAI [20].

3.2. Expression and purification of the recombinant enzyme

In order to express the L-AI, gene *araA* was cloned into pET-28a and successfully over-expressed in *E. coli* BL21 (DE3). The soluble recombinant protein reached to over 20% of the total protein in the cells. A single Ni^{2+} affinity chromatography step was used to purify the L-AI to more than 90% purity with a yield of 75%. SDS–PAGE analysis of the extracts of *E. coli* BL21 (DE3) harbouring pET-*araA* induced by IPTG, compared with that of the control *E. coli* BL21 (DE3) cells harbouring plasmid pET-28a, revealed the presence of large amounts of protein around 53 kDa (data not shown), which was in agreement with the predicted molecular

mass of the LFAI protein. The molecular masses of L-AIs in other bacteria were 55 kDa in *M. smegmatis* [15], 56 kDa in *E. coli* [1], 57 kDa in *T. neapolitana* [21] and 57 kDa in *T. maritima* [10]. The purified enzyme exhibited L-AI activity of 9.98 U/mg at optimal conditions which strongly supported the assumption that the putative *araA* gene in *L. fermentum* CGMCC2921 corresponded to the L-AI protein.

3.3. Effect of temperature and pH on enzyme activity

The optimum temperature of the purified LFAI was 65 °C, whereas 94% activity was remained at 60 °C (Fig. 2A). For industrial production of D-tagatose from D-galactose, isomerization performed at elevated temperatures (>60 °C) offers several advantages, such as higher conversion yield, better sugar solubility and lower risk of microbial contamination [2]. However, higher temperatures (>70 °C) introduce undesired effects such as browning and unwanted by-products formation [22]. Thus industrial D-tagatose production is suggested to be carried out at 60–65 °C [8,23].

Most previously characterized L-AIs have optimum pH in the range of 7.0–8.5. Nevertheless, a slightly acidic pH range (~6.0) can reduce browning and formation of by-products and lower cost for industrial applications, as observed in the case of acidophilic L-AIs from *Alicyclobacillus acidocaldarius* [12] and *Lactobacillus sakei* [24]. Investigation of the effect of pH on LFAI activity showed that while the enzyme was optimally active at pH 6.5, it exhibited 83% of its maximum activity at pH 6.0 (Fig. 2B). It should be noticed that besides *L. fermentum* CGMCC2921, other L-AIs from *Lactobacillus* genera also displayed high relative activities at acidic pH, such as *L. plantarum* NC8 L-AI (68% relative activity at pH 5.5) [17] and *L. sakei* L-AI (80% relative activity at pH 3.0) [24]. From the view of practical application, LFAI shows high activity at 60–65 °C and pH 6.0, making it a promising candidate for industrial D-tagatose production (Table 1).

3.4. Effect of divalent metal ions

In order to investigate the effect of divalent metal ions on LFAI activity, the purified enzyme was dialyzed against 50 mM phosphate buffer (pH 6.5) containing 10 mM EDTA. No activity was measurable in the absence of divalent metal ions. However, activity was recovered when Mn^{2+} or Co^{2+} was added. The effect of Mn^{2+} and Co^{2+} concentration on LFAI activity was investigated. As can be seen in Fig. 3, maximum stimulation of LFAI by Mn^{2+} and Co^{2+} occurred at a concentration of 1 mM and 2 mM, respectively. Compared to Mn^{2+} and Co^{2+}, Cu^{2+} strongly inhibited the D-galactose isomerization, while Zn^{2+} and Ba^{2+} slightly enhanced the enzyme activity. Other ions, such as Mg^{2+}, Ca^{2+} and Ni^{2+} had no effects on LFAI activity (Table 2).

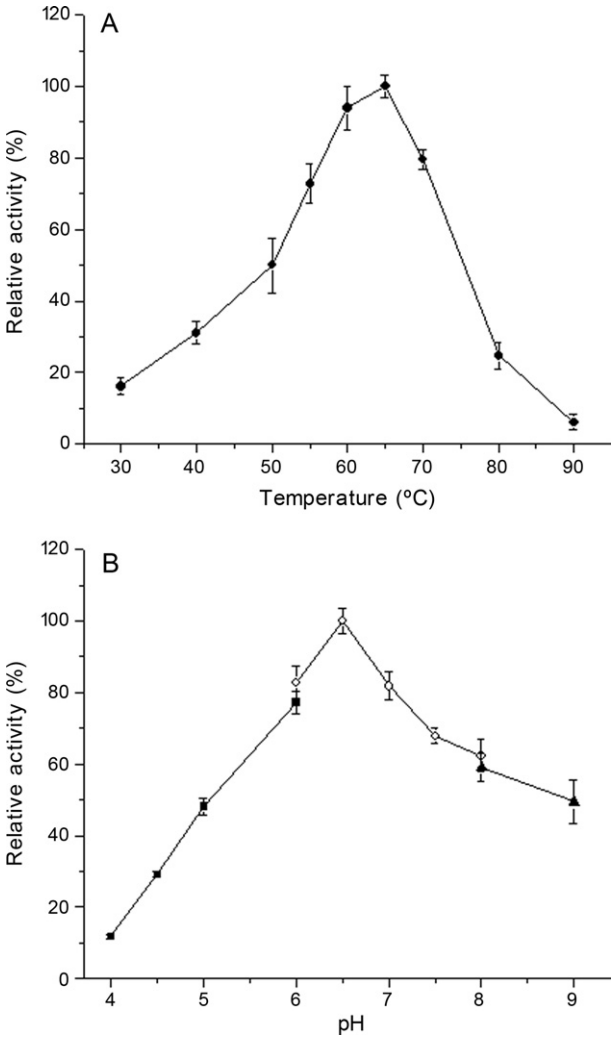

Fig. 2. Effect of temperaturen (A) and pH (B) on the activity of LFAI. Assays were carried out under standard conditions in the presence of 50 mM D-galactose. Activities at the optimal temperature and pH were defined as 100%. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%. *Filled square*: 50 mM sodium acetate buffer; *open circle*: 50 mM phosphate buffer; *closed triangle*: 50 mM Tris–HCl buffer.

The majority of L-AIs are metalloproteins involving metal ions for their optimal activity [20]. It was reported that mesophilic and thermophilic L-AIs require Mn^{2+} as a cofactor to enhance the isomerization reaction rate, while the hyperthermophilic L-AIs require Co^{2+} [1,2,10,21]. The addition of 1 mM Mn^{2+} plus 2 mM Co^{2+}

Table 1
Biochemical properties of LFAI and other reported microbial L-AIs.

Bacterium	Optimum temp. (°C)	Optimum pH	Metal ion requirement	Reference
Lactobacillus sakei	30–40	5.0–7.0	Mn^{2+}, Mg^{2+}	[24]
Alicyclobacillus acidocaldarius	65	6.0–6.5	Mn^{2+}, Co^{2+}, Mg^{2+}	[12]
Lactobacillus gayonii	30–40	6.0–7.0	Mn^{2+}	[14]
Lactobacillus plantarum SK-2	50	7.0	Mn^{2+}, Fe^{3+}	[29]
Mycobacterium smegmatis	45	7.0–7.5	Mn^{2+}, Co^{2+}, Mg^{2+}	[15]
Lactobacillus plantarum NC8	60	7.5	Mn^{2+}, Co^{2+}	[17]
Geobacillus stearothermophilus	70	7.0–7.5	Mn^{2+}, Co^{2+}, Mg^{2+}	[30]
Thermotoga maritima	90	7.5	Mn^{2+}, Co^{2+}	[10]
E. coli	30	8.0	Fe^{2+}, Mn^{2+}	[13]
Thermoanaerobacter mathranii	65	8.0	Mn^{2+}	[3]
Thermus sp.	60	8.5	Mn^{2+}	[11]
Geobacillus thermodenitrificans	70	8.5	Mn^{2+}	[9]
L. fermentum CGMCC2921	65	6.5	Mn^{2+}, Co^{2+}	This study

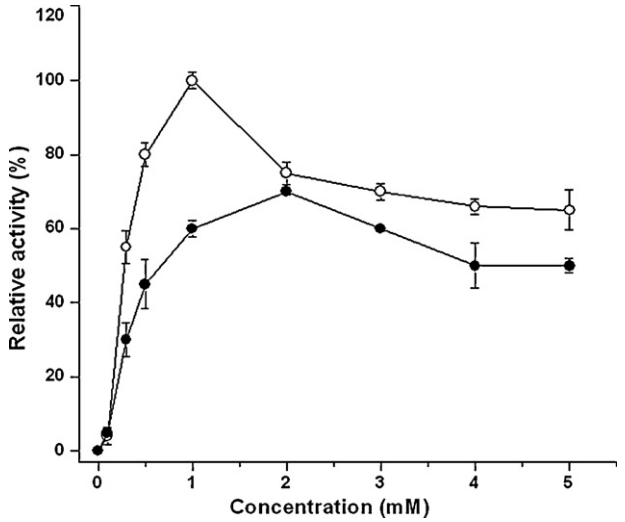

Fig. 3. Effect of Mn^{2+} and Co^{2+} addition on LFAI activity. *Open circle*: concentration of Mn^{2+} ion; *closed circle*: concentration of Co^{2+} ion. Activitiy at the optimal concentration of Mn^{2+} was defined as 100%. Each value represents the mean of triplicate measurements.

Table 2
Effect of different metal ions on the activity of LFAI.

Metal ion (1 mM)	Specific activity (U/mg protein)	Relative activity (%)
None[a]	2.0	100
EDTA	0	0
$MgCl_2$	2.2	109
$MnCl_2$	6.0	298
$CoCl_2$	4.0	201
$ZnCl_2$	2.6	129
$CaCl_2$	2.1	103
$CuCl_2$	0.4	20
$NiCl_2$	2.2	107
$BaCl_2$	2.4	120
$MnCl_2 + CoCl_2$[b]	8.5	420

The activity of EDTA-treated LFAI was assayed in the standard assay condition after incubating of 1 mM various metal ions. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

[a] The activity of native enzyme without EDTA treatment and metal ions addition was set as 100%.

[b] Mn^{2+} and Co^{2+} were added to the reaction mixture at 1 mM and 2 mM, respectively.

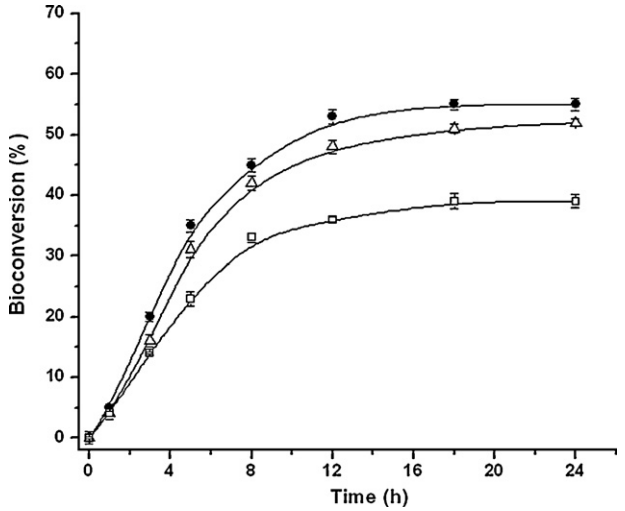

Fig. 4. Time course of D-tagatose production during LFAI-catalyzed isomerization of D-galactose. *Closed circle*: conversion curve at 65 °C; *open triangle*: conversion curve at 60 °C; *open square*: conversion curve at 70 °C.

Table 3
Half-life ($t_{1/2}$, min) of LFAI and other reported L-AIs at different temperatures.

Bacterium	Half-life ($t_{1/2}$, min)	Reference
Bacillus halodurans	20 (70 °C)	[30]
Bacillus licheniformis	120 (50 °C)	[25]
Geobacillus thermodenitrificans	30.5 (75 °C)	[9]
Geobacillus stearothermophilus	52 (80 °C)	[30]
Bacillus stearothermophilus US100	110 (75 °C)	[31]
Thermotoga neapolitana	120 (90 °C)	[21]
Thermotoga maritima	185 (90 °C)	[10]
L. fermentum CGMCC2921	30 (85 °C)/220 (75 °C)	This study

greatly improved LFAI activity (4.2-fold), suggesting that both these ions played important roles in the D-galactose isomerization by LFAI.

3.5. Thermal and pH stabilities

The thermostability of LFAI was proved to be Mn^{2+} dependent. Indeed, the enzyme was perfectly stable after a 2 h heating at 75 °C in the presence of either 1 mM Mn^{2+} or 1 mM Mn^{2+} plus 2 mM Co^{2+}, since 85% and 83% of its maximum activity were retained, respectively. On the contrary, in the absence of metal ions or in the presence of only 2 mM Co^{2+}, the enzyme was completely inactivated after 60 min. This result suggests the involvement of Mn^{2+} ion in the enzyme stabilization at high temperatures besides its role in the catalytic mechanism, whereas Co^{2+} seems to be essentially implicated in the isomerization reaction. Compared to previously reported L-AIs, LFAI showed a preferable thermostability in the presence of Mn^{2+}, with a half-life time of 30 min at 80 °C and 220 min at 75 °C (Table 3).

LFAI was stable at acidic pH since it retained 88% and 80% of its original activity after 24 h of incubation at pH 6.0 and 5.5, respectively. At pH 5.0, 55% of its activity was remained. In comparison, *L. plantarum* NC8 L-AI remained 89% of its activity after 24 h of incubation at pH 5.0 [17], and *L. sakei* L-AI had a half-life time of its activity of 49 h at pH 5.0 and 47 h at pH 6.0 (under 35 °C) [24].

3.6. Substrate specificity

The characterization of LFAI as an L-AI then allowed for the investigation of its substrate specificity for various aldoses. LFAI had a high preference for L-arabinose (220%) and D-galactose (relative activity: 100%). Other aldoses, such as D-xylose (2.7%), D-mannose (2.5%), L-xylose (1.9%), D-glucose (1.7%), and L-ribose (0.7%) did not serve as substrates for LFAI in the presence of Mn^{2+} or Co^{2+}. It was previously reported that L-AI from *Bacillus licheniformis* ATCC14580 showed 2% enzyme activity for D-galactose compared with L-arabinose [25], and *B. subtilis* str. 168 L-AI displayed substrate specificity only towards L-arabinose [26]. Different from LFAI, these L-AIs were ideal choice for enzymatic synthesis of L-ribulose from L-arabinose.

3.7. Kinetic parameters determination

Values of kinetic constants were determined on the basis of the Lineweaver–Burk plots. The K_m was 29.9 mM for L-arabinose and 60.2 mM for D-galactose. Besides, the catalytic efficiency (k_{cat}/K_m) and V_{max} was 19 mM^{-1} min^{-1}, 24.3 U/mg and 9.02 mM^{-1} min^{-1}, 9.8 U/mg for L-arabinose and D-galactose, respectively. Therefore, the catalytic efficiency of LFAI increased 2.1-fold using L-arabinose as a substrate compared with D-galactose. The LFAI catalyzes the isomerization of D-galactose with a relatively high catalytic efficiency (Table 4), showing a high substrate affinity towards D-galactose, which makes it potential for D-tagatose production.

Table 4
Comparison of L-arabinose isomerase kinetic constants from various microbial origins.

Bacterium	[a]V_{max} (U/mg)	[a]K_m (mM)	[a]k_{cat}/K_m (mM^{-1} min^{-1})	[b]k_{cat}/K_m (mM^{-1} min^{-1})	Reference
Bacillus halodurans	1.3	167	0.4	51.4	[30]
Geobacillus thermodenitrificans	6.9	408	0.5	48	[9]
Lactobacillus plantarum NC8	7.0	69.7	1.6	15.5	[17]
Geobacillus stearothermophilus (mutant enzyme)	37.6	578	2.1	65	[32]
Geobacillus thermodenitrificans (mutant enzyme)	NR	339	3.1	136	[33]
Thermotoga neapolitana	14.3	250	3.2	58.1	[21]
Alicyclobacillus acidocaldarius	7.5	129	3.3	41.5	[12]
Geobacillus stearothermophilus	7.8	145	1.2	61	[30]
Thermotoga maritima	8.9	60	8.5	74.8	[10]
Bacillus stearothermophilus US100	8.9	57	8.5	71	[31]
Acidothermus cellulolytics	4.9	28.9	9.3	NR	[34]
Lactobacillus sakei	76	59	10.3	64.8	[24]
L. fermentum CGMCC2921	9.8	60	9.0	19	This study

NR: not reported.
[a] V_{max}, K_m and k_{cat}/K_m for D-galactose.
[b] k_{cat}/K_m for L-arabinose.

Structure and mechanistic studies, to clarify the reason for substrate choosing of LFAI, are presently under way.

3.8. D-Tagatose production by LFAI

The study of isomerization of D-galactose (50 mM) to D-tagatose by LFAI at different temperatures at pH 6.5 demonstrated that the ratio of conversion of D-galactose to D-tagatose after 12 h was 52 and 36% at 60 °C and 70 °C, respectively. The highest amount of bioconversion was 55% at 65 °C with 1 mM Mn^{2+} (Fig. 4). The production of D-tagatose from D-galactose was further proved by HPLC analysis, and no by-products were observed (Fig. 5). The commercial process using xylose isomerase as an enzyme similar to L-AI is carried out at around 60 °C to limit color formation [27]. At this temperature, thermophilic L-AIs exhibit higher conversion yield than that of hyperthermophilic L-AIs [3,11,21]. Although hyperthermophilic L-AIs are more thermostable, their use in commercial D-tagatose production might be difficult because they require Co^{2+} ion as a cofactor and cobalt cannot be used in nutritional applications [2,6,10].

3.9. Purification and identification of D-tagatose

Hong et al. reported a method for isolating D-tagatose (ketose) from mixtures with D-galactose (aldose), instead of employing chemicals and organic solvents, ion-exchange chromatography was utilized and D-tagatose with high purity was obtained [28]. We use Amberite column with a water solvent system to separate D-tagatose so as to prevent environmental disadvantages. A total of 20 mL reaction mixture was applied to the column at a flow rate of 2 ml/min, then eluted by deionized water. Fractions containing pure D-tagatose (confirmed by HPLC) were pooled and concentrated by evaporation to dryness. The structure of purified D-tagatose was confirmed by ^1H NMR spectrometry, ^1H NMR

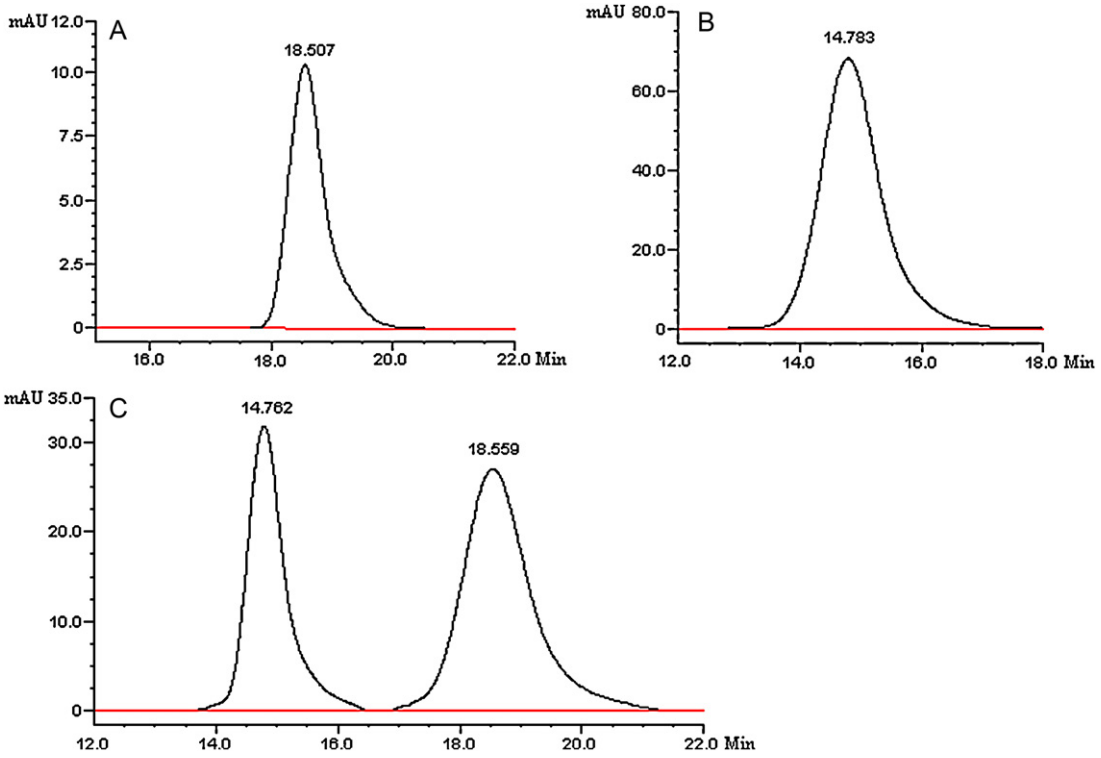

Fig. 5. HPLC analysis of the D-tagatose production. (A) D-Tagatose standard; (B) D-galactose standard; (C) products of isomerization by LFAI, the retention times of D-galactose and D-tagatose were 14.76 and 18.56 min, respectively.

(400 MHz, DMSO) δ 3.24 (m, 1H), 3.28 (d, 1H), 3.36 (t, 1H), 3.44 (d, 2H), 3.53 (s, 2H), 4.32 (d, 1H), 4.43 (d, 1H), 4.45 (t, 1H), 4.60 (d, 1H), 5.33 (s, 1H).

4. Conclusion

In summary, we have successfully cloned the *araA* gene from strain *L. fermentum* CGMCC2921 and expressed as a recombinant protein in *E. coli*. Compared with other L-AIs, LFAI exhibits not only preferable thermostability at higher temperatures with Mn^{2+}, but also behaves relatively high activity and stability at acidic pH. The successful identification and over-expression of the LFAI allows us to characterize a novel L-AI showing high specificity towards D-galactose and now sets the stage for more detailed investigation of this enzyme. In addition, a feasible and environmental friendly method for D-tagatose purification has been established. This work will be of great value to both the efficient expression and the large scale production of D-tagatose with *E. coli* as a host cell.

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